CHEMOTHERAPEUTIC AGENTS FOR INSECT MICROSPORIDIOSES

IN VITRO AND IN VIVO

by

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Thesis submitted for the Degree of Doctor of Philosophy

University of London

and for the Diploma of Membership of Imperial College (DIC)

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SEPTEMBER 1991

TO

MY WIFE, NAZNIN RAZZAQUE

AND

MY SON, RIDWAN AL AZIZ

ABSTRACT

The present study set out to investigate anti-microsporidial drugs. For this purpose, 20 drugs were selected to test against Nosema bombycis. Tested drugs were: albendazole, sinefungin, fumagillin, thimerosal, benzimidazole, pentostam, ciprofloxacin, toltrazuril, imidocarb dipropionate, metronidazole, sulphadimidine, sulphapyridine, sulphathiazole, rimocidin sulphate, cerulenin, itraconazole, compound 566C80, benomyl, novobiocin and streptolydigin. Drugs were first tested in an in vitro system using Spodoptera frugiperda cell culture and any that showed signs of anti-microsporidial activity were further tested in vivo using Heliothis zea as an experimental host. Except for benomyl, novobiocin and streptolydigin, all the drugs were tested in vitro. These three drugs could not be tested in vitro due to their insolubility in culture medium.

Among the drugs tested in vitro, seven drugs, namely albendazole, sinefungin, fumagillin, thimerosal, benzimidazole, pentostam and ciprofloxacin showed anti-microsporidial activity when infected cultures were exposed to the drug. Thimerosal inhibited development of the microsporidia relative to controls but did not prevent an increase in the percentage of infected cells. Exposure of host cells to drug concentrations which inhibited development for longer than 2 - 3 days was toxic to the cells. The others not only prevented further spread of infections between cells but also reduced the level of infection from the initial point. However, none of them completely eliminated the parasite from the cultures. The minimum concentration of the drugs which inhibited further spread of the parasite were: albendazole at 5.3µg/ml, sinefungin at 100µg/ml, fumagillin at 5.0µg/ml, benzimidazole at 250µg/ml, pentostam at 2,000µg/ml and ciprofloxacin at 250µg/ml.

When albendazole, sinefungin, fumagillin and benzimidazole, were tested in combination in vitro, none of the combinations showed any additive or synergistic effects except albendazole combined with sinefungin, which was additive when used up to 7 days.

With the exception of ciprofloxacin, all the drugs which showed anti-microsporidial activity *in vitro* were tested *in vivo*. In addition, benomyl, novobiocin, streptolydigin and toltrazuril were tested *in vivo*. In *in vivo* experiments, albendazole, benomyl, sinefungin and fumagillin showed marked anti-microsporidial activities in *H. zea* larvae. Albendazole and benomyl at 1 - 4mg/larva almost completely eliminated the parasite from the mature larvae but low level infections were recorded in the pupal stage. However, albendazole at 4mg/larva completely prevented development of the microsporidia when healthy larvae were exposed to drug and parasites concurrently. Fumagillin at 25 - 100µg/larva and sinefungin at 5 - 25µg/larva significantly reduced the production of spores in comparison with controls. The effects of these 4 drugs, albendazole, benomyl, sinefungin and fumagillin on *N. bombycis* at the subcellular level were observed by electron microscopy and their probable mechanisms of action were discussed. The effect of toltrazuril on *N. bombycis* was also studied by electron microscopy. It appeared that albendazole and benomyl interfered with nuclear division, possibly by interrupting microtubule formation, while fumagillin and sinefungin probably affected DNA synthesis. No ultrastructural alterations were seen after toltrazuril treatment and this was in accord with its observed lack of effect on growth *in vitro*. It was concluded that toltrazuril has no antimicrosporidial activity although these results were in disagreement with previous studies.

Albendazole at 1 - 4mg/larva had no adverse effects on larval and pupal development of *H. zea* while benomyl at 4mg/larva showed some toxicity.

ACKNOWLEDGEMENTS

I wish to express my gratitude to my supervisor Professor E.U.Canning for her help, guidance and encouragement througout this study, and also to thank my advisers Dr.C.Prior of the International Institute of Biological Control (IIBC) and Dr.W.S.Hollister for their valuable comments on the work.

I am grateful to Dr. R. Possee of the Institute of Virology, NERC, Oxford, England for supplying Spodoptera frugiperda cell line and to Professor R.Ishihara and Mr.H.Iwano of Nihon University, Japan for Antheraea eucalypti cell line and Nosema bombycis spores. Special thanks to Mr.T.Carty, Institute of Virology, NERC, Oxford, England for supplying Heliothis zea eggs.

I would also like to thank the followng people for supplying drugs as gift samples: Dr. R.J.Horton of Smith Kline Beecham Pharmaceuticals, England for albendazole; Dr. D.M.King of DuPont (U.K.)Ltd. for benomyl; Dr.J.B.McAlpine of Abbott Lab., U.S.A. for fumagillin; Dr.R.A.Neal, London School of Hygiene and Tropical Medicine, England, for pentostam; Dr.B.G.Gazzard, Westminster Hospital, London, England, for ciprofloxacin; Dr.H.D.Matthaei and Professor A. Haberkorn of Bayer AG., Germany, for toltrazuril; Mr.M.A.Wright of Rhône-Poulenc, England, for metronidazole, sulphadimidine, sulphapyridine and sulphathiazole; Dr.N.Belcher of Pfizer Inc. England, for rimocidin sulphate; Professor S.Ōmura of Kitasato Institute, Japan, for cerulenin; Dr.D.R.White of Upjohn Co., U.S.A. for novobiocin and streptolydigin. I also extend my thanks to The Wellcome Foundation Ltd., England, for supplying imidocarb dipropionate and compound 566C80 as gift samples.

I would also like to express my appreciation to Mr.S.A.Zakaria, Mr. J.P.Nicholas and Angela Willcox for their valuable technical assistance and Mrs.V.Walters for typing the thesis.

I am also grateful to the Commonwealth Scholarship Commission in the United Kingdom for finanacial assitance.

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1. INTRODUCTION

In the past few years the number of species and the quantity of insects reared in the laboratory has increased greatly in response to a variety of needs. Certain insects are now mass reared for release in autocidal control programmes, and more species probably will be used in the future. Insects are also reared for mass production of insect pathogens. Smaller colonies are maintained to study particular species and to develop specialized strains. Additionally, some economically important insects, like honey bees and silkworms, are mass reared for their products - honey and silk - which are the bases of thriving industries.

Many species of microsporidia are known to affect laboratory colonies either by destroying the colonies of insects or by temporarily interrupting the rearing schedules. The response of insects from such diseased colonies to experimental treatments may be erratic, which could result in misleading conclusions. Furthermore, in beneficial insects microsporidia may exert their effects by depressing the productivity of bees and silkworms and by reducing the efficacy of insect predators and parasitoids. The most important microsporidia involved in beneficial insects are Nosema apis in honey bees and Nosema bombycis in silkworms.

Many microsporidian species belonging to several genera can infect insect predators and parasitoids. There is evidence that microsporidian infection in the hosts of parasitoid insects may inhibit the development of the parasitoids and in some cases the infections may pass to the parasitoids themselves. Also insects are shipped regularly from biological control laboratories all over the world to initiate the control of pests. In order to avoid the spread of microsporidia from the control agents to other beneficial insects, these control insects should be microsporidia-free when shipped.

Furthermore, the rapid expansion of aquaculture has provided environmental conditions which favour transmission of microsporidia among fish and crustaceans. Microsporidian infections have also been reported from several mammals including pets and fur bearing species. These are important not only for their effects on economically important mammals but also because latent

infections in rodents may compromise results of laboratory experiments. Also immunosuppressive diseases, such as AIDS, have uncovered serious microsporidial infections in man for which treatment is required.

The present study sets out to investigate anti-microsporidial drugs which may be used to control or eliminate infections. Drugs were screened in an *in vitro* system and any that showed signs of anti-microsporidial activity were further tested *in vivo* in insects.

2. LITERATURE REVIEW

2.1 IMPORTANCE OF MICROSPORIDIA

Microsporidia are obligate intracellular parasites with a wide host range infecting both invertebrates and vertebrates. Since the discovery of microsporidia, a wide variety of organisms belonging to the phyla Apicomplexa, Ciliophora, Myxozoa, Coelenterata, Platyhelminthes, Nematoda, Porifera, Rotatoria, Annelida, Mollusca, Bryozoa, Arthropoda and Chordata have been recorded as hosts of microsporidia. The greatest number of species are known from arthropods, especially insects, and from fish.

2.1.1 Pathogenicity of microsporidia in beneficial insects

2.1.1.1 Effect on honey bees

Life span and behaviour

Nosema disease or nosemosis of honey bees (*Apis mellifera*) caused by the microsporidium, *Nosema apis*, is worldwide and causes considerable losses to the bee-keeping industry. This microsporidian parasite infects all castes of adult bees and has an adverse effect on colony development and honey production. Farrar (1947) reported that infection of *N. apis* had a depressing effect on brood rearing roughly proportional to the percentage of *N. apis* infection among the worker bees in package colonies and that honey yields were similarly varied. Thus colonies showing infection rates of 60 - 100% had about 100 - 125 square inches less brood than colonies showing 10% or less infection and colonies in the low-infection group averaged 54 pounds more surplus honey than those in the high-infection group. Lower honey yield and poorer colony development in *N. apis*-infected bee colonies were also reported by L'Arrivee (1966), Rossi *et al.* (1969) and Cornejo *et al.* (1970). Lower honey production and poorer colony development in infected colonies can be attributed to the effect of *N. apis* on life-span and foraging activities of worker bees, to the life-span (and thus the reproductive capacity) of queen's and to queen supersedure.

Burnside and Revell (1948) found that colonies with long-lived bees produced larger populations of field bees and larger honey crops than did colonies with relatively short-lived bees. Decreased life spans due to *N. apis* infection have been reported many times e.g. decreases of 10 - 40% (Burnside and Revell, 1948) and 22% (Kang *et al.*, 1976). Moffett and Lawson (1975)

reported that healthy bees lived 4.6% longer than infected bees and Poteikina (1960) gave the figure as 2.5 times as long. The life span of the queens is affected as well as that of the workers. L'Arrivee (1965) investigated the effect of dosage of N. apis, finding that life span of queens decreased as dosage levels increased. Thus, uninoculated queens lived 70.83 days compared to 33.98, 39.96 and 39.85 days for queens inoculated with 1.48×10^6, 0.78×10^6 and 0.53×10^6 N. apis spores per queen, respectively.

The decreased life span of infected queens in turn affects their productivity as evidenced by the findings of Moeller (1962), in which he reported 12% less sealed brood in infected colonies. Similarly, Loskotova et al. (1980) found that out of 17 diseased queens, 7 failed to oviposit and the other 10 started laying 3 days later than healthy queens.

The performance of colonies is also lessened by reduced foraging and pollinating activities of worker bees (Hassanein, 1952a). In this study disease-free workers spent about 29 minutes on each foraging expedition, whereas N. apis-infected bees spent 56 minutes so that disease-free bees made an average of 20 trips per day as compared to 11 trips for infected bees. The mean weights of pollen and nectar loads brought back to the hive by infected foragers were lower than corresponding weights of healthy bees.

Queen supersedure is a serious problem to the beekeeper. Nosema disease is largely responsible for the abnormal supersedure of queens. Farrar (1947) reported that out of 53 inoculated queens, only 3 escaped infection and the rest were superseded or stopped laying. He concluded that N. apis infection influenced queen supersedure. He also reported that when queens in the severely infected colonies were superseded, the resultant loss in honey production was 111 pounds. Furgala (1962) quantified the effect of Nosema inocula on queen supersedure and found that inoculation with as few as 1000 spores resulted in supersedure.

The reduction in hoarding behaviour of honey bees reported by Rinderer and Elliott (1977) might also account for the overall lower productivity of the colony.

Biochemical changes

N. apis infection alters the physiological conditions of honey bees. Roberts (1968) found qualitative differences in the types of fatty acids

between infected and healthy bees. The ratio of saturated to unsaturated fatty acids was higher in infected than uninfected bees. Saturated fatty acids comprised 32.2% of the fatty acids in infected bees but only 15.4% in uninfected bees. Wang and Moeller (1970) found higher amounts of amino acids in the haemolymph of normal female bees than in that of infected bees. Foti et al. (1971) also observed a decrease in the level of protein and free amino acids in the haemolymph of infected bees. A decrease in the total protein, lipids, glucose and fructose contents of the haemolymph of infected bees was reported by Tomaszewska (1979). Individual bees exposed to infection with N. apis 24 - 33 days before testing used 22% less oxygen, 33% less water and 14% more sucrose syrup than healthy bees (Moffett and Lawson, 1975). Zherebkin (1977) reported that body weight and nitrogen content of young diseased bees were higher than those of healthy bees but in older diseased bees, weight and nitrogen content were decreased. He added that there was less fat in diseased bees of all ages than in healthy bees.

Cellular abnormalities and histo-chemical changes of the organs affected in Nosema have been studied by several workers. Cantwell (1974) and Sprague (1977) reported that heavy N. apis infection caused the gut to swell and turn a light milky-white colour. Detailed ultrastructural changes in the midgut of worker honey bees infected with N. apis were studied by Liu (1984). He reported that midgut epithelial cells from healthy bees possessed numerous mitochondria, cisternae of endoplasmic reticulum, evenly distributed ribosomes, zymogen granules and two kinds of lipid inclusions. In heavily infected midguts, epithelial cells throughout the entire midgut were packed with mature spores. In some cases, spores were mixed with immature stages. The cytoplasm of the infected cells contained a large number of vacuoles, numerous large inclusion bodies, and aggregated ribosomes. The infected cells also exhibited extensive lysis. Duca et al. (1987a) observed that infected cells of the midgut epithelium of Apis mellifica carpathica became swollen and exfoliated and the peritrophic membrane was fragmented. In heavy infections, spores filled the cytoplasm of infected cells. Infected cells had reduced amounts of mucopolysaccharides and nucleic acid, particularly RNA.

Duca et al. (1987b) and Pápay et al. (1987) reported that the enzyme activities in the midgut of infected bees were greatly reduced or absent, which led to depression of the metabolic level and disrupted digestion and absorption of food. Altered midgut enzyme activities in infected bees were

also reported by Zherebkin (1977). He found that the activities of amylase, proteinase, invertase and catalase in diseased bees were much lower, slightly lower, about the same and slightly higher respectively, than in healthy bees; the differences were more pronounced as the bees grew older.

N. apis infection influences the development and secretory activity of the hypopharyngeal glands of the worker honey bees. These glands secrete the highly nitrogenous brood-food, royal jelly, for the youngest larvae in the colony. Hassanein (1952b) reported that N. apis infection considerably reduced the size of the lobes of the hypopharyngeal glands which caused reduction in the secretory activity of the glands. Wang and Moeller (1969) compared the morphology of the hypopharyngeal glands of Nosema-diseased and healthy worker honey bees. The glands of diseased bees suffered from regressive changes: the volume of the lobules of the glands decreased progressively, the nuclei disintegrated, the chromatin coalesced, and the cytoplasm and the size of the secretory globules were noticeably reduced. In later studies, Wang and Moeller (1971) reported on detailed ultrastructural changes in the hypopharyngeal glands of diseased worker honey bees. The hypopharyngeal glands of 5-day-old Nosema-infected worker bees had fewer ribosomes on the nuclear envelope, smaller cisternae of endoplasmic reticulum, smaller Golgi complex, myelin-like lysosome bodies, smaller mitochondria, fewer and smaller microvilli in the secretory mass of the outer layer of intracellular ductules, and the collapse of intracellular ductules. In the hypopharyngeal glands of 10-day-old bees severely infected with N. apis, the rough endoplasmic reticulum, Golgi complex and mitochondria disappeared and the myelin-like whorls of lysosome bodies became more pronounced. Ultimately the cytoplasm of the glands began to disintegrate and become vacuolated. The intracellular tracheoles in the hypopharyngeal glands showed malformation of structure. They concluded that the pathological changes in hypopharyngeal glands, caused by N. apis, inhibited royal jelly secretion. Liu (1990c) reported that the hypopharyngeal glands of healthy honey bees contained numerous electron-dense secretion granules, whereas in infected bees, these granules appeared to have increased in size and lost their electron density, possessing a core area that consisted of numerous smaller granules, and a slightly electron dense fringe, which in some cases had a crystalline structure. He suggested that these ultrastructural changes in the secretion granules of diseased bees was probably associated with a change in secretory

activity of the glands. In further studies Liu (1990d) confirmed that N. apis infection considerably altered the composition of the secretion products from the hypopharyngeal glands. The secretion products in the extracellular ducts of the hypopharyngeal glands of healthy bees consisted of a densely packed, faintly visible granular substance. Although the secretion products from the Nosema-infected honey bees exhibited the same granular structure, they were much less concentrated, so that the overall electron density of the secretion was reduced.

The development and secretory activity of endocrine glands such as the corpora cardiaca and corpora allata are also affected by N. apis infection. Liu (1990b) reported that the axons of corpora cardiaca in Nosema-infected honey bees contained numerous tightly packed neurosecretion granules of high electron density. The neurosecretion granules were of similar size in the axons of corpora cardiaca in healthy honey bees, but were of variable electron density: some had an electron-dense core, and the electron density decreased gradually from the core towards the fringe of the granules; other granules were broken down into a fine granular substance and yet others were completely broken down into very fine particles. He suggested that in the healthy honey bees, the release of neurosecretory products from the corpora cardiaca had taken place normally, whereas in the diseased bee, the release mechanism had been blocked, probably by the N. apis infection.

N. apis infection also alters the ultrastructure of mitochondria in the corpora allata of honey bees (Liu, 1990a). The mitochondria in the corpora allata of healthy honey bees were large, possessed well defined membranes, numerous mitochondrial granules and an electron-lucent matrix. In the corpora allata of Nosema-infected honey bees, mitochondria were small and the mitochondrial matrix had a higher electron density.

2.1.1.2 Effect on silkworms

Pebrine disease of silkworms, Bombyx mori caused by Nosema bombycis is one of the central problems in the silk industry. The microsporidium is transmitted transovarially to the progeny (Han and Watanabe, 1988) and almost all the races are susceptible to the parasite which causes tremendous loss in the silk industry (Jafri et al., 1978).

All the tissues of all stages of B. mori can be infected. Heavily infected organs are hypertrophied, distorted and have a milky-white appear-

ance. Brown or black spots appear on the cuticular surface of the infected larvae (Sprague, 1977; Jafri et al., 1978). Takizawa (1978) found black spots on the midgut of the infected larvae.

The pathogenicity of N. bombycis on the larval and pupal development of B. mori have been studied by some workers. Ohshima (1960) reported that the development of B. mori from larva to adult was accelerated when the insect was infected per os with N. bombycis in the larval stage. Ishihara (1963) found that, when early- and middle-aged pupae were infected with the spores of N. bombycis, the development of the pupae was accelerated and that this effect was enhanced as the inoculum size was increased. Ishihara and Fujiwara (1965) studied the spread of pebrine disease within a colony of silkworms and found that the rate of infection was high in the 1st, 2nd and 5th instars, but was low in the middle instars. A detailed study on the pathogenicity of N. bombycis on silkworms has been done by Fujiwara (1979). He reported that the susceptibility of B. mori to N. bombycis depends on the larval instars infected and on the size of the inoculum used. In one experiment, he infected 2nd, 3rd, 4th and 5th instar larvae on the first day after the moult and 5th instar larvae also on the 5th day after the moult. He found that early instars were more susceptible than later instars and none of the larvae emerged as adults when they were infected as 2nd or 3rd instars. When infection was initiated at the 4th instar, 26.32% died at the spinning stage and, though the remaining larvae pupated, only 26.32% emerged as adults. With the 5th instar larvae there was no difference between infections initiated immediately after the moult and those initiated 5 days later as all of them pupated and 93.33% and 91.67% emerged as adults, respectively. In another experiment, he infected 2nd instar larvae on day 0 with different levels of inocula (10^2 - 10^7) and found that mortality was quicker with the higher doses than with the lower doses. Han and Watanabe (1988) investigated the transovarial effect of N. bombycis on silkworm larvae. They found that in larval progeny of the F_1 generation that were infected transovarially, an acute lethal infection developed, and most larvae died by the end of the 3rd instar.

Veber and Jasič (1961) reported the effect of N. bombycis on the fecundity of B. mori. They found that infected females had a lower average fecundity, which was dependent both on spore dosage and on the larval instar in which infection was initiated. The mean fecundity of healthy females was 453 compared to 341 of females infected at 5th instar with a dose 5×10^3

spores/larva. The mean number of eggs laid per female was reduced to 332 by exposing the 5th larval instar to 5 x 10^4 spores/larva, while an even greater reduction in fecundity to 209 eggs/female was obtained when larvae were infected at the beginning of the 4th instars with 5 x 10^3 spores/larva.

2.1.1.3 Effect on parasitoids

A parasite is an organism which lives and feeds in or on another living organism. When the parasite lives on the surface of the host, it is called an ecto-parasite; and when it lives within the host's body, it is called an endo-parasite. Although there is no fundamental difference between parasite and parasitoid, the term parasitoid is usually applied to the parasitic insects. Both endo- and ecto-parasitoids are susceptible to microsporidian infection.

There are two principal ways in which a microsporidian parasite infecting a host insect may have an impact on a developing parasitoid. The parasitoid may itself become infected by the microsporidia while feeding on infected host tissues and infected parasitoids may die prematurely, or their adults may have low fecundity and short life span. Alternatively, the microsporidian parasite may affect the parasitoid indirectly by its detrimental effect on the host insect. The host insect may die before the development of the parasitoid is complete or there may be a nutritional imbalance in the infected host tissues or an imbalance in the parasitoid caused by the ingestion and accumulation of large numbers of nondigestible microsporidian spores in the midgut of the parasitoid larvae.

In 1945, Allen and Brunson first reported that a braconid parasitoid, Macrocentrus ancylivorus, used as a biological control agent against the oriental fruit moth, Grapholitha molesta, was infected with a microsporidian parasite. In their studies on the propagation of M. ancylivorus in the potato tuber worm, Gnorimoschema operculella, they observed that a considerable number of the adult parasitoids had swollen and malformed abdomens. Adults with such symptoms were scarcely able to fly and had a shorter life span with diseased females living about three-quarters as long as healthy ones and diseased males surviving only half as long. The fecundity of diseased parasitoids was about three times lower than that of healthy ones. Examination of these adult parasitoids showed that they were infected with a microsporidian parasite. Later (1947), they designated the microsporidian parasite as a

species of Nosema. Microsporidian infections of M. ancylivorus were also reported by Steinhaus and Hughes (1949) and Steinhaus (1951). The species involved were Plistophora californica and Nosema destructor. A detailed study of the effect of N. destructor on M. ancylivorus was done by Allen (1954). Diseased parasitoid adults were frequently weak or crippled at emergence, they had lowered vitality and produced fewer progeny. Diseased individuals of both sexes had significantly shorter life spans. The healthy females lived for an average of 13.0 days and the diseased females for 9.1 days. The reproductive capacity which was measured by the number of parasitoid cocoons produced, was significantly lower in the diseased group. The average for the non-diseased females was 97.4 cocoons and for the diseased females 36.0 cocoons.

Tanada (1955) reported that, although light and moderate infection by Perezia (=Nosema) mesnili within host cabbage worms, Pieris rapae, did not inhibit the braconid parasitoid Apanteles glomeratus from developing into adults, heavy infection in the cabbage worms prevented many of the A. glomeratus larvae from emerging and completing their development. Susceptibility of A. glomeratus to Nosema mesnili was also reported by Blunck et al. (1959), Steinhaus and Marsh (1962), Issi and Maslennikova (1966), and Hostounský (1970). Two other hymenopterous parasitoids of the cabbage worms, Hyposoter ebeninus and Pimpla instigator, were also found to be susceptible to N. mesnili and the infection was almost systemic in nature (Hostounský, 1970). Thomson (1958a) found that a significantly higher proportion of the parasitoid larvae of Apanteles fumiferanae and Glypta fumiferanae emerging from Perezia (=Nosema) fumiferanae-infected spruce budworm Choristoneura fumiferana, failed to complete their development. Laigo and Tamashiro (1967) also reported that Nosema infection in lawn army worm, Spodoptera mauritia acronyctoides, had deleterious effects on their internal parasitoid, Apanteles marginiventris. They observed high parasitoid larval and pupal mortality, greatly reduced adult emergence, and those few adults that did emerge were smaller and significantly shorter-lived than the parasitoids from uninfected hosts.

Another braconid parasitoid, Macrocentrus grandii used as a biological control agent against the European corn borer, Ostrinia nubilalis is susceptible to Nosema pyrausta infection. Andreadis (1980) found that all the stages of M. grandii were susceptible to N. pyrausta, which invaded the midgut epithelium, fat body, muscle, nerves and Malpighian tubules. N.

pyrausta infection reduced the adult emergence rate by more than 38% and longevity of infected adult survivors of both sexes was significantly shorter than that recorded for uninfected controls. Infected females were unable to transmit the microsporidian to additional corn borer hosts. In 1982, Andreadis confirmed the adverse effects of N. pyrausta by investigations on field populations of M. grandii. Siegel et al. (1986a) reported similar results. Cossentine and Lewis (1987) studied the impact of three microsporidia, N. pyrausta, Nosema sp. and Vairimorpha necatrix on M. grandii within infected European corn borer hosts. All three microsporidia decreased adult parasitoid eclosion. Female M. grandii which succeeded in becoming adult after development in hosts infected with V. necatrix or N. pyrausta lived for 10 - 14 days but did not transmit the pathogens transovarially to their offspring. Only male M. grandii adults emerged from hosts infected with Nosema sp.

The adverse effects of microsporidian infection on different parasitoids of European corn borers, O. nubilalis were also reported by York (1961). In laboratory rearings of the parasitoid species, Chelonus annulipes, the parasitoids emerged from the host and most of them pupated, but were not able to emerge as adults. In the case of Macrocentrus gifuensis, the only parasitoids to emerge from the microsporidian-infected host died before they pupated. A few dead larvae of the tachinid parasitoid, Lydella grisescens were also found within the host. An examination of these parasitoids showed heavy infections of microsporidia, probably of N. pyrausta. In contrast, Cossentine and Lewis (1988) reported that N. pyrausta infection had no adverse effect on Lydella thompsoni, a tachinid parasitoid of O. nubilalis but another species of Nosema had marked effects on the parasitoid. The parasitoids were unable to emerge as adults after development within Nosema sp.-infected O. nubilalis larvae. In earlier studies, Cossentine and Lewis (1986) had reported the impact of two microsporidia, V. necatrix and Vairimorpha sp. on a tachinid parasitoid, Bonnetia comta within Agrotis ipsilon hosts. Both microsporidia decreased the number of B. comta able to pupate and reduced the number of days required for adult parasitoid eclosion and the weights of the puparia. The effects were more detrimental to female B. comta than to male B. comta.

Brooks and Cranford (1972) observed that, although most of the hymenopterous parasitoids of Campoletis sonorensis, which developed in larvae of Heliothis zea infected with Nosema heliothidis, emerged as normal-appearing adults, a few adults were malformed. These were infected with N. heliothidis

and many (23%) of the infected F_1 individuals died as pupae. They also studied the development of C. sonorensis, infected with Nosema campoletidis, in larvae of H. zea which were themselves infected with N. heliothidis. The resultant parasitoids appeared to be infected by both microsporidian species. Some infected individuals became deformed adults, and others died as larvae or pupae. Susceptibility of another hymenopterous parasitoid, Cardiochiles nigriceps to N. cardiochilis were also reported although no details of the pathogenic effects were given. Own and Brooks (1986) reported that the hymenopterous parasitoid, Pediobius foveolatus was highly susceptible to Nosema epilachnae and Nosema varivestis, two naturally occurring microsporidia of the Mexican bean beetle, Epilachna varivestis. In heavily infected hosts, prevalence of infection with both microsporidia in the parasitoids progeny approached 100% and the percentage mortality was high. Mortality occurred primarily in the pupal stage and prevalence of infection and mortality were directly related to degree of host infection and microsporidian virulence. Most infected adults were normal in appearance but some had malformed wings, greatly swollen abdomens, or both. Infection did not adversely affect the developmental period of emerging adults but adult longevity was significantly reduced. Capability of oviposition and transovarial transmission of the pathogen by female parasitoids infected with N. epilachnae were markedly reduced.

A number of pupal parasitoids and egg parasitoids which are used in biological control programmes are also reported to be susceptible to microsporidia infecting their hosts.

Laigo and Paschke (1968) reported that the biology of Pteromalus puparum, a pupal parasitoid of cabbage worm, Pieris rapae, was adversely affected by Thelohania sp.. The adult emergence rate of the parasitoids from infected hosts was greatly reduced and those that successfully reached adulthood were smaller and generally shorter-lived than parasitoids from healthy hosts. The sex-ratio, especially of those bred from severely infected hosts, was also affected. The male-female ratio from microsporidiosis-treated hosts was 1:5.2 compared to 1:4.5 in the controls. Another pupal parasitoid, the chalcid wasp Dahlbominus fuscipennis was susceptible to the microsporidium, Thelohania pristiphorae when developing in microsporidian-infected pupae of Neodiprion swainei, N. lecontei, N. pratti banksianae and Pristiphora erichsonii. The microsporidium which infects tissues of the gut epithelium,

fat body and cerebral ganglial cells, shortened the adult lifespan by 5 - 6 days (Smirnoff, 1971).

The egg parasitoid, Trichogramma evanescens, a biocontrol agent of O. nubilalis is highly susceptible to N. pyrausta infection and the fecundity of the parasitoid is reduced by about half (Huger, 1984). Significant reduction in adult emergence rate of the egg parasitoid, Trichogramma nubilale due to N. pyrausta infection were also reported by Sajap and Lewis (1988). They observed that the adult emergence rate of the parasitoid was reduced by about 36% when they developed in N. pyrausta-infected egg masses.

Failure of the ecto-parasitoid, Bracon mellitor to emerge from the boll weevil, Anthonomus grandis larvae infected with Glugea gasti was reported by Bell and McGovern (1975). Examination of dead, immature B. mellitor extracted from infected hosts showed that all contained large numbers of G. gasti spores.

Parasitoids for release into the field for biocontrol should be microsporidia-free because the microsporidia can cause severe damage to the parasitoids, as revealed in the above survey. Drugs which might be used to prevent or eliminate microsporidia would not be useful in preventing parasitoids in field-infected hosts from acquiring these infections but could be used to ensure that healthy parasitoids are released and thus avoid introduction of microsporidian infections into the field.

2.1.1.4 Effect on insect predators

A predator is a free-living organism which feeds upon prey. Very few studies have so far been made on the pathogenicity of microsporidia in insect predators. Steinhaus and Hughes (1949) first reported that the green lace-wing predator, Chrysopa californica became infected with Pleistophora californica, a natural microsporidian parasite of Gnorimoschema operculella, when the larvae of the predator were fed spore suspensions of the microsporidian. Later, Finney (1950) briefly reported the pathogenic effect of P. californica on C. californica. He found that the parasites considerably shortened their lifespan and severely lowered the egg production. Lipa and Steinhaus (1959) described a microsporidian parasite, Nosema hippodamiae from the convergent ladybird beetle, Hippodamia convergens. They found that the parasite primarily invaded the midgut and fat body but in cases of severe infection, the parasite might invade other tissues as well. Sluss (1968) also found that about 50% of

H. convergens were infected with microsporidia, probably by N. hippodamiae during his studies on behavourial and anatomical responses of H. convergens to parasitism by Perilitus coccinellae, a braconid parasitoid. Smirnoff and Eichhorn (1970) reported that specimens of Laricobius erichsonii, a predator of Adelges sp. (Homoptera) were infected with Nosema sp., although no details of the infections were provided.

Van Essen and Anthony (1976) found that Notonecta undulata, a predator of mosquitoes was susceptible to Nosema algerae when fed upon diseased mosquito larvae, the normal hosts of N. algerae. The infection rates averaged 47.9%. Marti and Hamm (1985) reported that, although there was no sign of development of Vairimorpha sp. in a predator, Calleida decora when the predator larvae were allowed to feed on fall army worm, Spodoptera frugiperda larvae infected with the microsporidium, there were nevertheless some adverse effects on the predator. These were expressed as higher mortality of larvae, increased duration of larval period, greater age at adult eclosion, and an altered sex-ratio of adults, due to more deaths among female larvae than male larvae. Overall, 50% of treated larvae died compared to 14.3% of controls. They attributed these effects to the ingestion of a large, non-nutritive meal equivalent to a brief starvation period. These adverse effects of microsporidia on predators indicate that only microsporidia-free predators should be released into the enviornment for biological control of pests.

2.1.2 Pathogenicity of microsporidia in insects of agricultural and forestry importance.

Numerous studies have been carried out on the pathogenicity of microsporidia in insect pests. Most of the studies were limited to lepidopteran insects, but some relate to orthopteran, coleopteran and hymenopteran insects. In some of these studies insects have been collected from the field and reared to the adult stage in the laboratory, without knowledge of the initial intake of spores. In other studies laboratory reared insects have been infected with known doses of spores. In the majority of cases, it has been found that the microsporidia caused significant larval mortality and reduction of adult longevity and fecundity.

The pathogenic effects of microsporidia are mainly related to a reduction in food reserves in the fat tissue. This may occur directly by destruction of the fat cells or indirectly by interference with food

absorption. In insects, the midgut is important in digestion and absorption of food. The midgut is the primary site of infection of many microsporidian parasites and infection results in destruction of midgut cells, which become packed with spores. It is reasonable to suppose that cells in which the cytoplasm is replaced by tightly packed spores, must function inefficiently compared to healthy ones. Thus, in infected larvae there would be a reduction in the functional area of the gut, the amount of reduction depending on the degree of infection. Because of this, the amount of fat tissue would be less in infected larvae than in uninfected ones. As the adult stage in Lepidoptera is entirely dependent on fat body reserves, and in other insects, particularly in Orthoptera, reserves in the fat body must be replenished by absorbtion from the gut, it follows that the longevity of adults will be affected. As females convert a large proportion of their reserves into yolk in the formation of eggs, the effect is more noticeable than in males.

In addition, it has been proposed that microsporidia may act on the hormonal system of the host and may stimulate the production of an analogue of the insect juvenile hormone which either delays metamorphosis (Metspalu, 1976, 1980; Metspalu and Khiïesaar, 1980; Metspalu *et al.*, 1980; Khansen *et al.*, 1982) or hasten the growth of infected insects and induce supernumerary moults (Fisher and Sanborn, 1962, 1964).

2.1.2.1 Effect on development and longevity

Larvae with microsporidioses are not as vigorous and develop more slowly than healthy ones. Heavily infected larvae may not be able to survive to the adult stage and those which emerge as adults die quickly.

There are many examples, some of which are summarised as follows. European corn borers, *Ostrinia nubilalis* are susceptible to two species of microsporidia, *Nosema pyrausta* , infecting Malpighian tubules and silk glands, and *Vairimorpha necatrix* infecting fat body and haemolymph. Bacterial septicaemia is common in larvae, especially the early instars, infected with *V. necatrix*, probably as a result of gut bacteria invading the haemocoel following damage to the gut wall by the polar tubes (Pilley, 1976). Lewis *et al.* (1982) found that early instar larvae were more susceptible to *V. necatrix* than the later instars. When 1st instar larvae were exposed to infection, 51% died. However, when infection was initiated in later instars, there was lower mortality of larvae but there was a corresponding increase in mortality at the pupal stage and in the number of abnormal adults.

Zimmack et al. (1954), Zimmack and Brindley (1957) and Kramer (1959) reported that N. pyrausta infection in O. nubilalis caused reduction in the survival rate and adult longevity. Reduced longevity of adult moths after emergence from larvae infected with N. pyrausta was also reported by Van Denburgh and Burbutis (1962), and Windels et al. (1976). In experimental infections with N. pyrausta, Pezzutti and Bolchi (1977) found delayed pupation and considerably reduced adult emergence. Siegel et al. (1986b) studied the effect of transovarial transmission of N. pyrausta. They found that transovarially infected larvae experienced higher mortality than uninfected larvae and that the mortality was linked to the ovipositional sequence of the egg masses: larvae from infected egg masses laid after the 4th day of oviposition experienced greater mortality than those from infected egg masses laid during the first 2 days of oviposition.

The effects of microsporidian infection on spruce budworm, Choristoneura fumiferana have been studied by several workers. Nosema fumiferanae is a parasite of the midgut which spreads to other tissues including the fat body. As expected, young larvae are more susceptible to N. fumiferanae than older larvae (Thomson, 1955; Wilson, 1974a, 1982). The effects compared with controls are seen during larval and pupal stages as dose-dependent higher mortality (Wilson, 1974a, 1982, 1983, 1984a, 1986), retardation of larval and pupal development (Thomson, 1958b) and lowered pupal weight (Wilson, 1980; Bauer and Nordin, 1988). Wilson (1983, 1984a) compared pupal weights of C. fumiferana when infected with N. fumiferanae and another microsporidium, Pleistophora schubergi, which infects the midgut only: a low dose of $5x10^3$ spores of P. schubergi reduced only the female pupal weights but doses of $5x10^4$ spores of P. schubergi or $3x10^7$ spores of N. fumiferanae reduced the weights of both male and female pupae. Both parasites reduced the adult longevity (Thomson, 1958b; Wilson, 1980, 1983, 1984a).

Wilson (1977b, 1979, 1984b) studied the pathogenicity of 3 species of microsporidia in the forest tent caterpillar, Malacosoma disstria. These were Nosema disstriae infecting the silkglands and midgut but spreading to other tissues, P. schubergi and V. necatrix. The effects observed were dose-dependent higher mortality, malformed pupae, lowered pupal weight and reduced adult longevity.

Nosema heliothidis causes a generalised infection in most tissues of the corn earworm, Heliothis zea. It is not surprising that the effects of infection are seen as lowered activity (Lipa, 1968), longer developmental period and deformed pupae, or failure of adults to emerge (Gaugler and Brooks,

1975). Slower growth was also reported by Teakle (1977) and Darwish (1985). N. heliothidis also reduced longevity of H. zea moths (Gaugler and Brooks, 1975; Brooks and Cranford, 1978; Darwish, 1985).

Tanabe and Tamashiro (1967) found that all the larvae of cabbage looper, Trichoplusia ni, which were transovarially infected with Nosema trichoplusiae, died before completing the larval cycle. N. trichoplusiae causes generalised infections in all organs. Chu and Jaques (1979) found that a spore dose of $5x10^6$ of V. necatrix caused death of T. ni within 3-4.5 days. Fuxa (1981) reported that large spore doses of V. necatrix caused quick death and small doses caused death at pupation of T. ni, H. zea, Heliothis virescens, Pseudoplusia includens, Diatraea saccharalis and Spodoptera frugiperda. All these results emphasise the damage caused by V. necatrix to the gut of early instars which had been reported by Pilley (1976).

Some researchers studied the effects of additional feeding of microsporidian spores to naturally infected insects. Wilson (1977a, 1978) reported that, when larvae of C. fumiferana naturally infected with N. fumiferanae were fed additional spores either of N. fumiferanae or P. schubergi, there was higher mortality, significantly reduced female pupal weights and further shortening of their adult life. Lewis et al. (1983) found that additional spores of N. pyrausta or V. necatrix fed to larvae of O. nubilalis naturally infected with N. pyrausta caused high larval and pupal mortality and significantly fewer of the insects pupated and emerged in the V. necatrix and N. pyrausta treated insects than in either of the single treatment controls.

In contrast to lepidopteran insects, very little work has been done regarding the pathogenicity of microsporidia in orthopteran and coleopteran insects. Two of the species of microsporidia infecting orthopteran insect pests are Nosema locustae infecting the fat body, and Nosema cuneatum infecting the pericardium, fat body, gonads, tracheal matrix, midgut epithelium, Malpighian tubules, and neural tissues of grasshoppers. Nosema locustae prolonged the nymphal period and reduced the survival percentage of the African migratory locust, Locusta migratoria migratorioides (Canning, 1962) and of desert locust, Schistocerca gregaria (Srivastava and Bhanotar, 1986a). Henry (1971) reported that grasshopper nymphs infected with N. locustae during the 3rd instar or earlier developed slowly and usually died during the 4th or 5th instars. However, nymphs surviving from infection at the 4th and 5th instars usually developed further and persisted for prolonged

periods as adults. In contrast, Erlandson et al. (1986) reported that infection of N. cuneatum in 5th instar nymphs of Melanoplus sanguinipes reduced adult longevity. They also reported that 2nd and 3rd instar nymphs were more susceptible to the parasite than 5th instar nymphs.

The effects of Nosema whitei on Tribolium castaneum have been studied by several workers. N. whitei is a parasite of fat body. Milner (1972b) found that infected larvae grew more slowly and moulted not more than 5 times out of a normal 7 times. The mean weights of 5th moult infected larvae and of infected pupae were much lighter than those of uninfected ones. Armstrong (1978, 1979) also found that infected larvae gained less body weight than uninfected larvae. In further studies, (1982) he reported that N. whitei reduced the survival of T. castaneum by about 50%.

Brooks (1986) reported the effects of Nosema epilachnae and Nosema varivestis which generally produce systemic infections, on the adult longevity of the Mexican bean beetle, Epilachna varivestis. Both microsporidian species produced significant reductions in adult longevity although N. epilachnae was reported to be more virulent than N. varivestis.

2.1.2.2 Effect on fecundity and fertility

The influence of microsporidian infections on insect fecundity has been investigated by several workers, most of them concluding that microsporidia reduce insect fecundity. Again, examples serve to illustrate the effects.

The most notable effect of microsporidian infections on insect fecundity is the reduction in the numbers of eggs laid. Such observations have been reported for O. nubilalis infected with N. pyrausta (Zimmack et al., 1954; Zimmack and Brindley, 1957; Kramer, 1959; Van Denburgh and Burbutis, 1962; Pezzutti and Balchi, 1977; Siegel et al., 1986b); C. fumiferana infected with N. fumiferanae (Thomson, 1958b; Wilson, 1977; 1980); Hyphantria cunea infected with N. bombycis (Veber and Jasič, 1961); Pristiphora erichsonii infected with Thelohania pristiphorae (Smirnoff and Chu, 1968); T. castaneum infected with N. whitei (Milner, 1972b); H. zea infected with N. heliothidis (Gaugler and Brooks, 1975; Teakle, 1977; Darwish, 1985); E. varivestis infected with N. epilachnae or N. varivestis (Brooks, 1986). Wilson (1977a, 1978) found that feeding additional spores either of N. fumiferanae or P. schubergi to larva of C. fumiferana naturally infected with N. fumiferanae caused a substantial reduction in the number of eggs.

In general diseased female moths not only lay fewer eggs but often fail altogether to lay eggs. Microsporidian infections reduce the capability of females to lay fertile eggs and also reduce the hatching rates. Kramer (1959) found that the frequency of non producers of eggs of O. nubilalis was significantly greater in a N. pyrausta-infected group than in an uninfected group. Infected females which produced one or more egg masses were less productive than their uninfected counterparts. Van Denburgh and Burbutis (1962) found that only 52% of O. nubilalis female moths infected with N. pyrausta laid eggs, as compared with 100% of the uninfected females. Zimmack and Brindley (1957) reported that 4 eggs in 160 were infertile in eggs laid by N. pyrausta-infected females of O. nubilalis whereas only 1 egg in 301 was infertile of those laid by uninfected females. Diseased females laying higher numbers of infertile eggs and exhibiting lower egg hatching rates were also reported by Zimmack et al (1954), Splittstoesser and McEwen (1968), Pezzutti and Bolchi (1977), Wilson (1980), Srivastava and Bhanotar (1986b) and Mercer and Wigley (1987).

Some workers have studied the effect of level of infections on insect fecundity. Tanabe and Tamashiro (1967) found that the reproductive capacity of T. ni infected with N. trichoplusiae varied with the intensity of infection in the larval stage. Adults from heavily infected T. ni larvae laid a quarter to one sixth as many eggs as uninfected adults and the viability of these eggs was very low. Darwish (1985) found a strong correlation between the spore loads of N. heliothidis in H. zea males and the percentage of egg hatching from that mating, i.e. the higher the spore load in the males the lower the percentage of eggs hatching from that mating.

Some research workers have reported that microsporidia-infected insects mated less frequently and showed false mating and reduced ovipositional periods. Gaugler and Brooks (1975) reported that N. heliothidis reduced the mating success by 16.2% and 23% in the per os- and transovarian-infected H. zea respectively. They also reported that the average ovipositional period of transovarian- and per os-infected females was 7.7 and 8.9 days, respectively, in contrast to 10.2 days for the uninfected controls. Srivastava and Bhanotar (1986b) found that infection of N. locustae caused false matings and reduced the mating period to 1 h from 3 - 4 h in S. gregaria. Malone (1987) reported that Microsporidium itiiti reduced the fecundity of Argentine stem weevils, Listronotus bonariensis by shortening the period of egg-laying activity by 6

weeks. Armstrong and Bass (1986) reported that N. whitei-infected T. castaneum beetles mated less frequently than uninfected beetles and infected adults had significantly fewer progeny than uninfected ones. Reduced mating success of S. cordalis infected with Nosema sp. was also observed by Mercer and Wigley (1987).

The fecundity is not only affected when both mating partners are infected but also when only one partner is infected. Fournier and Etienne (1981) reported that infection of females of Chilo sacchariphagus with a microsporidium, Nosema sp. reduced potential fecundity, oviposition, and fertility, while infection in males reduced mating and eggs fertility. Briese and Milner (1986) found that severe infection of females of Anaitis efformata with P. schubergi reduced the fecundity and mating success, while infection in males reduced mating success only.

2.1.2.3 Effect on laboratory insect colonies

Because numerous insects including those discussed above are serious pests in agriculture and forestry, many species have been established in laboratory colonies for basic research. These insects have been reared for a wide variety of purposes: for the production of patasitoids, predators and pathogens for biological control of pests, for basic studies on insect physiology and behaviour and for teaching. Unfortunately microsporidian infections have appeared in these colonies and have built up to epizootic proportions under the confined and often overcrowded rearing conditions. Sometimes entire colonies have to be destroyed but even at levels where colonies can be maintained for long periods, the infections will interfere with the results of laboratory experiments. Some examples are cited below.

Two microsporidian parasites, Nosema destructor amd Pleistophora californica have been reported from laboratory colonies of potato tuberworm, Gnorimoschema operculella (Steinhaus and Hughes, 1949). While propagating a hymenopterous parasitoid Macrocentrus ancylivorus on the potato tuberworm, Allen and Brunson (1945) found that most of the parasitoids were infected with a microsporidian parasite, probably N. destructor. In 1947 they found that the microsporidian parasite infected not only the hymenopterous parasitoid but also the host tuberworm. Finney et al. (1947) reported that infection of the microsporidian parasite in the insectary caused the emergence of tuberworm larvae to drop almost 50% and the M. ancylivorus emergence to

drop to 65 - 70% of the normal emergence. Additionally, 45% more tuberworm larvae infected with the microsporidian parasite remained within the potatoes.

A microsporidian parasite of the genus Nosema, which greatly interfered with the mass rearing of the boll weevil Anthonomus grandis, was reported by Gast (1966). Up to and including the F_7 generation, mortality among the weevils averaged less than 10% but, in the F_8 generation, egg production fell from 12,000 eggs/day to 70 eggs/day and more than 95% of the adult weevils died. The colony was finally discarded. Flint et al. (1972) reported another microsporidian species designated as "1069" from laboratory colonies of A. grandis. They found that this microsporidian parasite often occurred in less than 10% of the weevils in the colonies without causing symptoms of disease. However, during epizootics, the proportion of infected weevils reached 80 - 100% and resulted in reduced oviposition. McLaughlin (1969) reported a microsporidian parasite, Glugea gasti which also interfered in the rearing schedules of A. grandis.

Infections with a species of Nosema have been reported in several species of fruit flies, such as the Mediterranean fruit fly, Ceratitis capitata, the Oriental fruit fly, Dacus dorsalis and the melon fly, Dacus cucurbitae, which are maintained in laboratory colonies. In the melon fly, the disease became epizootic on several occasions and decimated the laboratory stock of this fly. The disease was troublesome for several years in the USDA rearing laboratory (Kamasaki, 1970). Finney (1956) also faced serious problems with rearing the melon fly because of infection with Nosema and finally, the entire stocks were wiped out by the disease.

Steinhaus and Marsh (1962) recorded Nosema sp. from laboratory colonies of Heliothis zea. The Nosema disease reduced the egg hatchability in laboratory cultures of the host insect (Snow et al., 1970). Steinhaus and Marsh (1962) also reported the microsporidian infections in the laboratory colonies of Ostrinia nubilalis, Pieris brassicae, Melanoplus bivittatus, Melanoplus dawsoni, Melanoplus mexicanus, Tribolium castaneum and Tribolium confusum.

Unwanted microsporidian infections in laboratory experiments have sometimes hampered research on the hosts. During investigating feeding of the cinabar moth, Hypocrita jacobaeae, Bucher and Harris (1961) found that the larvae suffered heavy mortality from a microsporidian disease, probably caused by Nosema cerasivoranae or Nosema bombycis. Also experiments on the

behavioural and anatomical responses of the convergent lady beetle, Hippodamia convergens to parasitism by Perilitus coccinellae, Sluss (1968) found that a considerable number of parasitoid larvae failed to complete their development due to disease. Diseased parasitoid larvae died in all stages of development, most succumbed in the last larval instar or in the pupal stage. Occasionally small, very weak and short lived adults emerged from the pupa. Microscopic examination of dead or morabund P. coccinellae confirmed that they were infected with a microsporidian parasite, probably by Nosema hippodamiae. He recorded 10 - 20% in the parasitoid and about 50% in the host insect were infected with the microsporidian parasite.

2.1.3 Effect of microsporidia in Crustacea

Among the most obvious and most harmful diseases of Crustacea are those caused by microsporidia. The peculiar chalky-white or abnormal colour changes resulting from infection make the infected individuals commercially valueless. Numerous microsporidia attributed to 10 genera have been recorded from decapod Crustacea (Sprague, 1977; Vivarès et al. 1977a; Azevedo, 1987). Among invertebrates, Crustacea are probably the second most common hosts, next to insects and several economically important members such as shrimps, prawns, crayfishes and crabs are reported infected with microsporidia.

Some examples of infections in shrimps and prawns serve to illustrate the different sites of infection and variations in the overt effects of different microsporidia in these hosts. Baxter et al. (1970) reported a microsporidium of the genus Pleistophora from white shrimp, Penaeus setiferus and brown shrimp, Penaeus aztecus. The parasite invaded the hepatopancreas, cardiac muscle and intestinal tract of these shrimps. In addition, Sprague and Couch (1971), in their list of protozoan parasites of decapods, mentioned two more species of Pleistophora from shrimps. These were: Pleistophora miyairii from the digestive tract of the shrimp, Atyephira sp. and Pleistophora sp. from the muscles of the grass shrimp, Palaemonetes pugio. Street and Sprague (1974) named the latter species as Pleistophora lintoni. The pathogenicity of the microsporidian parasite, Pleistophora crangoni in three species of crangonid sand shrimps, Crangon franciscorum, Crangon nigricauda and Crangon stylirostris, was described by Breed and Olson (1977). They found that, although the microsporidian infections were limited to the skeletal muscle of the host, they exerted a marked impact on the sex ratio and on the biology of

infected shrimps. Ovaries of infected shrimps did not develop beyond a very early stage and shifted the sex ratio towards the female by interfering with the secretory activities of the androgenic gland. Infected shrimps were unusally large and grew faster or lived longer than uninfected shrimps.

Kelly (1979) described the pathology of pink shrimp, Penaeus duorarum infected with the microsporidians Thelohania duorara, Agmasoma penaei and Pleistophora sp.. Infections of T. duorara were widespread in most tissues; spores were located throughout the haemocoel, at the periphery of all striated muscle bundles, and in muscle and connective tissue surrounding the digestive tract. Agmasoma penaei infections invaded only dorsal abdominal muscles, muscles adjacent to blood vessels, and ovaries. Infected muscles and ovaries were eventually completely destroyed. Masses of A. penaei spores were often engulfed by haemocytes. Pleistophora sp. infected the interior of all striated muscles. Infected muscles were never completely destroyed but were often atrophied. Microsporidian epizootics in two species of fairy shrimps, Branchinecta gigas and Branchinecta mackini caused by Nosema sp. were reported by Dabron (1976).

Significant mortalities of dwarf crayfishes caused by microsporidian infection were reported by Sogandares-Bernal (1962). These were caused by two microsporidian species: Thelohania sp. from Cambarellus shufeldti, and Plistophora sp. from Cambarellus puer. The latter species was identified by Sprague (1966) and named as Plistophora sogandaresi.

Sprague (1977) listed as many as 11 microsporidia from crabs and three additional microsporidia have been reported since. Vivarès et al. (1977a) described Ormieresia carcini from Carcinus mediterraneus, Azevedo (1987) described Abelspora portucalensis from Carcinus maenas, and Vivarès and Azevedo (1988) described Ameson atlanticum from Cancer pagurus. Again, the sites of infection and general pathogenicity were described for each species. Also infections in crabs have been used to analyse the effects of microsporidia on the physiology of the hosts. Vivarès et al. (1977b) observed differences in the zymograms of healthy crabs, Carcinus mediterraneus, and those of crabs parasitized by the muscular microsporidia Thelohania maenadis. The zymograms of the parasitized crabs showed an increase in chymotrypsin activity and variations in the zones of esterase activity. Vivarès and Cuq (1981) also reported physiological and metabolic variations in C. mediterraneus parasitized in the muscles by T. maenadis. They found that in

parasitized crabs, total protein and glucose levels in haemolymph, as well as muscular glycogen, were significantly reduced. Muscle lactate concentration was substantially increased, whereas lactate dehydrogenase activity was reduced by almost one-half.

The need to prevent microsporidian infections in Crustacea is especially important because most of the Crustacea are used as food in different parts of the world. Furthermore, the rapid expansion of aquaculture has provided environmental conditions which favour transmission of microsporidia among crustaceans.

2.1.4 Effect of microsporidia on vertebrates

Microsporidia infect host species in all five classes of vertebrates. Much of the works devoted to microsporidiosis of vertebrates is limited to taxonomic and biological studies of the parasites. However, some important investigations have been carried out on the pathogenicity of several microsporidia in fish. Fish are the commonest hosts among vertebrates and serious pathogenic conditions both in marine and freshwater fishes have been reported. About 70 species of microsporidia belonging to 11 genera and a further 12 species assigned temporarily to the collective genus Microsporidium have been reported as parasites of fish. About 30 others have been recorded in fish but not named (Canning and Lom, 1986).

Some examples serve to illustrate the serious effects of microsporidia may have on fish populations. Legault and Delisle (1967) reported vast mortalities of rainbow smelt, Osmerus eperlanus mordax due to Glugea hertwigi infection in Canadian lakes. Fatal infections of plaice, Pleuronectes platessa due to Glugea stephani were also described by McVicar (1975). Lom (1976) reported that invasions by microsporidia caused growth retardation and dysfunction, deformation or complete destruction of vital body organs, often ended in mortality. Matthews and Matthews (1980) found that the swimming efficiency of turbot, Scophthalmus maximus was imparied by severe infections of Tetramicra brevifilum leading to lowered growth rate and increased mortality in wild populations due to predation and starvation. A microsporidian epizootic of the golden shiner, Notemigonus crysoleucas due to Plistophora ovariae infection was reported by Summerfelt and Warner (1970), and Michel et al. (1989) reported that Heterosporis finki completely destroyed a stock of angel fish, Pterophyllum scalare.

A few species of microsporidia have been recorded from amphibia, reptiles and birds. Whether infections are less common in these hosts or have simply not been looked for is not known.

One species, Encephalitozoon cuniculi has a wide host range among mammals, reported hosts are: rabbits, mice, rats, guinea pigs, hamsters, shrews, goats, sheep, swine, horse, dog, fox, pole cat, suricats, cats, leopard, lion, monkey, baboon and man (Canning and Lom, 1986). These authors also listed seven other species infecting mammals. Torres (1927) was the first to report a microsporidian infection in man but this diagnosis has never been confirmed. However, several species of microsporidia have been implicated as agents of human diseases. To date, four genera of microsporidia identified as Encephalitozoon, Nosema, Enterocytozoon and Pleistophora have been reported in humans (Shadduck and Greeley, 1989). Numerous infections have been diagnosed in patients with AIDS (Modigliani et al., 1985, Desportes et al., 1985; Terada et al., 1987; Curry et al., 1988; Orenstein et al., 1990; Yee et al., 1991). Apart from, Encephalitozoon hellem which causes keratoconjunctivitis in AIDS patients, infections in ocular tissue by micropsoridia have been reported several times (Ashton and Wirasinha, 1973; Pinnolis et al., 1981).

Enterocytozoon bieneusi, which was first reported by Desportes et al. (1985), is apparently unique to humans and causes diarrhoea by infecting the small intestine. Eeftinck Schattenkerk et al. (1991) reported that 27% of patients infected with HIV-1 who suffered unexplained diarrhoea were found to be infected with Enterocytozoon bieneusi when examined carefully by small intestinal biopsy. The presence of E. bieneusi in small intestinal biopsy samples from AIDS patients with chronic diarrhoea were also reported by Rijpstra et al. (1988) and Orenstein et al. (1990).

Effective chemotherapy for microsporidiosis in vertebrates is badly needed for several reasons: (1) microsporidia are a serious threat to fish farming and with the increasing development of aquaculture, their effects will be more widespread and ruinous, (2) many animals used for experimental research carry latent infection which may interfere with the experimental results, (3) finally, immunosuppressive diseases such as AIDS have uncovered serious microsporidial infections in man for which treatment is urgently required.

2.2 BIOLOGY AND DEVELOPMENT OF MICROSPORIDIA

2.2.1 General development

Microsporidia are small unicellular organisms, all of which are obligate intracellular parasites. They generally complete their life cycles in a single host. Two phases of development are recognised: merogony also known as schizogony, which is the phase of proliferation, and sporogony which culminates in the production of sporoblasts, which undergo morphogenesis into highly characteristic spores.

Certain features of microsporidia, such as the absence of mitochondria and the presence of prokaryotic-like ribosomes, as well as unique structures such as the polar tube make them distinctive among eukaryotic organisms. These features are important because drugs selected to act at the level of these characters would be harmless to the host cells.

Spores

Microsporidian spores are a variety of shapes with sizes ranging from 1μm to 20μm. However, they are unique in being Gram-positive and in having three basic components: the spore wall, the extrusion apparatus and the sporoplasm.

The spore wall consists of two layers: an outer amorphous electron-dense layer, the exospore and an inner electron transparent middle layer, the endospore. Within the wall the plasma membrane surrounds the cytoplasmic structures and nucleus (sometimes two nuclei). In the endospore, the presence of chitin, an extremely non-reactive compound renders the spores very resistant.

The extrusion apparatus consists of the polar tube with its anchoring disc or polar sac, the polaroplast and the posterior vacuole. The polar tube is a thread-like structure attached to the centre of an umbrella-shape organelle located at the apex of the spore. This anchoring disc for the polar tube is called the polar sac. The polar tube runs an oblique course backward narrowing slightly in diameter along its length, and forms a coil in the peripheral layers of the cytoplasm. In some genera there is an abrupt change of diameter in the tube about half way along its length. The polaroplast is a membranous structure surrounding the straight part of the polar tube. Near the posterior pole of the mature spore is the posterior vacuole.

The remaining space within the spore wall is occupied by relatively undifferentiated cytoplasm containing one or two nuclei, some rough endoplasmic reticulum and free ribosomes. This is the infective agent or sporoplasm.

Meronts

Meronts, which develop from the sporoplasm are rounded, irregular or elongate cells. The cytoplasm contains one nucleus or a diplokaryotic pair of nuclei. There is some rough and smooth endoplasmic reticulum and the plasma membrane is simple. Meronts divide repeatedly by binary or multiple fission or by plasmotomy.

Sporonts

The internal organisation of sporonts may not be very different from that of meronts, except in the extent of development of endoplasmic reticulum. Sporonts are usually distinguished from meronts by the presence of an electron-dense surface coat secreted on to the plasma membrane during transition from merogony to sporogony. Nuclei may again be isolated or diplokaryotic. The products of sporogonic division are called sporoblasts. Sporonts may divide directly into sporoblasts by binary fission or become multinucleate stages and pass through a multiple or sequential series of divisions. The spores are either packaged within sporophorous vesicles, or are dispersed freely in the host cell cytoplasm.

Sporoblasts

Sporoblasts, the intermediary stage of sporonts and spores, are distinguished from other developmental stages by their ovoid shape and still greater development of smooth and rough endoplasmic reticulum. Their development is a process of maturation into spores, i.e. synthesis of spore organelles.

2.2.2 Special features of microsporidian cells

Microsporidia are eukaryotic organisms with a classically organized nucleus endowed with a nuclear envelope, but some of its peculiar characteristics such as lack of mitochondria, as well as the presence of a primitive Golgi apparatus and prokaryotic-like ribosomal structure and nucleotide

sequence suggest that microsporidia are intermediate between prokaryotes and eukaryotes. There are no reserve substances in microsporidia nor structures comparable to kinetosomes, peroxisomes or typical lysosomes.

Microsporidia are unusual in lacking mitochondria at all stages of development (Vávra, 1965) and this fact has been confirmed by all investigators of microsporidian fine structure.

The presence of a Golgi apparatus in microsporidian cells was first reported by Vávra (1965) and he used the term "primitive" because the structure had little resemblance to the stacks of flattened saccules of typical Golgi. Since then, several investigators have reported the presence of Golgi vesicles in microsporidian developmental stages (Vávra, 1968; Milner, 1972; Colley et al. 1975; Vivarès and Azevedo, 1988; Larsson, 1989). Vávra (1976) has reported several groups of Golgi vesicles but some other authors have failed to observe any vesicles which could be interpreted as Golgi (Lom and Corliss, 1967; Sprague and Vernick, 1968). There is a single record of Golgi vesicles in mature spores (Gassouma and Ellis, 1973). Freshly extruded sporoplasms also contain no trace of this organelle (Ishihara, 1968; Weidner, 1972; Wiedner and Trager, 1973).

In Metchnikovella sp., the Golgi vesicles are closely associated with the spindle plaques (Hildebrand, 1974) and increase in number during plasmotomy (Vivier and Schrevel, 1973). In meronts of N. apis, the Golgi complexes are located at opposite poles of the nucleus or lie adjacent to each nucleus of the diplokaryon. In multinucleate meronts of this species, the Golgi vesicles are more developed than in earlier stages in the sense that vesicles are joined to form a tubular network closely associated with channels of endoplasmic reticulum (Youssef and Hammond, 1971). In Hrabyeia xerkophora, a Golgi-like reticulum was found located near the nuclei of the sporont (Lom and Dyková, 1990).

The role of the Golgi apparatus in microsporidian cells has been studied by several workers, all of them concluding that it is involved in the formation of various spore organelles during spore morphogenesis. Only exceptionally has the Golgi apparatus been implicated in the synthesis of the surface coat of sporonts as suggested for Unikaryon legeri (Canning and Nicholas, 1974). Development of the polar tube from the Golgi complex has been reported by several investigators (Vávra, 1965; Devauchelle et al. 1970; Youssef and Hammond, 1971; Jensen and Wellings, 1972; Liu and Davies, 1972;

Walker and Hinsch, 1972; Vivarès, 1980; Durfort and Vallmitjana, 1982). Sprague and Vernick (1969) believed that the Golgi apparatus of Nosema nelsoni was directly transformed into the polaroplast complex, certain membranous investments of the polar filament, the polar sac and perhaps part of the posterior vacuolar system. Canning and Sinden (1973) also observed that Golgi apparatus might be responsible for the development of the polar filament, the polaroplast membranes and perhaps the polar sac in N. algerae. Street and Henry (1987) reported that the Golgi apparatus is apparently responsible for polar cap and polar tube formation in N. cuneatum.

Ribosomes are found in all stages of microsporidia which are either attached to the endoplasmic reticulum or are freely dispersed in the cytoplasm. Polyribosomes arranged in tight spirals sometimes occur in young spores (Canning and Sinden, 1973; Canning and Nicholas, 1974; Azevedo, 1987; Azevedo and Canning, 1987), but the physico-chemical properties of microsporidian ribosomes indicate that they are prokaryotic in nature.

Ishihara and Hayashi (1968) isolated ribosomes from sporoplasms of N. bombycis and found that there were only 70S monoribosomes with two subunits of 50S and 30S. These values are similar to those of prokaryotic ribosomes. Curgy et al. (1980) further confirmed the prokaryotic nature of microsporidian ribosomes by reporting that the ribosomal RNAs of the species of microsporidia, Thelohania maenadis and Inodosporus sp. had sedimentation coefficients of 23S and 16S for the large and small subunits respectively. Recent sequence data on V. necatrix has revealed even closer prokaryotic characters; the sequence of the 5.8S rRNA, which forms a separate molecule in most eukaroytes, is incorporated into the 23S rRNA (Vossbrinck and Woese, 1986) and the 16S rRNA shows little sequence homology with small subunit rRNA of a range of eukaryotes (Vossbrinck et al., 1987). The sequence differences indicated that microsporidia probably arose as an early branch of the line leading from the prokaryotes to the eukaryotes (Vossbrinck et al., 1987).

Microtubules have been observed in the nucleus of many microsporidia, e.g. N. bombycis (Ishihara, 1970), N. apis (Youssef and Hammond, 1971), N. algerae (Canning and Sinden, 1973), Metchnikovella sp. (Vivier and Schrevel, 1973), Nosema herpobdellae (Spelling and Young, 1983), Abelspora portucalensis (Azevedo, 1987) and N. cuneatum (Street and Henry, 1987).

Another interesting feature of microsporidian structure is the cell limiting membrane. Usually its configuration changes with the advancement of

the developmental stages. The sporoplasm and meront are limited by a thin single unit membrane which becomes overlain by an electron dense surface coat in sporogony. The electron dense coat becomes the exospore layer of the spore wall, after interpolation of the electron lucent endospore between it and the plasma membrane. Only exceptionally has the meront membrane been reported to have a more complicated structure. Milner (1972a) observed a multi-layered outer coat in N. whitei and Canning and Sinden (1973) found that the plasma membrane of N. algerae was overlain by a thin layer of electron-dense amorphous material. Modifications in the exospore of the spore wall have been reported by several researchers. Multiple layers have been distinguished in the exospore: four or five layers in N. locustae (Huger, 1960); three layers in Plistophora hyphessobryconis (Lom and Corliss, 1967) and two dense layers separated by a transparent one in Nosema spelotremae (Stanier et al., 1968). Furthermore, some researchers reported on the presence of an additional coat surrounding a complicated multi-layered exospore. Larsson (1986) reported that the multi-layered exospore of Episeptum inversum was surrounded by a thick reticulated or chambered translucent coat. In 1989, he further reported that the spore of Jirovecia involuta was enclosed by a 10nm thick double layered envelope. Voronin (1989) found that the complicated exospore of Lanatospora macrocyclopis had two electron-dense layers and a woolskin-like coat. The exospore of Hrabyeia xerkophora is covered by a glycocalyx-like layer of 21 - 35nm thick (Lom and Dyková, 1990).

2.2.3 Life cycle of the microsporidium used in this study

The microsporidium Nosema bombycis isolated from the lawn grass cutworm, Spodoptera depravata in Japan, is a strain of the type species, Nosema bombycis Naegeli, 1857. The life cycle of this strain was studied by Iwano and Ishihara (1988) in Antheraea eucalypti cell cultures. They found that sporoplasms released in culture cells just after inoculation grew into schizonts 12 h post-inoculation. The number of schizonts increased during the first 24 h after inoculation and at the same time, some of the parasites entered into sporogony. Sporonts divided into two sporoblasts and spores began to appear 30 h after inoculation. Spores had two compact nuclei, cytoplasm of high electron density, a polar filament and a thick wall. However, they observed two types of spores developing in the cell cultures, which they maintained for many weeks after inoculation. The first spores which developed

36 h post-inoculation were larger and had a smooth, thin wall, inside of which the contents were poorly preserved. At 72 h spores were smaller, with a thicker wall and the nuclei were hard to differentiate. They advanced their studies in 1989 and 1991, when they again observed two types of spore in the same cell culture system. The spores formed in cultured cells 36 h post-inoculation had a polar tube coil of 3-5 turns under a thin spore wall, while spores from cultured cells 72 h post-inoculation had a coil 10-12 turns under a thick wall. They speculated that the first type of spore possibly germinates in cultured cells immediately after formation, while the second type required conditioning for germination. The first type could serve for autoinfection in the same host as that in which it was produced while the second type could serve for transmission between hosts.

2.3 CHEMOTHERAPY OF MICROSPORIDIOSIS

The word 'chemotherapy' was introduced by the famous German pharmacologist, Paul Ehrlich to mean the killing of obnoxious organisms residing inside a host, by administration of drugs to the host. The study of chemotherapy of microsporidiosis in insects began in the early part of the 20th century. At the beginning, chemotherapy was limited to the control of microsporidiosis of beneficial insects, particularly of Nosema disease of honey bees. As the importance of microsporidia increased and its importance was not limited to beneficial insects but also to other insects, other invertebrates and vertebrates, the field of chemotherapy was extended to these sections as well. To date numerous drugs have been tested against microsporidia both in vitro and in vivo, but none has completely eradicated the diseases although a few have given partial success. Many of the drugs are also toxic to the hosts.

2.3.1 Chemotherapy of microsporidiosis in beneficial insects

Most of the research on chemotherapy of microsporidiosis has been conducted on Nosema disease in honey bees but there have been a few studies on silkworms. White (1919) reported that betanaphthol and salol were effective in controlling Nosema disease of honey bees when fed to bees in diluted honey, but that eucalyptus and quinine were not (quoted by Katznelson and Jamieson, 1952a). Numerous other substances, including antibiotics, sulphadrugs, arsenicals and anti-protozoan agents, were investigated without finding a drug with significant action (Haller, 1948; Palmer-Jones, 1949). In view of these failures to control the disease, the announcement of McCowen et al. (1951) of the striking amoebicidal action of a new antibiotic, fumagillin, isolated from Aspergillus fumigatus cultures by Hanson and Eble (1949), aroused great interest amongst microsporidiologists. Accordingly, Katznelson and Jamieson (1952a) carried out experiments with fumagillin against N. apis in honey bees and found marked anti-microsporidial action of the compound. Subsequently, the anti-microsporidial properties of fumagillin against N. apis were confirmed in other studies (Katznelson and Jamieson, 1952b; Bailey, 1953a).

In general, results of tests with fumagillin against N. apis in both laboratory and field trials have fallen short of complete eradication although a few workers have claimed that it eliminates Nosema infection from honey bees.

Katznelson et al. (1955) reported that concentrations of fumagillin as low as 8.3mg/l reduced the percentage infection to 3%, compared to 93% in the controls at 24 days. Furgala and Boch (1970) observed that fumagillin at 12.6mg/l almost completely suppressed Nosema development within 17 days. Nearly complete elimination of Nosema disease was also reported by Gochnauer and Furgala (1962), Mussen and Furgala (1975), and Sugden and Furgala (1979). Lehnert (1977) achieved complete eradication of Nosema infection from queens by feeding 26.3mg fumagillin/l for 7 days. However, he failed to eradicate the Nosema infection completely from infected worker bees with the same treatment. Moffett et al. (1969) treated bees with 20ppm fumagillin for 2 days before infection and for 14 days after infection and found that treated bees had light infections and only 18% developed Nosema disease compared with 98% in the control.

In the field trials, researchers usually treated the bee colonies with drugs in the autumn and assessed their effects the following spring. Bailey (1953b) reported that treatment with fumagillin completely eliminated the Nosema infection by February and March of the following year but infection reappeared in April. In later studies, Bailey (1955) reported that fumagillin alone could not completely eliminate the Nosema disease by the following spring, and in order to achieve complete eradication, he stressed the need to transfer the colonies to sterile combs in combination with the fumagillin treatment. Mommers (1957) found that treatment with Nosemack (a preparation of thimerosal) and Fumidil-B (a preparation of fumagillin) significantly reduced the Nosema disease in colonies of Dutch bees and confirmed that the drug did not prevent an increase in Nosema infection by the next spring. However, the increase was significantly less in drug-treated colonies than in control colonies. Furgala and Boch (1970) found that treatment with Nosemack and Humatin (paramomycin) suppressed Nosema infection only slightly, whereas Fumidil-B (fumagillin) markedly suppressed Nosema infection, and infections which persisted throughout the following year were at a very low level.

Alekseenok (1986) studied the effect of nosematol in aerosol form against N. apis in honey bees and N. bombycis in silkworms. In nosematol-treated hives, the prevalence of infection with N. apis fell by 75 - 100%, and the intensity of infection by 90 - 100% within 10 - 15 days. Nosematol reduced the infection of N. bombycis in artificially-infected 4th and 5th instar larvae of B. mori by 5 - 10% of that of the control. Atabekova (1981) fed

monomycin (500u/ml water), primaquine (0.054mg/ml), sulphapyridazine (2mg/ml) plus furazolidone (0.15mg/ml), trichopol (3mg/ml) twice daily to B. mori larvae between larval stages 3rd and 5th. She found that none of these drugs were fully effective but the best was primaquine which reduced the prevalence of imaginal infection to 4% compared to 17 - 19% in untreated controls.

Drugs which have been found ineffective in controlling Nosema disease in honey bees are summarized in Table 1.

Table 1 Name of drugs ineffective against Nosema disease of honey bees.

Name of drugs	Reference
Streptomycin, Aureomycin, Sulphametazine, Atabrine, Paludrine, Carbarsone, Emetine, Ceepryn (QAC), Chiniofon, Diodoquin, Chloroquine	Katznelson & Jamieson, 1952a
Win 5047, Vi-oxaline, Erythromycin	Katznelson et al. 1955.
Streptimidone, Protomycin, Decatetraenedioic acid	Gochnauer & Furgala, 1962
Amprolium, Anthelmycin, Buquinolate, Chlorine dioxide, Compound 356C31, Dapsone, Doxycyline, Eucalyptus oil, Hydroxychloroquine, Hygromycin, Kanamycin, Methacycline, Methenamine, Monensin, Nalidixic acid, Neomycin, Nitrofurazone, Papain, Primaquine, Pyrimethamine, Trimethoprim.	Moffett et al., 1969
Metronidazole	Moffett et al., 1969; Hitchcock, 1972
Benomyl	Mussen & Furgala, 1975
Enteroseptol	Sugden & Furgala, 1979

2.3.2 Chemotherapy of microsporidiosis in other insects

2.3.2.1 Fumagillin (Fumidil-B)

Lewis and Lynch (1970) reported that Fumidil-B at 200-12,000ppm significantly reduced the level of N. pyrausta infection in O. nubilalis larvae. Lynch and Lewis (1971) found that Fumidil-B suppressed the level of infection only while larvae were feeding on diet containing the drug, and that, when feeding ceased and the gut was emptied of diet containing the drug, the level of infection increased throughout the pupal and adult stages. As Fumidil-B failed to eliminate N. pyrausta completely from O. nubilalis in a single generation, Lewis et al. (1971) carried out an experiment in which the larvae were exposed continuously both individually and in mass to the diet containing 200-1500ppm of Fumidil-B for several successive generations. From the observations of six successive generations of individually-reared insects and from two successive generations of mass-reared insects, they reported that Fumidil-B failed to eliminate the parasite completely but kept the level of infection significantly low in all generations.

Flint et al. (1972) investigated the effect of Fumidil-B on a microsporidian infection (designated 1069) in Anthonomus grandis. A dose of 0.25% in the adult diet resulted in an average of 2% infected weevils at 2 weeks post emergence, compared with 21% infection of similar weevils fed diet without Fumidil-B. Weevils from a diseased colony fed larval and then adult diets containing 0.15% and 0.0025% Fumidil-B, respectively, were 12% diseased compared with 86% of weevils reared and maintained on untreated diets. Hsiao and Hsiao (1973) also reported that fumagillin, at concentrations as high as 20ppm suppressed the level of infection of Nosema sp. in alfalfa weevil, Hypera postica.

Some researchers concluded that Fumidil-B could completely eliminate microsporidian infections if used at high enough doses. Wilson (1974b) found that Fumidil-B at 7000ppm completely eliminated N. fumiferanae from C. fumiferana but a concentration of 500ppm only reduced the level of infection in larvae. Intermediate concentrations of 1000 and 3000ppm eliminated the parasite from the larvae but infections resurged in adults. Similarly, Armstrong (1976) reported that Fumidil-B at concentrations of 250-4000mg/l only suppressed infection of Nosema kingi in adult Drosophila willistoni but a concentration of 8000mg/l completely eliminated the parasite from the host. Badowska-Czubik et al. (1984) observed that fumagillin reduced the prevalence

of Nosema carpocapsae to 11.3% at 200ppm in codling moth, Laspeyresia pomonella and to zero at 800ppm, compared with 52.2% on an untreated diet.

Surprisingly, some investigators found that fumagillin had no effect against the microsporidia investigated. Thus Briese and Milner (1986) found that fumagillin was ineffective even to suppress P. schubergi infections in Anaitis efformata and Shinholster (1975) found the same for the drug against Nosema whitei and a coccidium (Adelina tribolii) in T. castaneum.

2.3.2.2 Benomyl

The anti-microsporidial properties of benomyl, a selective fungicide, was accidentally discovered by Hsiao and Hsiao (1973), when they tried to develop an artificial diet for laboratory rearing of the alfalfa weevil, Hypera postica. They found that benomyl at a low level (250ppm) was sufficient to eliminate Nosema sp. from the weevil when infected larvae were fed for 3 days on diet into which the drug was incorporated. Since then, a number of research workers have investigated the anti-microsporidial properties of benomyl but found that like fumagillin, it can only suppress, not eliminate, infections. Some researchers have found no effect at all.

Armstrong (1976) reported that benomyl at concentrations of 250-500 mg/l suppressed the development of N. kingi in D. willistoni. Harvey and Gaudet (1977) observed that benomyl reduced the prevalence of infection with N. fumiferanae in adults of C. fumiferana. They observed some reduction in microsporidian levels using 25ppm benomyl and that higher concentrations were more effective. However, even 250ppm failed to reduce the incidence of microsporidia below 29% in a heavily infected stock. Brooks et al. (1978) found that, even at 1000ppm, benomyl failed to eliminate the microsporidian, N. heliothidis completely from H. zea colonies. When newly hatched, transovarially infected larvae were placed on artificial diets containing 250, 500, or 1000ppm benomyl, infections were not detected in late-stage larvae but low-level infections were found in pupae and newly emerged adults. Similar observations were also made by Briese and Milner (1986) against P. schubergi in A. efformata. They found that benomyl suppressed infection as long as treatment was continued, but the microsporidian infection resurged during the non-feeding pupal stage.

Shinholster (1975) found that benomyl was active against N. whitei in T. castaneum but inactive against a coccidium, Adelina tribolii. Badowska-Czubik et al. (1984) found that benomyl had no effect against N. carpocapsae in L. pomonella.

2.3.2.3 Toltrazuril

Toltrazuril, an established anti-coccidial drug, was tested by Mehlhorn *et al.* (1988) against *Nosema* sp. in different insects. They found that when naturally infected bees (*Apis mellifera*), wasps (*Pimpla turionellae*) and flies (*Drosophila melanogaster*) were fed toltrazuril at a concentration 20µg/ml in sugar solution the parasites were killed and the hosts survived, whereas infected but non-treated insects died within three days.

In chemotherapy experiments researchers usually fed the infected larvae on drug-incorporated diet throughout their larval period either individually or in mass, or exposed a group of adults to drug-treated food for certain periods. There is no measurement of amount of food consumed by each individual or by the group treated. Thus, there was no measurement of the amount of drug required to control the disease.

2.3.3 Chemotherapy of microsporidiosis in other invertebrates and vertebrates

In contrast to the work on microsporidian infections in insects, research on chemotherapy of microsporidioses in other invertebrates and in vertebrates is limited.

Overstreet (1975) studied the chemotherapy of microsporidiosis in crabs. He found that buquinolate, an anti-coccidial drug prevented microsporidiosis caused by *Nosema michaelis* in the blue crab, *Callinectes sapidus*, while benomyl and fumagillin were ineffective. When spores of *N. michaelis* and buquinolate were administered together blue crabs were less likely to acquire spore-ridden muscle tissue than individuals not allowed the drug. Furthermore, if the crabs were given the drug for 48 h preceeding or following the introduction of spores, there was minimal prevalence of infection and even after two months, spores were not observed in the musculature of most crabs.

Kano and Fukui (1982) reported that fumagillin prevented the development of *Pleistophora* infections in eels, *Anguilla japonica*, when administered orally at 250mg/kg body weight for 30 days immediately after inoculation of spores. Infection was also prevented if eels were immersed in water containing 60.5ppm for 120 h from the 2nd day after inoculation. In their later studies Kano *et al.* (1982) made detailed observations on the effect of different doses of fumagillin. They found that when fumagillin was given to eels in their feed

at 5mg/kg/day for 60 days, 7.2-14.4mg/kg/day for 30 days, or 50mg/kg/day for 20 days the prevalence of Pleistophora was limited to 0-15%. Intermittent treatment for 20 days (5 days on, 5 days off) at 50mg/kg/day was also effective. When treatment was begun 6 days or more after inoculation it was too late. When given to symptomatic fish, fumagillin inhibited the further development of lesions, but the disease recurred about one month after treatment stopped. They also added that fumagillin helped prevent the transmission of the disease from infected to healthy fish in the same aquarium. In eels held at a low temperature (13-14°C for over 30 days) after infection, fumagillin prevented parasite development as long as it was administered before the temperature was raised. Mehlhorn et al. (1988) reported the effect of toltrazuril on Glugea spp. in naturally infected fish such as carp, trout and eel as well as ornamental fish. They found that toltrazuril at 10µg/ml killed most of the parasites within 2 - 4 h.

Recently, the occurrence of human diseases has stimulated interest in chemotherapy but no satisfactory drugs have been found. Matsubayashi et al (1959) found that a 9-year old child with Encephalitozoon sp. recovered after an illness of several weeks during which sulphonamide antibiotic treatment was given. There is no certainty that the drug played a part in his recovery. Ledford et al. (1985) reported that the myositis suffered by their patient slightly improved after administration of trimethoprim/sulfisoxazole and sulfadiazine. In contrast, Yee et al. (1991) found that trimethoprim/sulfisoxazole had no effect on the epithelial keratopathy caused by Encephalitozoon hellem in a patient with AIDS, but itraconazole in association with corneal scraping was effective and a complete resolution of symptoms and signs were achieved. Recently, Eeftinck Schattenkerk et al. (1991) reported that metronidazole was effective in controlling diarrhoea caused by microsporidial infection in HIV-1-infected individuals with pronounced cellular immune deficiency.

2.3.4 Chemotherapy of microsporidia in cell culture

Cell cultures have rarely been used to investigate chemotherapy of microsporidia. Bayne et al. (1975) exposed cell cultures derived from snails, Biomphalaria glabarata infected with Pleistophora-like microsporidia to Fumidil-B at 25 and 50µg/ml for 15 weeks and to benomyl at 25, 50 and 100µg/ml for 8 weeks but neither drug eliminated the infection from the cultures. Kurtti and Brooks (1976, 1977) added fumagillin at a concentration of 1 ppm to

cell cultures of Heliothis zea within 24 h of inoculation with Nosema disstriae and found that development of the parasite was blocked: no infections were observed as long as the fumagillin was present but infections developed within several days if the medium was replaced without fumagillin. If addition of fumagillin was delayed until three days after inoculation, infections developed but were arrested. They concluded that fumagillin was microsporidia-static not microsporidicidal.

Shadduck (1980) investigated the multiplication of Encephalitozoon cuniculi in rabbit kidney and canine kidney cell culture in the presence of fumagillin at a concentraion of 5µg/ml added to the culture medium after 4 days. He found that development was inhibited within 48 h and for as long as the fumagillin remained in the culture medium.

Greater success was reported by Sohi and Wilson (1979) who found that fumagillin at 2, 5 and 10µg/ml and benomyl at 25µg/ml permanently eliminated the microsporidium, N. disstriae within 35 days from continuous haemocyte cultures established from Malacosoma disstria. They also reported that gentamycin at 50 and 100µg/ml used for 35 days did not inhibit the development of N. disstriae.

Waller (1979) tested seven antibiotics and chemotherapeutic agents against E. cuniculi in canine kidney cell culture. These drugs were: chloroquine phosphate, chloramphenicol, dimetridazol, oxitetracycline, spiramycin, sulfonamide and sulfadoxin + trimethoprim. The most effective was chloroquine phosphate which, at a concentration of 12.5mg per 1000ml culture medium and during a test period of eight weeks, reduced the harvest of spores to 31% of that from inoculated untreated cultures.

2.4 MODE OF ACTION OF DRUGS USED AGAINST MICROSPORIDIA

2.4.1 Fumagillin

The mechanism of action of fumagillin is still incompletely understood and different opinions have been expressed. Katznelson and Jamieson (1952b) theorized that fumagillin exerted its effect upon the "early motile stages" (sporoplasms) of N. apis prior to cell invasion. Sporoplasms are now thought to be injected into host cells via the polar tube without having a free phase. Bailey (1953a) observed that the early vegetative stages (meronts) were killed by the drug. Some researchers have studied the action of fumagillin at a biochemical level. Vandermeer and Gochnauer (1971) suggested that fumagillin might

interfere with fatty acid metabolism and Hartwig and Przelecka (1971) concluded, from their cytochemical and autoradiographical studies, that fumagillin arrests DNA replication. On the other hand, Jaronski (1972) demonstrated cytochemically that fumagillin inhibits RNA synthesis using the microsporidium, Octosporea muscaedomesticae in black blow fly, Phormia regina. Liu (1973) demonstrated ultrastructural alterations in the spore membrane, especially of young spores of N. apis after treating infected bees with Fumidil-B. Shadduck (1980) also observed vesicular distortion of the plasma membrane in fumagillin-treated spores of E. cuniculi. In addition, he reported severe cytoplasmic swelling, and a marked reduction in the number of cytoplasmic ribosomes.

2.4.2 Benomyl

The mode of action of benomyl against microsporidia has yet to be investigated. There are few observations even on its gross effects on different stages of microsporidia. Hsiao and Hsiao (1973) suggested that benomyl exerted its chemical effects on sporogenesis of the Nosema sp. in H. postica while Brooks et al. (1978) reported that both merogonic and sporogonic stages of N. heliothidis in H. zea were affected by the drug.

2.4.3 Toltrazuril

The mode of action of toltrazuril was studied by Schmahl and Mehlhorn (1989) against Glugea anomala in sticklebacks, Gasterosteus aculeatus. They found that the drug was toxic to all the stages except mature spores, and the extent of damage was correlated with the dose of the drug administered. After a single treatment the drug caused significant damage to uni- or multinucleate meronts, sporogonial plasmodia, sporoblasts and immature spores. The damage mainly consisted of a decrease in the number of ribosomes, a reduction of the multinucleate meronts, disturbance in the formation of the sporophorous vesicles, general lysis of the karyoplasm and malformation of the polaroplast. After intermittent therapy the damage was intensified, and the multinucleate meronts and sporogonial plasmodia disappeared.

2.4.4 Thimerosal

Liu (1988) studied the mechanism of action of thimerosal on N. apis by exposing infected and excised honey bees midgut to the drug in vitro. He found that thimerosal was toxic to the vegetative stages but not the spores of

the parasite. The depletion of nuclear material and ribosomes as well as the decrease in electron density of the cytoplasm of sporonts and sporoblasts indicated that thimerosal might act on the nucleic acid and protein metabolism of the parasite.

2.4.5 Itraconazole

Liu and Myrick (1989) observed ultrastructural changes in the spores of N. apis after infected honey bees were treated with itraconazole. They reported that itraconazole inhibited the development of the polar filament in the spore. The drug also appeared to interfere with lipid metabolism and disrupted the integrity of the plasma membrane within the spore wall.

2.5 EFFECT OF ANTI-MICROSPORIDIAL DRUGS ON THE BIOLOGY OF INSECTS

An effective anti-microsporidial drug should be highly selective, i.e. it should be antagonistic for the microsporidia, but not for the hosts. Drugs with such properties have so far not been reported. Fumagillin and benomyl are partially effective as anti-microsporidial drugs, but the dose levels, which are needed to eliminate or suppress the parasites, are toxic to the host, affecting growth, development and fecundity.

2.5.1 Fumagillin

Lewis and Lynch (1970) reported that, even at doses of 12,000 ppm Fumidil-B did not completely eliminate N. pyrausta from O. nubilalis but at this level, the drug was toxic to the host: it increased the time to pupation and reduced the pupal weights of both male and female insects. Time to pupation was increased by 2.2 days in male and by 3.5 days in female insects. The mean pupal weights of males decreased from 68.5mg in the control to 56.3mg, and those of the females decreased from 91.9mg in the controls to 72.5mg. Lynch and Lewis (1971) also reported that O. nubilalis larvae reared on diet containing lower levels of Fumidil-B developed faster than larvae reared on the higher drug levels. Wilson (1974) observed that drug levels greater than 7000 ppm of Fumidil-B which were required to eliminate N. fumiferanae completely from C. fumiferana were toxic to the host and caused significant decrease in male and female pupal weights and increased larval and pupal mortality and developmental time. A dose of 11,000ppm reduced the male and female pupal weights by 24 and

50mg, respectively and increased the developmental time of the insects by about 9 days. He also recorded 12.5% and 12.1% larval and pupal mortality, respectively in comparison with 2.2% and 0% mortality in the controls. Thomas (1977) found that there was a survival difference between sexes of C. fumiferana when infected 2nd-instar larvae were reared with Fumidil-B incorporated into the diet. Female insects were more susceptible to the drug than male insects.

Flint et al. (1972) reported that Fumidil-B reduced the fecundity and egg hatch of A. grandis. A dose of 0.25% Fumidil-B reduced the fecundity and egg hatch to 32% and 76% of the control, respectively. Lesser amounts of 0.125%, 0.025%, and 0.0125% Fumidil-B reduced fecundity to 48%, 79% and 74% of the control values, respectively. Reduced fecundity in the females of C. fumiferana at a concentration greater than 2,709ppm Fumidil-B was reported by Thomas (1977).

2.5.2 Benomyl

Hsiao and Hsiao (1973) reported that benomyl at 250ppm in the larval diet completely eliminated the Nosema parasite from alfalfa weevil without exerting any harmful effect on the host, although they did not determine the level of drug which was toxic to the host. In contrast, Harvey and Gaudet (1977) reported that benomyl was not only ineffective in eliminating N. fumiferanae from C. fumiferana, but the fungicide had serious deleterious effects on the insect hosts. Growth and fertility of C. fumiferana were reduced when the drug was used at concentrations of 75ppm and above. The most notable effect of benomyl was the reduction of fertile matings and of percentage of eclosion of eggs. Males were more sensitive to benomyl than females. Benomyl also significantly reduced pupal weights and produced smaller adults. Livingston et al. (1978) observed that larval exposure to 3,075ppm benomyl resulted in almost total sterility of T. ni males and larval exposure to 5,536ppm caused a 40% sterility in Pseudoplusia includens males. In T. ni, fecundity was significantly lower with crosses involving treated males than those with untreated males.

3. MATERIALS AND METHODS

3.1 MICROSPORIDIUM

The microsporidium used in this study was isolated from the lawn grass cutworm, Spodoptera depravata and established in vitro in Antheraea eucalypti cells. A culture was kindly provided by Professor R. Ishihara and Mr. H. Iwano of Nihon University, Japan. The microsporidian was identified by the Japanese authors as a strain of the type species, Nosema bombycis Naegeli, 1857.

3.2 DRUGS TESTED

Twenty drugs were selected for testing against N. bombycis. The name, specification and source of the drugs are given in Table 2. Except for benzimidazole and cerulenin, the drugs were selected for testing because (a) they had shown some effect against other protozoa, (b) anti-microsporidial properties had been claimed for them previously, or (c) they were reported to act on prokaryotic ribosomes. Benzimidazole, the nucleus component of benomyl was chosen because benomyl, the well known anti-microsporidial drug was insoluble in culture medium and could not be tested in vitro. Cerulenin, an antifungal agent which interrupts the growth of fungi by affecting lipid metabolism (Nomura et al., 1972) was chosen because other antifungal agents had been effective against microsporidia in previous work and some of these affected lipid metabolism in microsporidia (Vandermeer and Gochnauer, 1971; Liu, 1973; Liu and Myrick, 1989). All the drugs except benomyl, novobiocin and streptolydigin were tested in vitro. Those drugs which had exhibited anti-microsporidial activity in vitro were considered for in vivo tests. Benomyl, novobiocin and streptolydigin, which could not be tested in vitro due to their insolubility in the culture medium, were tested in vivo. In addition, toltrazuril which had previously been reported as an anti-microsporidial drug, was also tested in vivo.

Table 2 Name, specification and source of drugs tested against *N. bombycis*

Serial No.	Name of drug	Specification	Source
1	Albendazole	B/87 D2728	Smith Kline Beecham Pharmaceuticals
2	Sinefungin	Lot 78F-0284	Sigma Chemical Co.
3	Fumagillin	Sample 6029-236-11	Abbott Laboratories
4	Thimerosal	Lot 68F-0282	Sigma Chemical Co.
5	Benzimidazole	Lot 128F-7701	Sigma Chemical Co.
6	Pentostam	Lot 22056	Dr.R.A.Neal, London School of Hygiene and Tropical Medicine, England.
7	Ciprofloxacin	Batch ER111	Bayer AG
8	Toltrazuril	Batch 500095	Bayer AG
9	Imidocarb dipropionate	QA.No.0682	Wellcome Foundation Ltd.
10	Metronidazole (RP 8823)	Batch AN27518	Rhône-Poulenc Ltd.
11	Sulphadimidine (M&B 1070)	Batch L 1326	Rhône-Poulenc Ltd.
12	Sulphapyridine (M&B 693)	Batch P654	Rhône-Poulenc Ltd.
13	Sulphathiazole (M&B 760)	Batch RD 12333	Rhône-Poulenc Ltd.
14	Rimocidin sulphate	Lot 6638-211	Pfizer Inc.
15	Itraconazole	Batch A091	Janssen Biochimica
16	Cerulenin	-	Professor Satoshi Ōmura, Kitasato Institute, Japan.
17	Compound 566C80	Ref. XRF90-437	Wellcome Foundation Ltd.
18	Benomyl	CAS NO 17804-35-2	Du Pont (U.K.) Ltd.
19	Novobiocin	Lot 873CC	Upjohn Company
20	Streptolydigin	Lot 1400x42-A	Upjohn Company

3.3 IN VITRO EXPERIMENTS

3.3.1 Maintenance of the insect cell line

The cell line of the fall armyworm, Spodoptera frugiperda was kindly supplied by Dr. R. Possee, NERC, Institute of Virology, Oxford, England. The cells were grown as monolayers in $25cm^2$ disposable plastic tissue culture flasks (sterilin) containing 5ml TC-100 medium (Gibco). The growth medium was TC-100 with 10% heat-inactivated foetal calf serum (FCS) (Gibco) and the maintenance medium was TC-100 with 5% FCS. Penicillin, streptomycin (Gibco) and kanamycin (Sigma) were supplemented to both growth and maintenance media at concentrations of 100 i.u., 100µg and 100µg per ml, respectively.

Cultures were incubated at $25^{\circ}C$ and were subcultured every 5-7 days. To subculture the monolayers, cells were dislodged from the bottom of the culture flask by gentle flushing with a pasteur pipette and the medium containing the free cells was centrifuged in universal tubes at 150g for 5 min. The supernatant was discarded and the cell pellet was resuspended into 5ml fresh growth medium. From this cell suspension, 1-2 drops were transferred, using a pasteur pipette, into each replicate culture flask containing 5ml of fresh growth medium.

3.3.2 Infection of S. frugiperda cell culture

The Antheraea eucalypti cell line infected with N. bombycis was used as the source of parasitized cells. The A. eucalypti cells were propagated at $25^{\circ}C$ in $25cm^2$ disposable plastic tissue culture flasks containing 5ml Grace's medium (Gibco) supplemented with 10% FCS, 100 i.u. penicillin/ml, 100µg streptomycin/ml and 100µg kanamycin/ml. These cells are slightly larger and more elongated than the cells of S. frugiperda and grow suspended in the medium.

To infect the S. frugiperda cell cultures, 1ml suspension of infected A. eucalypti cells was added to 2ml suspension of uninfected S. frugiperda cells and the mixture was centrifuged at 150g for 5 min. The pellet was resuspended in 3ml of fresh growth medium and 1ml of this suspension was introduced into each culture flask containing 4ml of fresh growth medium. Incubation was at $25^{\circ}C$.

Since the A. eucalypti cells always float in the medium, they were removed when the medium was changed. Furthermore, the growth of A. eucalypti cells was very poor in TC-100 medium. Thus any residual cells were outgrown in

the subcultures. In this manner N. bombycis infected S. frugiperda cell culture was achieved without re-introducing the A. eucalypti cells and was maintained for more than 1.5 years.

3.3.3 Maintenance of microsporidia in S. frugiperda cell cultures

To maintain the parasitized S. frugiperda cell cultures, the spent medium was changed weekly and replaced with fresh maintenance medium. When the culture became heavily infected, and only a few healthy cells remained, subculture was carried out. For subculture, the cells were loosened from the bottom of the flask by gentle flushing with a pasteur pipette and 1ml of the suspension was mixed with 3 - 5ml of a suspension of cells from uninfected cultures. The mixed cell suspension was centrifuged at 150g for 5 min, the pellet was resuspended in 5ml fresh growth medium, and 1ml aliquots were seeded into each replicate culture flask with 4ml of fresh growth medium.

3.3.4 Preparation of cell cultures for drug testing

For drug testing, infected cells and uninfected cells were mixed 1:10 and 1ml, containing about 1×10^5 cells was placed into each well of a 24-well tissue culture plate (Nunc). It was found that S. frugiperda cells would not adhere to glass coverslips placed in the tissue culture trays. Growth on coverslips would have facilitated examination of the effects of drugs. In order to prevent the cells adhering to the plastic base of the wells, glass coverslips were inserted, so that the cells remained in suspension. Cells were allowed to grow for 3 - 5 days in different experiments.

3.3.5 Preparation of drugs

All the drugs were obtained in powdered form except ciprofloxacin which was obtained as a sterile solution from which a series of concentrations were prepared in maintenance medium.

Powdered drugs were dissolved in specific solvents. The minimum amount of solvent was used just to dissolve the drug. These drug solutions were further diluted in TC-100 medium and sterilized by passing through 0.22µm millipore membrane filters. From these stock, desired concentrations were made up by dilution in maintenance medium.

In the case of albendazole, toltrazuril, itraconazole and compound 566C80, the drugs dissolved in solvent precipitated out when added to medium

and were partially redissolved by sonication. The highest concentrations achieved in the maintenance medium were 5.3, 29.94, 5.9 and 11.88 µg/ml for albendazole, toltrazuril, itraconazole and compound 566C80, respectively. Lower concentrations of these drugs were prepared as before by further dilution in maintenance medium. As relatively large amounts of solvent were needed to dissolve benzimidazole, pentostam, metronidazole and sulphadimidine, these drug-solvent solutions were sterilized directly without further dilution in medium. From this stock, desired concentrations were made up in maintenance medium. In each experiment the amount of solvent was kept constant in all the concentrations of drugs including the control.

A list of the chemical solvents used to dissolve the drugs and the amounts used are given in Table 3.

Table 3 Name of solvent used to dissolve the drug and amount of solvent used in the experiment

Serial No.	Name of drug	Name of solvent	Amount of solvent used
1	Albendazole	Dimethyl sulfoxide (DMSO)	0.02%
2	Toltrazuril	DMSO	0.06%
3	Rimocidin sulphate	DMSO	0.2%
4	Itraconazole	DMSO	0.05%
5	Compound 566C80	DMSO	0.09%
6	Sulphapyridine	2N NaOH	0.5%
7	Sulphathiazole	Hydrochloric acid	5%
8	Cerulenin	Absolute ethanol	Expt.1 0.2% Expt.2 0.02%

3.3.6 Assays for drug activity

3.3.6.1 Single drug treatment

N. bombycis infected *S. frugiperda* cell cultures prepared according to section 3.3.4 were exposed to medium containing drugs for different periods. The spent medium was removed carefully from the wells of the tissue culture plates and was replaced immediately by 0.5ml of fresh medium containing the drug. The spent medium was centrifuged at 150g for 5 min. The supernatant was discarded, and the pellet was resuspended in 0.5ml of drug-

treated medium which was returned to the original culture well. Thus, each culture well received 1ml of medium containing drug and infected cells. As controls, infected cultures were exposed to maintenance medium containing the same amount of solvent used in the drug treatments. There were 2 to 3 replicates for each treatment and the medium was renewed after 3 days by centrifuging the cells as before. Uninfected cell cultures were also included in the experiments to assess the effects of drug and solvent on host cells. Usually cultures were exposed to the drug up to 7 days.

3.3.6.2. Combined drug treatment.

An experiment was conducted to assess the combined effects of pairs of drugs on N. bombycis in S. frugiperda cell culture. For this purpose, 4 drugs, sinefungin (100µg/ml), albendazole (2.5µg/ml), benzimidazole (250µg/ml) and fumagillin (5µg/ml) were considered. Initially, drugs were made up individually as before at twice the desired concentrations, then the drugs were mixed 1:1. Cultures, prepared as in section 3.3.4, were exposed to the combined drugs for 7 days. The drug treatment protocol was as with the single drug treatment. There were two replicates for each treatment. Control (infected but untreated and uninfected cultures) were included in the experiments.

3.3.6.3 Longer term exposure

An attempt was made to assess the effect of longer term exposure of drugs on N. bombycis in S. frugiperda cell culture. One part of infected cells was mixed with 5 parts of uninfected cells and 1ml of suspension was seeded in a $25cm^2$ flask containing 4ml of drug-free growth medium. These were allowed to grow for 3 - 4 days. Cultures were exposed to benzimidazole at 250 and 500µg/ml for 10 days; to pentostam at 500 and 1,000µg/ml for 10 days; to fumagillin at 5µg/ml for 35 days; and to albendazole at 5.3µg/ml for 21 days. Drug preparation and treatment procedure was as before. The effects of drugs were assessed weekly (section 3.3.7) and the medium was renewed weekly in the remaining flasks. Two flasks for benzimidazole, 2 flasks for pentostam, 1 flask for fumagillin and 3 flasks for albendazole were used for each exposure and for the controls. Only 1 flask of uninfected culture was included with each treatment to assess the effect of drugs and solvent on host cells. After exposing the cell cultures to the drugs for desired periods, drugs were

withdrawn and the cultures were exposed to drug-free maintenance medium for another 7 - 14 days, to determine subsequent levels of infection.

3.3.7 Assessment

Cell suspensions harvested from 24 well tissue culture trays after exposure to drug(s) were centrifuged at 150g for 5 min. The supernatant was discarded and the pellet was resuspended in 500µl or less of TC-100 medium. The cells were spun on to glass slides using a cytospin centrifuge. The smears were air dried, fixed in methanol for 4 - 5 min and stained in 10% (v/v) Giemsa stain for 45 min. The effect of drug(s) was assessed by estimating the percentage of infected cells. In order to remove bias, the number of infected cells was counted per 200 cells in each of 5 - 10 areas on the slide. These areas were marked by the vernier on the microscope stage and the cells in the same areas were examined for the control and drug treated samples, at each of the time intervals. In some cases, where the cell number in the selected area was less than 200 the total cells were counted.

3.4 IN VIVO EXPERIMENTS

3.4.1 Microsporidian host

Corn earworm, Heliothis zea was used as an experimental host for N. bombycis. Eggs of H. zea were kindly supplied by Mr. Tim Carty, NERC Institute of Virology, Oxford, England. Surface sterilized eggs which were received attached to pieces of muslin or filter paper were allowed to hatch in well-aerated sealed plastic boxes at 27°C. The larvae were reared on semi-synthetic diet (Appendix 1), the 1st and 2nd instar larvae being reared en mass and the remaining instars being reared individually. The insects were kept in controlled chambers at 27° ± 1°C, 55% R.H. and 16L:8D. The frass was removed when required.

3.4.2 Assays for drug activity

The activity of drugs on the microsporidia was investigated either by exposing larvae to drug after they had been infected with microsporidia (post-exposure experiments) or by exposing uninfected larvae to the drug and microsporidian spores simultaneously (concurrent experiments).

3.4.2.1 Post-exposure experiments

Several late 2nd instar larvae were individually transferred to 4.0 x 1.5cm vials and starved for 6 h. Meanwhile, from a highly concentrated spore suspension 2.5 - 3.5µl drops of suspension, each containing 1 x 10^5 purified spores were dispensed on to the surface of $4mm^3$ pieces of the drug-free diet. The liquid was absorbed in to the diet. A piece of spore-treated diet was introduced into each vial and the larvae were allowed to feed until the whole piece of diet had been consumed. They were then individually transferred to 30ml plastic cups containing 4ml of drug-treated or drug-free diet (controls). Drugs were added to the liquid diet at 60^oC to achieve the desired concentrations. Thirty-five to 65 larvae were used for each treatment including the controls. All the drugs which were screened for the *in vivo* tests, except sinefungin and fumagillin, were tested in the post-exposure experiments.

3.4.2.2 Concurrent experiments

Drugs tested in post-exposure experiments were also simultaneously tested in concurrent experiments. Plastic cups containing 4ml of drug-treated or drug-free diet prepared as in post-exposure experiment were used as the source of microsporidian infection and drug treatment. The spore suspensions containing 1 x 10^5 spores in 25µl drops were dispensed on to the surface of the food and spread evenly by rotating the cup. The liquid was absorbed into the diet. Newly emerged 3rd instar larvae, starved for 6 h, were individually transferred to the cups and reared as before. Twenty to 35 larvae were used for each treatment.

For sinefungin and fumagillin, $6mm^3$ pieces of diet were first treated with 1 x 10^5 spores and then with 5µl drops of drug solution. For the controls $6mm^3$ pieces of food were treated with 1 x 10^5 spores only. Each piece was individually transferred into 4.0 x 1.5cm vials and newly emerged 3rd and 4th instar larvae, starved for 6 h, were individually introduced into each vial. After consuming the whole piece of the treated food each larva was individually transferred to a plastic cup containing drug-free diet. Twenty-five to 30 larvae were used for each treatment.

3.4.3 Assessment

When all of the drug-treated diet had been consumed, 5 - 10 larvae were randomly selected from each treatment and checked for infection. Drug

activity was assessed by spore harvest from larvae or pupae. Infections were determined on 6th instar larvae by examination of fresh smears of the fat body and midgut tissues for spores by phase-contrast microscopy. In all cases except with benomyl and albendazole (Expt. 2), infections were established and evidence was provided by the presence of numerous spores in control and drug-treated insects. Subsequently another 10 larvae from post-exposure experiments and 5 larvae from concurrent experiments were randomly selected from each treatment. These were weighed before determination of spore burden. Each larva was disrupted in 1ml distilled water in a tissue grinder, the resulting suspension diluted and the spores were counted using a haemocytometer. The number of spores was expressed per milligram body weight. Observations were made at 8 days post-exposure to drugs except with streptolydigin, benzimidazole (expt. 2) and thimerosal (expt. 2) when observations were made on days 10, 10 and 11 post exposure to drugs, respectively. With benomyl and albendazole (expt. 2 and expt. 1 at highest dose), as very few spores or none were observed, larvae were allowed to pupate in saw-dust in well-aerated boxes. On the 22nd day of infection, 10 pupae from post-exposure experiments and 5 pupae from concurrent experiments were randomly selected from each treatment, weighed, the spores counted, and expressed per milligram body weight. For albendazole (post-exposure expt.) as none of the control larvae pupated, same aged larvae were examined for comparison of spore numbers with drug treated pupae.

3.5 HISTOLOGICAL STUDIES

3.5.1 Preparation for light microscopy

Larvae infected with *N. bombycis* which had consumed 4ml of diet containing 100µg of toltrazuril per ml of diet, were dissected in saline solution. The midgut was cut into pieces 10mm long, which were fixed, dehydrated, embedded, sectioned and stained as in Appendix 2.

3.5.2 Preparation for electron microscopy

Midgut tissues of *N. bombycis*-infected larvae treated with benomyl, albendazole, sinefungin, fumagillin and toltrazuril were processed for electron microscopy. Except for toltrazuril, larvae were infected at late 2nd instar stage and exposed to the drug-treated diet at 5th instar stage. In the case of toltrazuril, larvae were infected at early 4th instar stage and

exposed to the drug-treated diet as soon as the spore treated food was consumed. For benomyl and albendazole, infected 5th instar larvae were exposed to diet containing 250µg of benomyl or 500µg of albendazole per ml of diet for 48 h. For toltrazuril infected larvae were exposed to 4ml of diet containing 100µg toltrazuril per ml of food and reared until the whole amount of food had been consumed, about 5 - 6 days. For sinefungin and fumagillin, infected 5th instar larvae were each exposed to a diet treated with 10µg of sinefungin or 100µg of fumagillin. As soon as the drug-treated diet had been consumed, larvae were transferred to drug-free diet for 24 h.

About 1mm cubes of midgut tissue obtained from infected-drug-treated larvae as described above were fixed in Karnovsky's fixative for 10 min at room temperature then for 1 h at 4°C. The specimens were dehydrated in graded acetone solution and embedded in Spurr's resin. Midgut tissues from last-instar infected but untreated larvae which were initially infected at early 4th instar stage, were similarly prepared. Ultrathin sections stained in uranyl acetate and Reynold's lead citrate were viewed with a Philips EM300 electron microscope at an accelerating voltage of 80KV.

A detailed schedule of fixation, dehydration, embedding and staining is given in Appendix 3.

3.6 EFFECT OF DRUGS ON THE LARVAL AND PUPAL DEVELOPMENT OF *Heliothis zea*

In addition to the study of the activity of albendazole and benomyl on *N. bombycis*, the effect of the drugs was also studied on the biology of the microsporidian host, *H. zea*. Observations were made on larval mortality, larval period, development time (from hatch to emergence) and percent pupation. After sacrificing some of the pupae for the assessment of drug effects on *N. bombycis*, the remaining pupae were individually kept in 30ml cups covered by a perforated lid for adult emergence. Pupal weight, and percent adult emergence were recorded. In the case of albendazole, larvae which were uninfected and untreated and larvae which were uninfected but treated with the highest dose of the drug were also observed for drug effects.

4. RESULTS

4.1 EFFECT OF DRUGS ON *Nosema bombycis* IN *Spodoptera frugiperda* CELL CULTURE

4.1.1 Albendazole

Albendazole was first tested at 3 concentrations, 1.0, 2.5 and 5.3µg/ml in culture medium, for 7 days and the results are presented in Table 4. The concentration 5.3µg/ml significantly reduced the percentage infected cells from the initial level of 40.7% to 26.9% ($P<0.01$) (F test). In the untreated control cultures, the percentage of infected cells increased to 63.6%. Albendazole at 1.0 and 2.5µg/ml had no adverse effect on *N. bombycis*.

Albendazole at 5.3µg/ml in culture medium was then tested for prolonged exposure of infected cells to the drug (Table 5). The percentage of infected cells had declined sharply from 40% to 24.8% by 7 days post-exposure and then remained unchanged for the remaining 14 days of the test period. In the control cultures the number of infected cells gradually increased from 40% to 80.8% during the corresponding period of 21 days. After 21 days when the drug containing medium was replaced with drug-free maintenance medium, the infection increased from 25.9% to 48.5% within 7 days. By this time, control cultures were almost completely destroyed. Albendazole at 5.3µg/ml of culture medium had no adverse effect on the gross structure of either infected or uninfected *S. frugiperda* cells during the test period of 21 days, as determined by light microscopy.

4.1.2 Sinefungin

The initial level of infection of 26.6% infected cells rose to 73.2% in untreated cultures by 7 days (Table 6). All concentrations of sinefungin (100-500µg/ml in culture medium) significantly reduced the percentage of infected cells ($P<0.01$) (F test). At 250 and 500µg/ml the percentage of infected cells decreased respectively to 19% and 19.2% by 3 days and to 21.7% and 20.2% by 7 days. At 100µg/ml the drug prevented increase of infection above the initial level throughout the experimental period. No adverse effects of sinefungin on the host cells were observed with any of the doses tested up to 3 days post-exposure. However, on the 7 day post-exposure to doses $\geq$250µg/ml cells became clumped. This was noted in infected cultures and in uninfected cultures used as controls for drug effects.

Table 4 Number of S. frugiperda cells infected with N. bombycis before and after treatment with albendazole for 7 days

Concentration (µg/ml)	Percentage of infected cells* (Mean ± S.E.)		% Reduction
	0 days	7 days	
0	40.7	63.6 ± 2.0 a+	
1.0	40.7	66.8 ± 1.6 a	
2.5	40.7	59.1 ± 1.3 a	7.1
5.3	40.7	26.9 ± 1.3 b	57.7

* For analysis of variance, percentage figures were transformed by arcsin $\sqrt{\%}$

\+ Means followed by the same letter are not significantly different from each other

Table 5 Number of S. frugiperda cells infected with N. bombycis during exposure to albendazole and after withdrawal of drug

Concentration (µg/ml)	Percentage of infected cells (Mean ± S.E.)				7 days after withdrawal of drug
	0 days	7 days	14 days	21 days	
0	40	65.3 ± 3.4	67.3 ± 1.0	80.8 ± 1.1	Massive infection
5.3	40	24.8 ± 1.3	26.9 ± 2.2	25.9 ± 2.5	48.5 ± 2.2
% Reduction		62.0	60.0	67.9	

Table 6 Number of S. frugiperda cells infected with N. bombycis before and after treatment with sinefungin for 3 and 7 days

Concentration (µg/ml)	Percentage of infected cells* (Mean ± S.E.)				
	0 days	3 days	% Reduction	7 days	% Reduction
0	26.6	60.9 ± 1.6 a		73.2 ± 0.3 a+	
100	26.6	29.0 ± 1.7 b	52.4	26.4 ± 0.4 b	63.9
250	26.6	19.0 ± 0.7 b	68.8	21.7 ± 0.2 b	70.4
500	26.6	19.2 ± 0.3 b	68.5	20.2 ± 1.0 b	72.4

* For analysis of variance, percentage figures were transformed by arcsin $\sqrt{\%}$

\+ Means followed by the same letter are not significantly different from each other

4.1.3 Fumagillin

Fumagillin was tested at 6 concentrations, 5, 10, 25, 50, 100 and 200 µg/ml in culture medium added to *N. bombycis*-infected cultures for 7 days (Table 7). Compared with control cultures receiving no drug, there were significant decreases in the percentage of infected cells from the initial level of 20% at all 6 concentrations of fumagillin ($P<0.01$) (F test). There were no significant differences in the effects of the drug at any of the concentrations used from the lowest concentration (5µg/ml) to the highest concentration (200µg/ml), the percentage of infected cells ranging from $5.2 \pm 0.3\%$ to $6.9 \pm 0.4\%$ after 7 days. In the control cultures the percentage of infected cells rose to 50.7% in the same period.

In an experiment on the prolonged exposure of microsporidia to the drug, fumagillin at 5µg/ml in culture medium was tested for 35 days (Table 8). There was a sharp decline from the initial level of infection of 20.3% of infected cells to 9.4% on day 7 and 4.3% on day 14 of treatment. Thereafter the level was virtually unchanged for the remainder of the experimental period. The infection in the control cultures gradually increased and the monolayer was almost completely destroyed by the parasites within 28 days. When, at 35 days exposure to drug, the drug-treated medium was replaced by drug-free maintenance medium, the infection increased from 4.1% to 25.9% over 14 days. No adverse effects of 5µg of fumagillin/ml in culture medium on the gross structure of cells were observed in infected or uninfected cultures by light microscopy during the test period of 35 days.

4.1.4 Thimerosal

The effects of thimerosal on the microsporidian growth are presented in Tables 9 and 10. At 5µg/ml in culture medium the percentage of infected cells rose above the initial level of 25.1% but at 2 days was significantly lower ($P<0.01$) (F test) than the control level (35.6% compared with 55.6%). However, on day 3 cells in the cultures exposed to drug had begun to die, indicating that the drug was at a toxic level. Higher doses were toxic at 2 days post exposure. In a 2nd experiment doses below 5µg/ml were used. As expected these did not prevent an increase in the percentage of cells above the initial level (19.3%). Doses below 2µg/ml did not affect the development of the microsporidia compared with the controls during the 7-day period of observtion. At 2µg/ml there was a significant decrease in the percentage of infected cells at 3 days but the toxic effects were such that the cultures were destroyed at 7 days.

Table 7 Number of S. frugiperda cells infected with N. bombycis before and after treatment with fumagillin for 7 days

Concentration (µg/ml)	Percentage of infected cells* (Mean ± S.E.)			% Reduction
	0 days	7 days		
0	20	50.7 ± 0.6	a+	
5	20	6.9 ± 0.4	b	86.4
10	20	5.8 ± 0.1	b	88.6
25	20	5.2 ± 0.3	b	89.7
50	20	5.4 ± 0.2	b	89.3
100	20	5.6 ± 0.3	b	89.0
200	20	6.2 ± 0.3	b	87.8

* For analysis of variance, percentage figures were transformed by arcsin $\sqrt{\%}$

+ Means followed by the same letter are not significantly different from each other

Table 8 Number of S. frugiperda cells infected with N. bombycis during exposure to fumagillin and after withdrawal of drug

Concentration (µg/ml)	Percentage of infected cells						14 days after withdrawal of drug
	0 days	7 days	14 days	21 days	28 days	35 days	
0	20.3	59.0	80.2	68.1	-	-	-
5.0	20.3	9.4	4.3	3.5	3.3	4.1	25.9
% Reduction		84.1	94.6	94.9			

- Cultures almost completely destroyed

Table 9 Number of S. frugiperda cells infected with N. bombycis before and after treatment with thimerosal for 2 days (Expt. 1)

Concentration (µg/ml)	Percentage of infected cells* (Mean ± S.E.)		% Reduction
	0 days	2 days	
0	25.1	55.6 ± 1.3 a+	
5	25.1	35.6 ± 0.6 b	36.0
10	25.1	Toxic	
20	25.1	Toxic	

* For analysis of variance, percentage figures were transformed by arcsin $\sqrt{\%}$

+ Means followed by the same letter are not significantly different from each other

Table 10 Number of S. frugiperda cells infected with N. bombycis before and after treatment with thimerosal for 3 and 7 days (Expt. 2)

Concentration (µg/ml)	Percentage of infected cells* (Mean ± S.E.)			
	0 days	3 days	% Reduction	7 days
0	19.3	58.4 ± 1.2 a+		69.8 ± 0.3
0.5	19.3	57.7 ± 1.5 a	1.2	70.7 ± 0.8
1.0	19.3	53.5 ± 1.7 a	8.4	70.9 ± 0.8
2.0	19.3	36.6 ± 1.6 b	37.3	Toxic

* For analysis of variance, percentage figures were transformed by arcsin $\sqrt{\%}$

+ Means followed by the same letter are not significantly different from each other

4.1.5 Benzimidazole

Doses of 750μg/ml and 1,000μg/ml were toxic to the host cells. The only doses which prevented multiplication of the microsporidia were 250μg/ml and 500μg/ml: at 7 days the levels of infection were 27.6% and 17% compared with the initial level of 25.1%. At all other drug levels the numbers of infected cells rose during the experiment about the initial levels of infection of 34% (Table 11) and 25.1% (Table 12). Doses of 10μg/ml and 25μg/ml did not produce significant changes in the numbers of infected cells and at 50μg/ml and 100 μg/ml the differences were significant ($P<0.01$) (F test) but minimal.

Examination of infected and uninfected cultures exposed to benzimidazole at 250 and 500μg/ml showed that all host cells were dead by 10 days. Cultures not receiving drug were healthy.

4.1.6 Pentostam

Pentostam was first tested at 4 concentrations, 100, 250, 500 and 1,000μg/ml in culture medium for 7 days (Table 13). The spread of infection from an initial level of 31.5% was not prevented at any of these concentrations, although growth was inhibited relative to the control when cells were exposed to 500 or 1,000μg/ml ($P<0.01$) (F test) of drug. Compared with the control no significant decreases in infection in the culture were observed with doses of 100 or 250μg/ml.

Pentostam was then tested for antimicrosporidial activity at concentrations of 1,500-3,000μg/ml added to cultures for 7 days. The results are presented in Table 14. With 1,500 and 2,000μg/ml pentostam, the percentages of infected cells at 7 days were 46.8% and 42% respectively, which differed significantly from the control level of 82.8% ($P<0.01$) (F test). Additionally, 2,000μg/ml maintained the infection at about the original level of 40%. Concentrations of pentostam of 2,500 and 3,000μg/ml gave rise to gross changes and death of the host cells even after 24 h. Furthermore, the cultures were destroyed after the prolonged exposure of infected and uninfected cultures to 500 and 1,000μg/ml for 10 days.

Table 11 Number of S. frugiperda cells infected with N. bombycis before and after treatment with benzimidazole for 7 days (Expt. 1)

Concentration (µg/ml)	Percentage of infected cells* (Mean ± S.E.)		% Reduction
	0 days	7 days	
0	34	84.3 ± 0.2 a+	
10	34	82.5 ± 0.5 a	2.1
25	34	83.4 ± 0.3 a	1.1
50	34	75.2 ± 0.3 b	10.8
100	34	69.5 ± 0.4 c	17.6

* For analysis of variance, percentage figures were transformed by arcsin $\sqrt{\%}$

+ Means followed by the same letter are not significantly different from each other

Table 12 Number of S. frugiperda cells infected with N. bombycis before and after treatment with benzimidazole for 7 days (Expt. 2)

Concentration (µg/ml)	Percentage of infected cells* (Mean ± S.E.)		% Reduction
	0 days	7 days	
0	25.1	58.4 ± 1.2 a+	
250	25.1	27.6 ± 0.6 b	52.7
500	25.1	17.0 ± 0.7 b	70.9
750	25.1	Toxic	
1000	25.1	Toxic	

* For analysis of variance, percentage figures were transformed by arcsin $\sqrt{\%}$

+ Means followed by the same letter are not significantly different from each other

Table 13 Number of S. frugiperda cells infected with N. bombycis before and after treatment with pentostam for 7 days (Expt. 1)

Concentration (µg/ml)	Percentage of infected cells* (Mean ± S.E.)		% Reduction
	0 days	7 days	
0	31.5	63.6 ± 0.5 a+	
100	31.5	63.8 ± 1.9 a	
250	31.5	63.6 ± 2.4 a	
500	31.5	47.4 ± 1.0 b	25.5
1000	31.5	39.4 ± 0.5 b	38.1

* For analysis of variance, percentage figures were transformed by arcsin $\sqrt{\%}$

+ Means followed by the same letter are not significantly different from each other

Table 14 Number of S. frugiperda cells infected with N. bombycis before and after treatment with pentostam for 7 days (Expt. 2)

Concentration (µg/ml)	Percentage of infected cells* (Mean ± S.E.)		% Reduction
	0 days	7 days	
0	40	82.8 ± 1.7 a+	
1500	40	46.8 ± 2.3 b	43.5
2000	40	42.0 ± 1.2 b	49.3
2500	40	Toxic	
3000	40	Toxic	

* For analysis of variance, percentage figures were transformed by arcsin $\sqrt{\%}$

+ Means followed by the same letter are not significantly different from each other

4.1.7 Ciprofloxacin

The results of testing the exposure of cultures to ciprofloxacin ranging from 25μg/ml to 500μg/ml for periods up to 7 days are presented in Table 15. Spread of infection was only prevented by concentrations of 250 and 500μg/ml. However, all doses had significantly reduced the percentage of infected cells by the 2nd day post-exposure to drugs ($P<0.01$) (F test). At 25μg/ml and 50μg/ml there was a slight decrease in the percentage of infected cells from the control level but these differences were not significant when cultures were examined at 4 days and 7 days (F test). At 100μg/ml the percentages of infected cells were reduced to 77.2%, 70.6% and 71.6% compared to 86.2%, 86% and 85.3% respectively in the control cultures on days 2, 4 and 7 post-exposure. These reductions were significant on all 3 days ($P<0.01$) (F test). When the concentration was $\geqslant$250μg/ml, not only was there a significant reduction in the percentage of infected cells on all 3 days, but there was inhibition of the spread of microsporidia to new cells from the initial level of 57%. The effects of 250μg/ml and 500μg/ml concentrations were significantly different from the effects of 100μg/ml but were not significantly different from one another at any of the time points. None of the concentrations of ciprofloxacin had adverse effects on the gross structure of the host cells.

4.1.8 Toltrazuril

Toltrazuril at 29.94μg/ml in culture medium had no effect on *N. bombycis* in *S. frugiperda* cell cultures at any of the exposure times investigated (Fig.1). The number of infected cells increased with increase in time and there were no significant differences between control and drug-treated cultures (F test).

4.1.9 Imidocarb dipropionate

Figure 2 demonstrates that imidocarb dipropionate at 100μg/ml and 250μg/ml in medium slightly reduced the percentage of infected cells at 7 days post exposure to drug. However, the differences were not statistically significant (F test). The drug at higher concentrations $\geqslant$500μg/ml medium were toxic to the cultures by day 2 post-exposure and all the cells died during the test period.

Table 15 Number of S. frugiperda cells infected with N. bombycis before and after treatment with ciprofloxacin for 2, 4 and 7 days

Concentration (µg/ml)	Percentage of infected cells* (Mean ± S.E.)							
	0 days	2 days	% Redn.	4 days	% Redn.	7 days	% Redn.
0	57	86.2 ± 1.6 a+		86.0 ± 1.1 a		85.3 ± 0.1 a	
25	57	77.6 ± 0.9 b	10	84.4 ± 0.6 a	1.9	84.0 ± 0.2 a	1.5
50	57	78.7 ± 0.6 b	8.7	81.0 ± 2.0 a	5.8	84.1 ± 0.2 a	1.4
100	57	77.2 ± 3.2 b	10.4	70.6 ± 1.6 b	17.9	71.6 ± 1.5 b	16.1
250	57	58.2 ± 1.8 c	32.5	56.9 ± 0.5 c	33.8	54.2 ± 1.4 c	36.5
500	57	52.5 ± 0.2 c	39.1	49.8 ± 1.1 c	42.1	49.0 ± 1.3 c	42.3

* For analysis of variance, percentage figures were transformed by arcsin $\sqrt{\%}$

+ Means followed by the same letter are not significantly different from each other

Fig.1 Percentage of *S. frugiperda* cells infected with *N. bombycis* at different exposures to 29.94µg of toltrazuril per millilitre of culture medium (11% cells infected at day 0). Error bars represent 1 standard error of the mean.

Fig. 2 Percentage of *S. frugiperda* cells infected with *N. bombycis* after 7 days exposure to increasing doses of imidocarb dipropionate (36.5% cells infected at day 0 indicated on Y-axis). Error bars represent 1 standard error of the mean.

Fig. 1

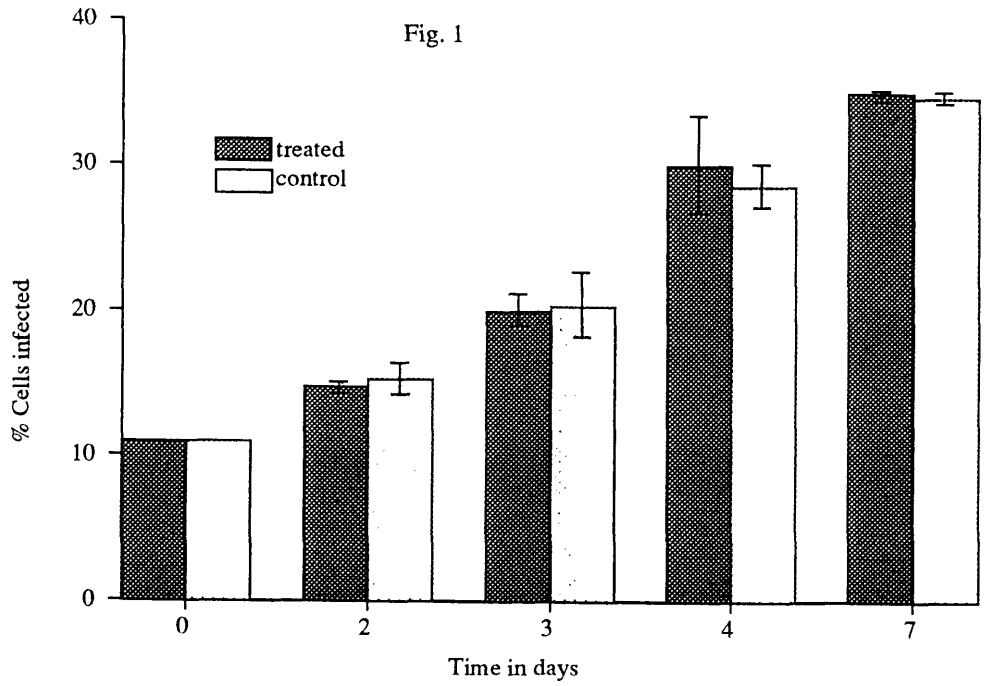
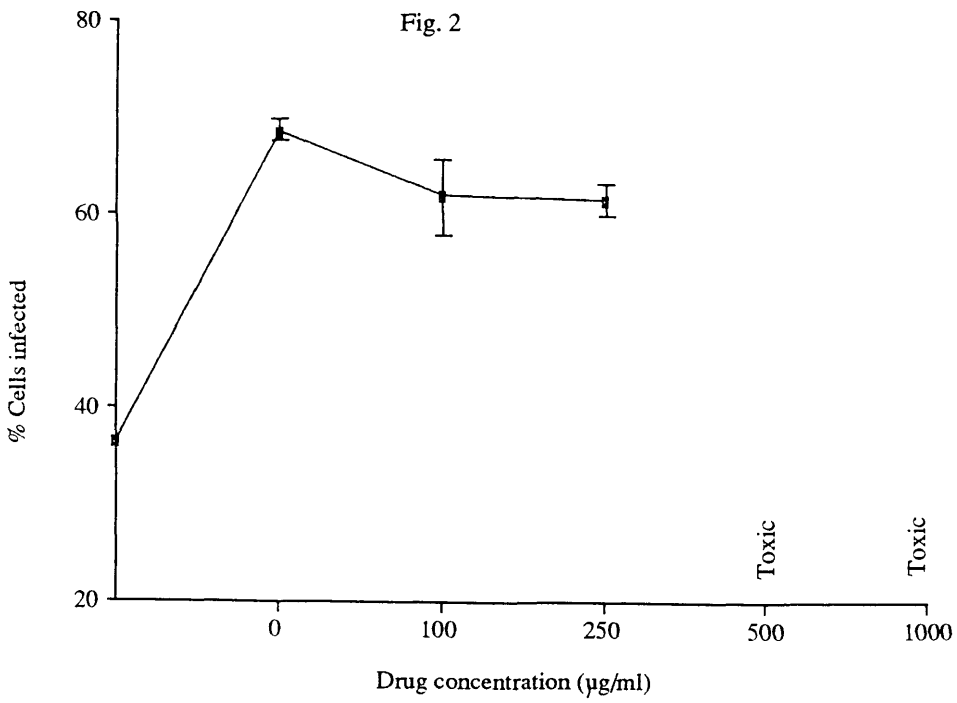

40
30
20
10
0
% Cells infected
treated
control
0
2
3
4
7
Time in days

Fig. 2


80
60
40
20
% Cells infected
Toxic
Toxic
0
100
250
500
1000
Drug concentration (µg/ml)

4.1.10 Metronidazole

A series of concentrations of metronidazole ranging from 50 to 3,000μg/ml were tested in 2 experiments by exposing the cultures for 7 days (Figs. 3 & 4). No effects were observed with concentrations up to 2,000μg/ml, above this the drugs were toxic within 24 h.

4.1.11 Sulphadimidine

Sulphadimidine was tested at 4 concentrations, 100, 300, 600 and 900μg/ml in medium and the results are presented in Figure 5. There were no differences in the percentages of infected cells between controls and drug treated cultures up to 600μg/ml when examined at 7 days post-exposure to drug. At 900μg/ml the drug was toxic to cultures within 24 h.

4.1.12 Sulphapyridine

The results with sulphapyridine ranging from 50μg/ml to 1,000μg/ml are shown in Figure 6. None of these doses inhibited the development of the microsporidia compared with the control on day 7 post-exposure to drug.

4.1.13 Sulphathiazole

Sulphathiazole was tested at concentrations of 50, 100, 250, 500 and 1,000μg/ml in medium. All cells in infected and in uninfected cultures died within 24 h even at the lowest concentration. Also, the control cultures containing the solvent for the drug also died within 24 h.

4.1.14 Rimocidin sulphate

Rimocidin sulphate was tested at 3 concentrations, 10, 25 and 100μg/ml in medium for 5 days. There were no differences in the percentage of infected cells betwen control and drug-treated cultures (Fig. 7).

4.1.15 Itraconazole

Itraconazole was tested at concentrations of 1.0, 2.5 and 5.9μg/ml in medium for 7 days. The results, illustrated in Figure 8 demonstrate that the drug had no effect on the microsporidian growth at any of these concentrations.

Figs.3 & 4 Percentage of *S. frugiperda* cells infected with *N. bombycis* after 7 days exposure to increasing doses of metronidazole (21% and 20% cells infected at day 0 indicated on Y-axis, respectively for Fig.3 and Fig.4). Error bars represent 1 standard error of the mean.

Fig. 3

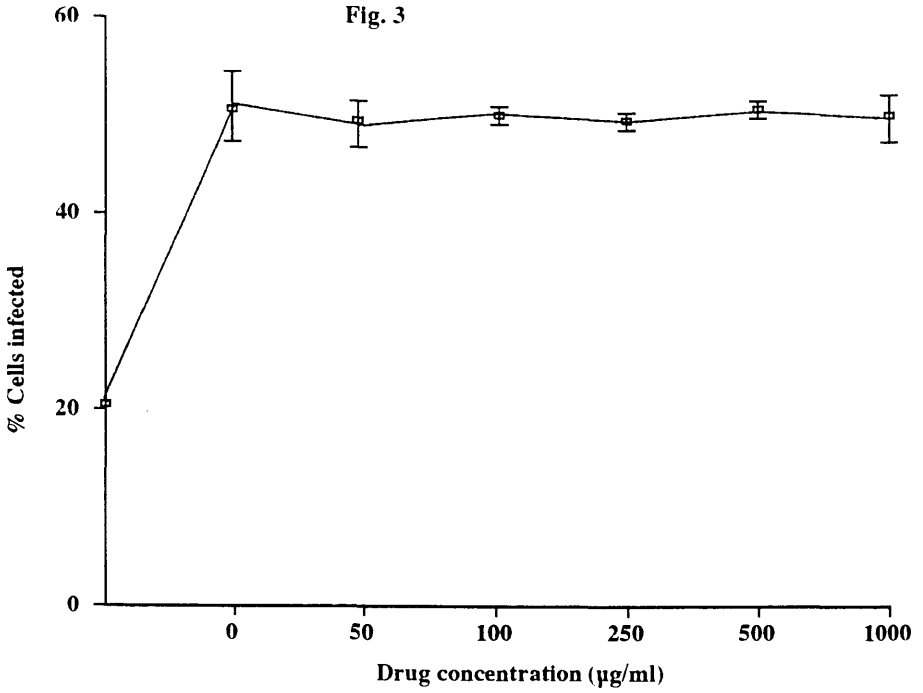

60
40
20
0
% Cells infected
0
50
100
250
500
1000
Drug concentration (µg/ml)

Fig. 4

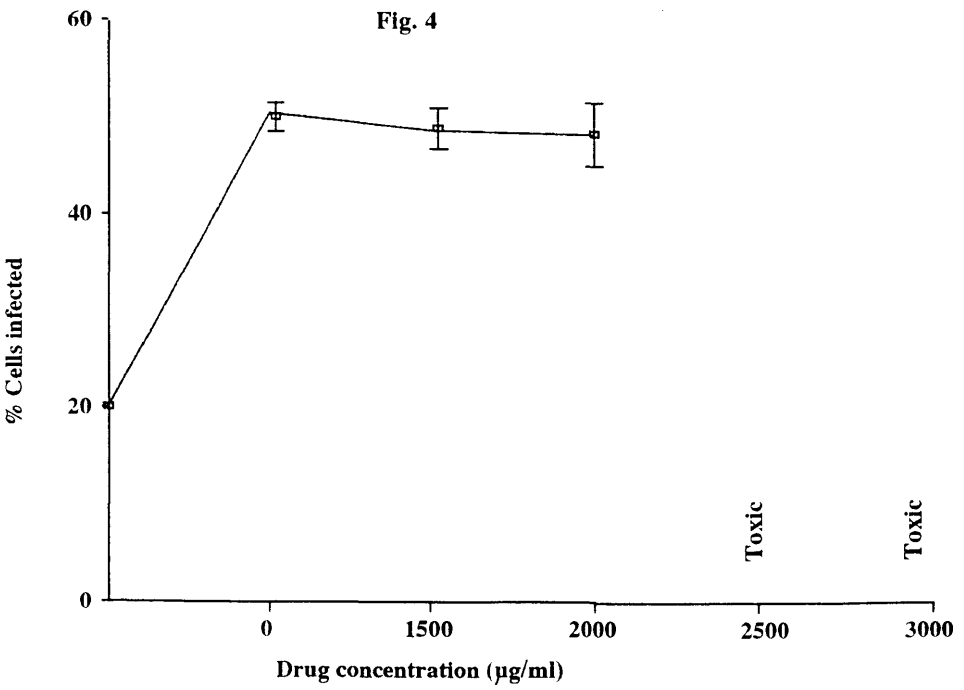

60
40
20
0
% Cells infected
0
1500
2000
2500
3000
Toxic
Toxic
Drug concentration (µg/ml)

Fig. 5 Percentage of S. frugiperda cells infected with N. bombycis after 7 days exposure to increasing doses of sulphadimidine (28.5% cells infected at day 0 indicated on Y-axis). Error bars represent 1 standard error of the mean.

Fig. 6 Percentage of S. frugiperda cells infected with N. bombycis after 7 days exposure to increasing doses of sulphapyridine (21% cells infected at day 0 indicated on Y-axis). Error bars represent 1 standard error of the mean.

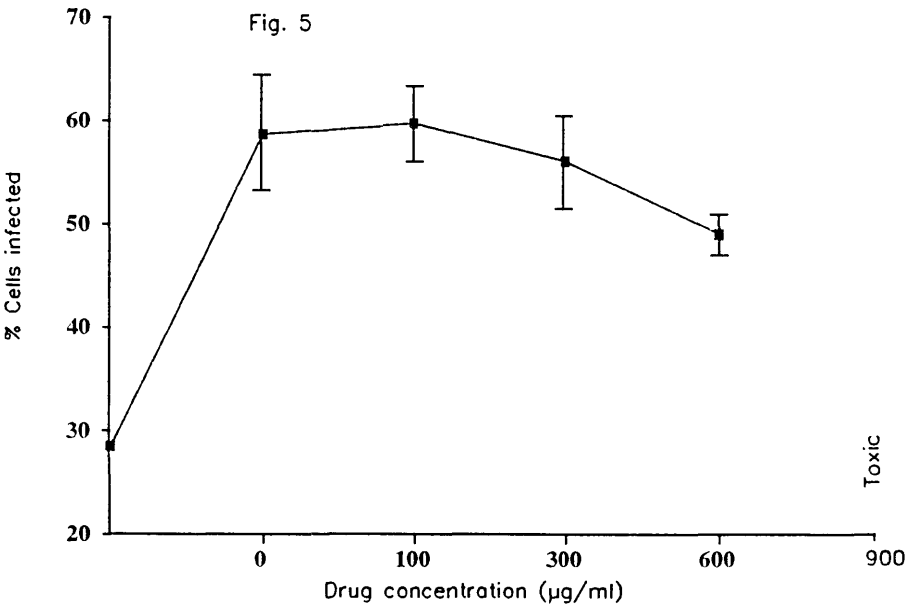


Fig. 5
70
60
50
40
30
20
% Cells infected
0
100
300
600
900
Toxic
Drug concentration (µg/ml)

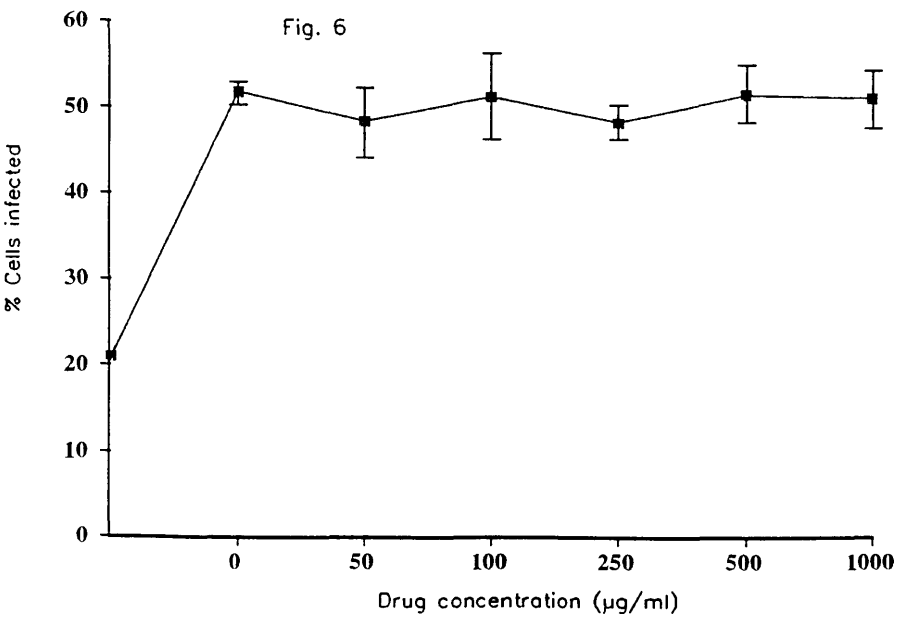


Fig. 6
60
50
40
30
20
10
0
% Cells infected
0
50
100
250
500
1000
Drug concentration (µg/ml)

Fig. 7 Percentage of *S. frugiperda* cells infected with *N. bombycis* after 5 days exposure to increasing doses of rimocidin sulphate (28.3% cells infected at day 0 indicated on Y-axis). Error bars represent 1 standard error of the mean.

Fig. 8 Percentage of *S. frugiperda* cells infected with *N. bombycis* after 7 days exposure to increasing doses of itraconazole (36.7% cells infected at day 0 indicated on Y-axis). Error bars represent 1 standard error of the mean.

Fig. 7

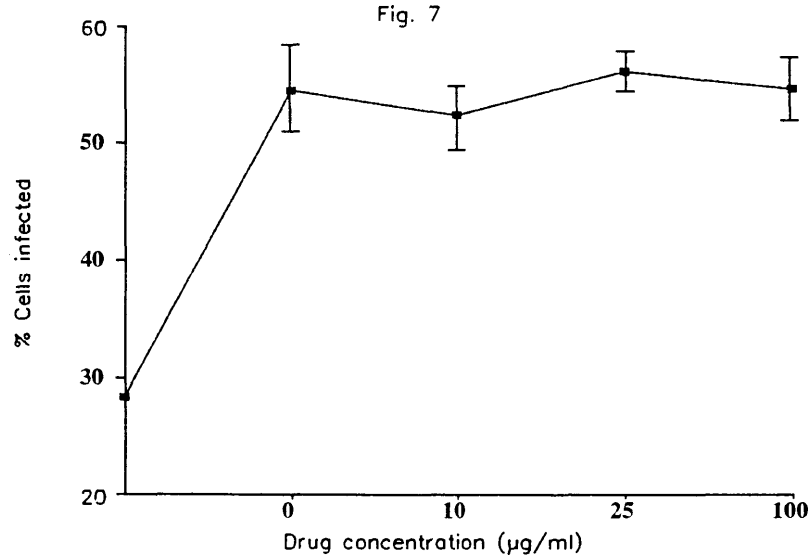
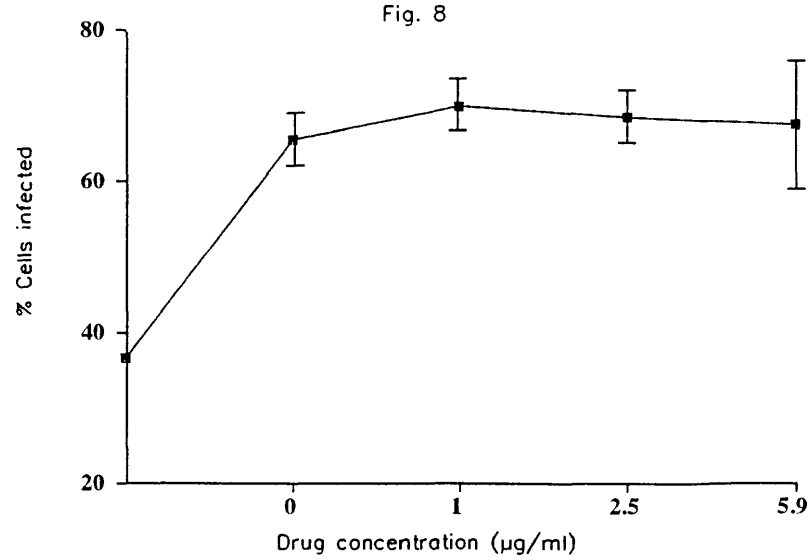

60
50
40
30
20
% Cells infected
0
10
25
100
Drug concentration (µg/ml)

Fig. 8


80
60
40
20
% Cells infected
0
1
2.5
5.9
Drug concentration (µg/ml)

4.1.16 Cerulenin

In one experiment cerulenin was tested at 25, 50, and 100µg/ml in medium but all the concentrations were toxic, and infected and uninfected cultures had died within 24 h. Infected and uninfected cultures were healthy in the absence of drugs. In another experiment cerulenin was tested at 2.5, 5.0 and 10µg/ml in medium and, although the host cells remained healthy, the drug had no effect on the microsporidian growth during the test period of 5 days (Fig. 9).

4.1.17 Compound 566C80

Three concentrations of 3.04, 6.08 and 11.88µg/ml of this compound were tested in the cultures for 7 days but no differences were observed in microsporidian growth relative to the control (Fig.10).

4.1.18 **Effect of combined drugs**

The results of using combinations of drugs on microsporidia-infected cultures for 7 days are presented in Table 16. All the drugs tested had previously shown anti-microsporidial activity when used alone. The only combination that failed to prevent spread of the infection was benzimidazole and albendazole, in the presence of which the percentage of infected cells rose from an initial level of 40% to 52.1%. This was a slight improvement on the effect of this concentration of albendazole alone, being 24.7% of the control value compared with 7.1% for albendazole alone (Table 4). However, the combination was antagonistic for benzimidazole as a reduction of 52.7% of the control value had been achieved with 250µg/ml benzimidazole alone (Table 12).

The combination of benzimidazole and sinefungin reduced the infection by 57.1% compared to the control value, although the reduction was a slight improvement for benzimidazole alone. However, the combination was antagonistic for sinefungin, as sinefungin alone reduced the infection by 63.9% (Table 6).

Compared to the control value, the combination of benzimidazole and fumagillin reduced the infection by 78.8%. The combination showed some improvement in favour of benzimidazole alone but antagonistic for fumagillin, because fumagillin alone reduced the infection by 86.4% (Table 7).

Fumagillin in combination with albendazole and sinefungin reduced the infection compared to the control value, by 79.6% and 67.5% respectively. Both combinations were antagonistic for fumagillin. However, the reductions were

Fig. 9 Percentage of *S. frugiperda* cells infected with *N. bombycis* after 5 days exposure to increasing doses of cerulenin (31.3% cells infected at day 0 indicated on Y-axis). Error bars represent 1 standard error of the mean.

Fig. 10 Percentage of *S. frugiperda* cells infected with *N. bombycis* after 7 days exposure to increasing doses of compound 566C80 (40% cells infected at day 0 indicated on Y-axis). Error bars represent 1 standard error of the mean.

Fig. 9

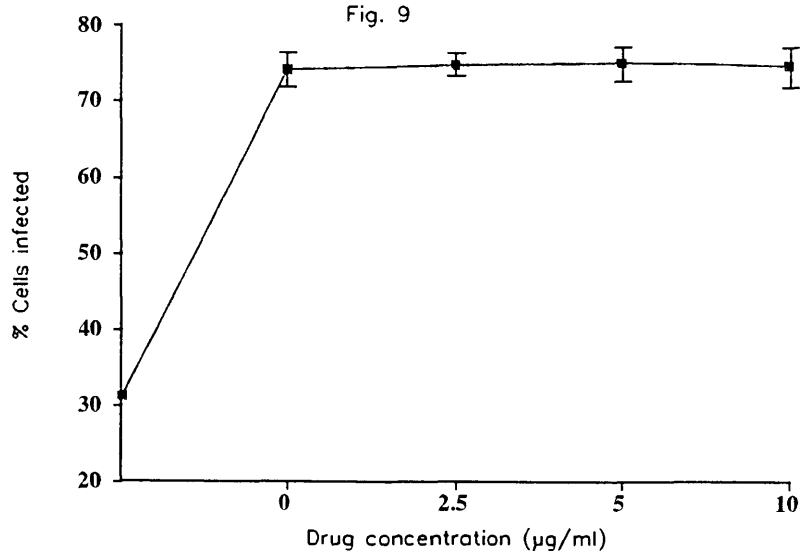

80
70
60
50
40
30
20
% Cells infected
0
2.5
5
10
Drug concentration (µg/ml)

Fig. 10

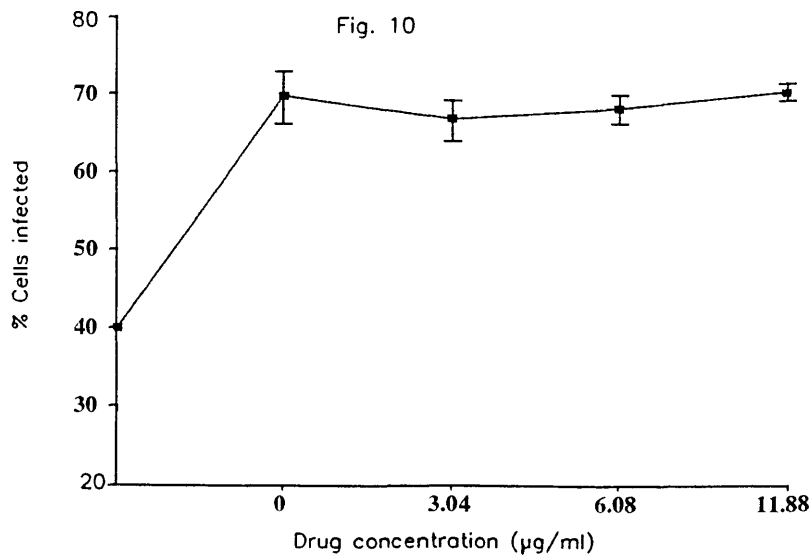

80
70
60
50
40
30
20
% Cells infected
0
3.04
6.08
11.88
Drug concentration (µg/ml)

Table 16 Number of S. frugiperda cells infected with N. bombycis before and after treatment with different combinations of drugs for 7 days

Drug Combination	Concentration (µg/ml)	Percentage of infected cells* (Mean ± S.E.)		
		0 days	7 days	% Redn.
Control	0 + 0	40	69.2 ± 4.1 a+	
Sinefungin + Benzimidazole	100 + 250	40	29.7 ± 2.0 b	57.1
Sinefungin + Albendazole	100 + 2.5	40	21.4 ± 1.0 b	69.1
Sinefungin + Fumagillin	100 + 5.0	40	22.5 ± 0.9 b	67.5
Benzimidazole + Albendazole	250 + 2.5	40	52.1 ± 2.8 a	24.7
Benzimidazole + Fumagillin	250 + 5.0	40	14.7 ± 0.7 b	78.8
Albendazole + Fumagillin	2.5 + 5.0	40	14.1 ± 0.2 b	79.6

* For analysis of variance, percentage figures were transformed by arcsin $\sqrt{\%}$

+ Means followed by the same letter are not significantly different from each other

much higher than albendazole alone and slighly higher than sinefungin alone.

The combination of sinefungin and albendazole reduced the infection by 69.1% compared to the control value. This was the only combination where higher reduction was achieved than recorded by the individual drug. However, the reduction was additive.

All the combinations tested were not toxic to cultures except sinefungin + benzimidazole and sinefungin + albendzaole, where cells of both infected and uninfected cultures started to clump on the 7th day.

4.2 EFFECT OF DRUGS ON *Nosema bombycis* IN *Heliothis zea*

4.2.1 Albendazole

In one experiment albendazole was tested at 3 concentrations, 0.2, 0.4 and 2.0mg/larva, given post-exposure or concurrently. Some were examined as 6th instar larvae when drug was given post-exposure, others were examined as pupae when the drug was given post-exposure or concurrently. Albendazole at 2.0mg/larva almost completely eliminated *N. bombycis* from the late-stage larvae, although some of them contained too few to provide a meaningful count. The controls were heavily infected. Albendazole at 0.4 and 0.2mg/larva significantly reduced the number of spores in late-stage larvae by 73.3% and 43.3% compared to the control value, respectively (Table 17) (Student t-test). The reduction was significant at the 1% level with the 0.4mg/larva treatment and at the 5% level in the 0.2mg/larva treatment.

Observations made on pupae surviving from the same experiment showed that, although there was an increase in the number of spores over the number estimated in late-stage larvae, the number was nevertheless significantly lower ($P<0.01$) at all 3 concentrations of drug than in the controls. This was true for the post-exposure and concurrent experiments except with 0.2mg/larva in the post-exposure experiment, when the number of spores was not significantly different from that of the control (Table 18) (Student t-test). The drug at 0.2, 0.4 and 2.0 mg/larva, compared to the control value, reduced the number of spores by 33.3%, 59.3% and 99.9% in the post-exposure experiment (Table 18) and by 54.5%, 72.7% and 99.9% in the concurrent experiment (Table 19) respectively.

In another experiment, albendazole was tested at 1.0, 2.0, and 4.0mg/larva, given post-exposure and concurrently. Some estimates of spore numbers

Table 17 Effect of albendazole on production of N. bombycis spores in H. zea larvae (post-exposure treatment: Expt. 1)

Albendazole (mg/larva)	Mean (range) ± S.E./mg x 10^5	% Reduction
0	6.0 (3.0 - 12.5) ± 0.9	
0.2	3.4 (1.6 - 6.5) ± 0.6 *	43.3
0.4	1.6 (0.8 - 2.5) ± 0.2 **	73.3
2.0	Very few	

* Significant at the 5% level

** Significant at the 1% level

Table 18 Effect of albendazole on production of N. bombycis spores in H. zea pupae (post-exposure treatment: Expt. 1)

Albendazole (mg/larva)	Mean (range) ± S.E./mg x 10^6	% Reduction
0	2.7 (0.9 - 4.2) ± 0.4	
0.2	1.8 (0.8 - 3.3) ± 0.4	33.3
0.4	1.1 (0.5 - 1.6) ± 0.3 **	59.3
2.0	0.002 (0.001 - 0.004) ± 0.0003**	99.9

** Significant at the 1% level

Table 19 Effect of albendazole on production of *N. bombycis* spores in *H. zea* pupae (concurrent treatment: Expt. 1)

Albendazole (mg/larva)	Mean (range) ± S.E./mg x 10^6	% Reduction
0	1.1 (0.9 - 1.3) ± 0.1	
0.2	0.5 (0.3 - 0.6) ± 0.1 **	54.5
0.4	0.3 (0.2 - 0.4) ± 0.03 **	72.7
2.0	0.001 (0.0005 - 0.002) ± 0.0002**	99.9

** Significant at the 1% level

were made on 6th instar larvae and the rest on surviving pupae. In the post-exposure experiment, albendazole at 2.0 and 4.0mg/larva almost completely eliminated the parasite from the late-stage larvae and a greatly decreased level of infection compared with the control was observed with the 1.0mg/larva treatment (data not shown). Observations of pupae showed that there was some increase in the number of spores over the level of larvae. The numbers of spores were significantly lower than in the controls in all treatments ($P<0.01$) (Student t-test) (Table 20). In the concurrent experiment, albendazole at 1.0 and 2.0mg/larva almost completely eliminated the parasite from the late-stage larvae (data not shown) and low-level infections were observed in pupae. However, the number of spores was significantly lower than in the control ($P<0.01$) (Student t-test) (Table 21). Using albendazole at 4.0mg/larva no infections were detected in larvae and there was no resurgence in the pupae. No spores were found in tissue of late-stage larvae or pupae when examined under phase-contrast microscopy. Giemsa stained preparations from the tissue of pupae also showed them to be free of infection.

4.2.2 Benomyl

Benomyl, given post exposure to infection or concurrently, was tested at 3 concentrations, 1.0, 2.0 and 4.0mg/larva. All the doses almost completely eliminated the parasite from the late-stage larvae. Although some larvae contained very few spores the numbers were too low for accurate examination. The controls were heavily infected. Despite the suppression of the parasite development in larvae, low-level infections were also observed in pupae. Compared to the control values, benomyl at 1.0, 2.0 and 4.0mg/larva reduced the number of spores by 89.2%, 89.7% and 89.2% in post-exposure experiment (Table 22) and by 99.2%, 99.3% and 99.2% in concurrent experiment (Table 23). At all doses the reductions in spore number compared with controls, both in post-exposure and concurrent experiments, were significant ($P<0.01$) (Student t-test).

4.2.3 Sinefungin

In one experiment sinefungin was tested at 2.5, 5.0, 25, 50 and 1,000µg/larva by exposing early 3rd instar larvae to a piece of food treated with both drug and spores (Table 24). Sinefungin at $\geq$5µg/larva was toxic to larvae; all larvae died between the 2nd and 4th days even before the whole drug-treated piece of diet had been consumed. No mortality was observed with

Table 20 Effect of albendazole on production of N. bombycis spores in H. zea pupae (post-exposure treatment: Expt. 2)

Albendazole (mg/larva)	Mean (range) ± S.E./mg x 10^6	% Reduction
0	2.8 (1.2 - 4.3) ± 0.5	
1	0.3 (0.2 - 0.4) ± 0.03 **	89.3
2	0.003 (0.0009-0.005) ± 0.0005**	99.89
4	0.001 (0.0004-0.002) ± 0.0001**	99.96

** Significant at the 1% level

Table 21 Effect of albendazole on production of N. bombycis spores in H. zea pupae (concurrent treatment: Expt. 2)

Albendazole (mg/larva)	Mean (range) ± S.E./mg x 10^6	% Reduction
0	1.1 (0.9 - 1.7) ± 0.1	
1	0.014 (0.008-0.022) ± 0.003 **	98.7
2	0.0009(0.0006-0.0013) ± 0.0001**	99.9
4	No infection	100.0

** Significant at the 1% level

Table 22 Effect of benomyl on production of *N. bombycis* spores in *H. zea* pupae (post-exposure treatment)

Benomyl (mg/larva)	Mean (range) ± S.E./mg x 10^5	% Reduction
0	18.5 (10.3 - 28.1) ± 2.0	
1	2.0 (1.1 - 3.1) ± 0.2 **	89.2
2	1.9 (1.1 - 2.9) ± 0.2 **	89.7
4	2.0 (1.2 - 2.9) ± 0.2 **	89.2

** Significant at the 1% level

Table 23 Effect of benomyl on production of *N. bombycis* spores in *H. zea* pupae (concurrent treatment)

Benomyl (mg/larva)	Mean (range) ± S.E./mg x 10^5	% Reduction
0	13.5 (9.2 - 18.4) ± 1.5	
1	0.112 (0.082-0.170) ± 0.015 **	99.2
2	0.100 (0.054-0.151) ± 0.016 **	99.3
4	0.103 (0.072-0.150) ± 0.013 **	99.2

** Significant at the 1% level

Table 24 Effect of sinefungin on production of *N. bombycis* spores in *H. zea* larvae (exposing early 3rd instar larvae to drug and spores concurrently)

Sinefungin (µg/larva)	Mean (range) ± S.E./mg x 10^5	% Reduction
0	4.1 (1.8 - 7.3) ± 0.5	
2.5	3.8 (2.3 - 7.0) ± 0.5	7.3
5	Toxic	
25	Toxic	
50	Toxic	
100	Toxic	

sinefungin at 2.5µg/larva. The dose reduced the number of spores by 7.3% compared to the control value on 8th day post-exposure. However, the reduction was not significant (Student t-test). In a 2nd experiment sinefungin was tested at 5.0, 10 and 25µg/larva by exposing early 4th instar larvae to a small piece of food treated with drug and spores (Table 25,A). On the 8th day post-exposure estimation of spore numbers showed that all the doses significantly reduced the number of spores (Student t-test). At 10µg and 25µg/larva there were reductions of 65.7% and 73.5% respectively of spores compared to the control value, and the differences were significant at the 1% level. At 5 µg/larva there was a percentage reduction of 41.0% compared with the controls and the difference was significant at the 5% level. In the 25 µg/larva treatment, 3 out of 25 larvae died during the test period but no deaths were recorded in the other groups.

In another experiment sinefungin at 10µg and 25µg/larva were tested again (Table 25,B). Both the doses significantly reduced the number of spores compared to the control on the 8th day post-exposure to drug and spores ($P<0.01$) (Student t-test), the reductions for the 10µg and 25µg concentrations being 77.1% and 97.4% respectively. In this experiment, sinefungin was toxic to the larvae. Out of 25 larvae, 2 larvae in 10µg/larva treatment and 23 larva in 25µg/larva treatment died during the test period.

4.2.4 Fumagillin

Fumagillin was tested at 3 concentrations, 10, 25, and 100µg/larva by exposing early 4th instar larvae to a small piece of food treated with both drug and spores (Table 26). On the 8th day post-exposure, estimates of the number of spores in 5th instar larvae showed that fumagillin at 100 and 25µg/larva significantly reduced the number of spores compared to the controls (Student t-test). The dose 100µg/larva reduced the number of spores by 56.5% compared to the control value and the difference was at 1% level significant. The dose 25µg/larva reduced the number of spores by 34.8% compared to the control value and the difference was at 5% level significant. Although fumagillin at 10µg/larva, slightly reduced the number of spores by 26.1% the reduction was not significant (Student t-test).

Table 25 Effect of sinefungin on production of *N. bombycis* spores in *H. zea* larvae (exposing early 4th instar larvae to drug and spores concurrently)

Sinefungin (μg/larva)	A		B	
	Mean (range) $\pm$ S.E./mg x 10^4	% Redn.	Mean (range) $\pm$ S.E./mg x 10^4	% Redn.
0	16.6 (10.1-27.5) $\pm$ 1.8		22.7 (9.6-38.4) $\pm$ 3.2	
5	9.8 (4.3-22.7) $\pm$ 1.7*	41.0	-	
10	5.7 (0.8-12.7) $\pm$ 1.2**	65.7	5.2 (1.4-9.5) $\pm$ 1.0**	77.1
25	4.4 (1.5-8.6) $\pm$ 0.8**	73.5	0.6 (0.5-0.7) $\pm$ 0.1**	97.4

* Significant at the 5% level
** Significant at the 1% level

Table 26 Effect of fumagillin on production of *N. bombycis* spores in *H. zea* larvae (exposing early 4th instar larvae to drug and spores concurrently)

Fumagillin (μg/larva)	Mean (range) $\pm$ S.E./mg x 10^5	% Reduction
0	2.3 (1.0 - 3.8) $\pm$ 0.3	
10	1.7 (1.2 - 2.6) $\pm$ 0.2	26.1
25	1.5 (0.8 - 2.6) $\pm$ 0.2 *	34.8
100	1.0 (0.6 - 1.9) $\pm$ 0.1 **	56.5

* Significant at the 5% level
** Significant at the 1% level

4.2.5 Thimerosal

Thimerosal was tested at 4 doses, 20, 100, 200 and 400µg/larva in two experiments. In the first experiment, thimerosal at 20µg/larva, given concurrent and post-exposure treatment to early 3rd instar and infected early 4th instar larvae, respectively showed no effect on microsporidian development over 8 days (Table 27). In the second experiment, thimerosal at 100, 200 and 400µg/larva was tested only in post-exposure treatment by exposing infected early 4th instar larva. All 3 concentrations were toxic to larvae. All the larvae died within 4 days in the 200 and 400µg/larva treatments. Using 100µg/larva 30/35 larvae died and the remaining 5 larvae failed to consume the whole amount of drug-treated food by 11 days. However, spore counts from the 5 surviving larvae showed no reduction from the control (Table 28).

4.2.6 Benzimidazole

In one experiment benzimidazole at 1.0 and 2.0mg/larva was tested either by exposing infected early 4th instar larvae to the drug or by exposing early 3rd instar larvae to the drug and spores concurrently. Observations made on 8 days post-exposure showed no effects of the drug on spore numbers (Table 29). In another experiment, benzimidazole was tested at 4.0 and 10mg/larva, given post-exposure or concurrently as before. The drug-treated food was unpalatable to larvae. All the larvae eventually consumed all the food containing 4mg of drug but some larvae failed to finish the food containing 10mg of drug during the 10 day period. Observations made 10 days post-exposure showed that neither dose had any effect on the microsporidian development (Table 30).

4.2.7 Pentostam

Pentostam was tested at 4.0 and 8.0mg/larva both in post-exposure and concurrent treatments. In the post-exposure experiment infected early 4th instar larvae were exposed to the drug and in the concurrent experiment, early 3rd instar larvae were exposed to the drug and spores simultaneously. Spore numbers were estimated at 8 days. Both the concentrations failed to reduce the number of spores relative to the controls when given post-exposure and concurrently (Table 31).

Table 27 Effect of thimerosal on production of *N. bombycis* spores in *H. zea* larvae (Expt. 1)

Thimerosal	Post-exposure	Concurrent-exposure
(µg/larva)	Mean (range) ± S.E./mg x 10^5	Mean (range) ± S.E./mg x 10^5
0	3.4 (1.8 - 6.2) ± 0.4	1.1 (0.8 - 1.6) ± 0.1
20	3.9 (1.7 - 8.6) ± 0.6	1.3 (0.8 - 2.1) ± 0.2

Table 28 Effect of thimerosal on production of *N. bombycis* spores in *H. zea* larvae (Expt. 2)

Thimerosal (µg/larva)	Post-exposure Mean (range) ± S.E./mg x 10^5
0	8.5 (6.6 - 12.1) ± 1.0
100	8.9 (7.7 - 10.9) ± 0.6
200	Toxic
400	Toxic

Table 29 Effect of benzimidazole on production of *N. bombycis* spores in *H. zea* larvae (Expt. 1)

Benzimidazole	Post-exposure	Concurrent-exposure
(mg/larva)	Mean (range) ± S.E./mg x 10^5	Mean (range) ± S.E./mg x 10^5
0	1.9 (1.4 - 2.8) ± 0.1	1.1 (0.8 - 1.6) ± 0.1
1	1.9 (1.4 - 2.4) ± 0.1	1.3 (0.7 - 2.3) ± 0.3
2	1.8 (1.1 - 2.6) ± 0.1	1.1 (0.7 - 1.5) ± 0.1

Table 30 Effect of benzimidazole on production of *N. bombycis* spores in *H. zea* larvae (Expt. 2)

Benzimadazole	Post-exposure	Concurrent-exposure
(mg/larva)	Mean (range) ± S.E./mg x 10^5	Mean (range) ± S.E./mg x 10^5
0	6.7 (5.2 - 8.3) ± 0.3	2.2 (1.5 - 3.1) ± 0.3
4	5.6 (3.7 - 6.9) ± 0.3	2.0 (1.4 - 2.4) ± 0.2
10	5.7 (1.0 - 7.9) ± 0.6	2.3 (1.7 - 2.6) ± 0.2

Table 31 Effect of pentostam on production of *N. bombycis* spores in *H. zea* larvae

Pentostam	Post-exposure	Concurrent-exposure
(mg/larva)	Mean (range) ± S.E./mg x 10^5	Mean (range) ± S.E./mg x 10^5
0	3.4 (1.8 - 6.2) ± 0.4	1.1 (0.8 - 1.6) ± 0.1
4	3.6 (1.9 - 5.6) ± 0.4	1.4 (0.9 - 2.0) ± 0.2
8	3.3 (2.5 - 4.7) ± 0.2	1.3 (0.9 - 2.0) ± 0.2

4.2.8 Toltrazuril

Toltrazuril was tested at 3 concentrations, 100, 200 and 400µg/larva, given post-exposure treatment by exposing infected early 4th instar larvae or concurrently by exposing early 3rd instar larvae to drug and spores simultaneously. Spore numbers were estimated at 8 days. Toltrazuril at all concentrations failed to eliminate the microsporidium from its experimental host. No differences were detected in production of spores between control and drug-treated groups either in post-exposure or in concurrent experiments (Table 32).

4.2.9 Novobiocin

Novobiocin was tested at 4.0 and 10mg/larva given post-exposure treatment by exposing infected early 4th instar larvae or concurrently by exposing early 3rd instar larvae to the drug and spores concurrently. Observations made on day 8 showed no differences in production of spores between control and drug-treated groups either both in post-exposure or in concurrent experiments (Table 33).

4.2.10 Streptolydigin

Streptolydigin was tested at 2.0 and 6.0mg/larva given post-exposure to the infected early 4th instar larvae and concurrently to the early 3rd instar larvae. The results are presented in Table 34. Both concentrations reduced the number of spores in the 6th instar larvae after 10 days post-exposure. The doses 2.0 and 6.0mg/larva reduced the number of spores by 16.7% and 28.8% respectively compared to the control value in the post-exposure experiment and by 27.3% and 30.3% in the concurrent experiments. However, these differences were not significant (Student t-test).

4.3 HISTOLOGICAL OBSERVATIONS

4.3.1 Light microscopy

Histological studies by light microscopy from midgut tissues of *N. bombycis*-infected *H. zea* larvae which had consumed 400µg of toltrazuril showed massive infections of the parasite (Fig. 11). Infection was also widespread in body tissues.

Table 32 Effect of toltrazuril on production of *N. bombycis* spores in *H. zea* larvae

Toltrazuril	Post-exposure	Concurrent-exposure
(µg/larva)	Mean (range) ± S.E./mg x 10^5	Mean (range) ± S.E./mg x 10^5
0	2.8 (1.5 - 4.2) ± 0.3	6.1 (3.7 - 9.9) ± 1.1
100	2.5 (1.2 - 5.5) ± 0.4	6.6 (4.5 - 7.8) ± 0.6
200	3.0 (1.1 - 4.9) ± 0.4	7.3 (6.2 - 8.0) ± 0.4
400	2.5 (1.5 - 3.6) ± 0.2	6.7 (4.1 - 9.8) ± 1.1

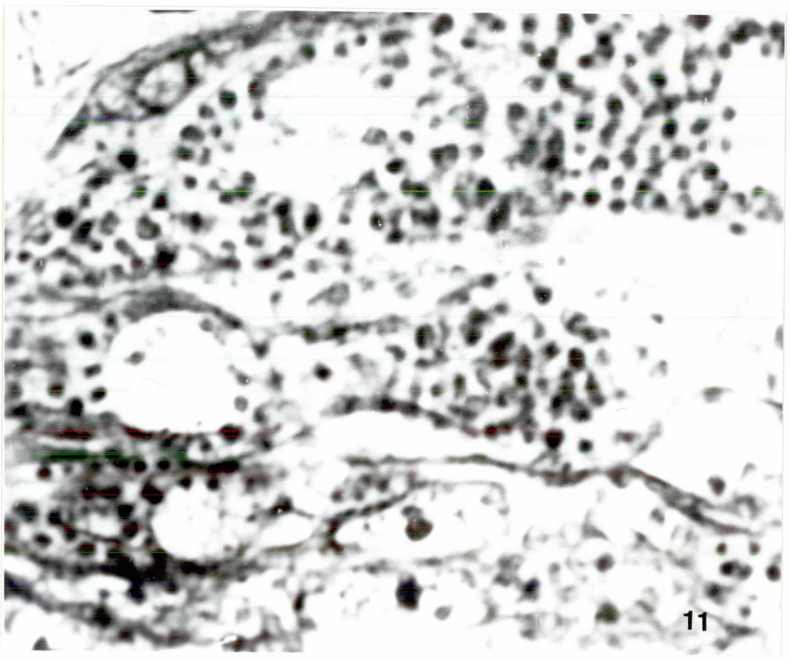
Table 33 Effect of novobiocin on production of *N. bombycis* spores in *H. zea* larvae

Novobiocin	Post-exposure	Concurrent-exposure
(mg/larva)	Mean (range) ± S.E./mg x 10^5	Mean (range) ± S.E./mg x 10^5
0	3.0 (2.1 - 5.1) ± 0.3	1.2 (0.7 - 1.9) ± 0.2
4	2.4 (1.6 - 4.1) ± 0.2	1.1 (1.0 - 1.4) ± 0.1
10	2.7 (2.0 - 3.7) ± 0.2	1.0 (0.9 - 1.0) ± 0.02

Table 34 Effect of streptolydigin on production of *N. bombycis* spores in *H. zea* larvae

Streptolydigin	Post-exposure		Concurrent-exposure	
(mg/larva)	Mean (range) ± S.E./mg x 10^5	% Redn.	Mean (range) ± S.E./mg x 10^5	% Redn.
0	6.6 (5.3 - 8.1) ± 0.3		3.3 (2.3 - 4.2) ± 0.4	
2	5.5 (3.0 - 9.2) ± 0.7	16.7	2.4 (1.7 - 3.0) ± 0.2	27.3
6	4.7 (3.0 - 6.1) ± 0.3	28.8	2.3 (2.0 - 2.9) ± 0.2	30.3

Fig. 11 Midgut tissues from *N. bombycis*-infected *H. zea* larva which had consumed 400μg of toltrazuril. Note massive infections of the parasites. x1000


11

4.3.2 Electron microscopy

Effect of albendazole, benomyl, sinefungin, fumagillin and toltrazuril on N. bombycis at the subcellular level were studied by electron microscopy. For albendazole, benomyl, sinefungin and fumagillin, midgut tissues from 5th instar larvae which were initially infected at late 2nd-instar stage were processed for electron microscopy after 7 days post-exposure of infection and 2 days post-exposure of drug-treatments. In the case of toltrazuril, midgut tissues from last-instar larvae which were initially infected at early 4th instar stage were processed for electron microscopy after 7 days post-exposure of infection and 5-6 days post-exposure of drug treatment. For controls, midgut tissues from last instar larvae which were also initially infected at early 4th instar stage were processed for ultrastructure study after 7 days post-exposure of infection.

In a few cases, ultrastructural observations revealed the disruption of host tissues which might be due to heavy infections of the parasites.

4.3.2.1 Controls

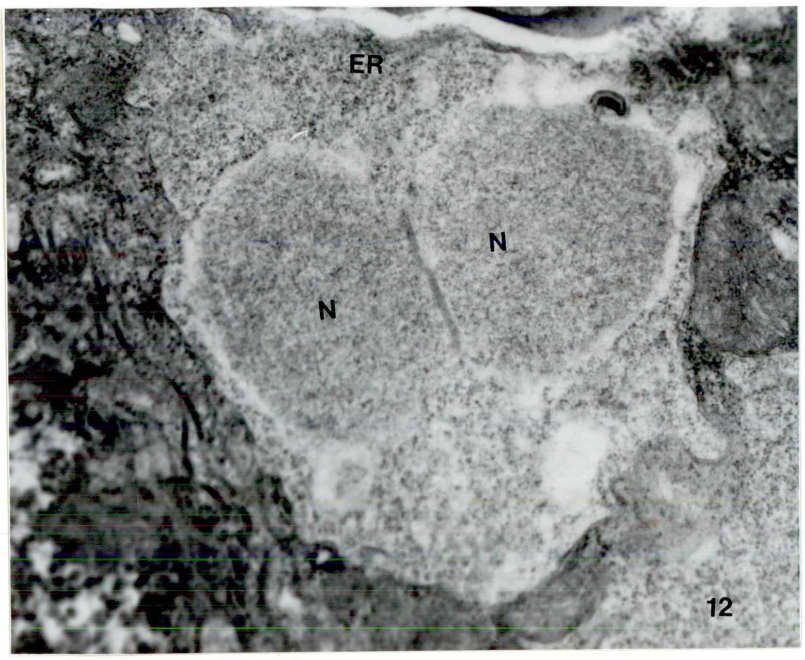
In the untreated group, ultrastructural observations of the midgut tissues of infected larvae showed typical meronts, sporonts, sporoblasts and spores of N. bombycis (Figs. 12-17). Meronts have a simple plasma membrane, usually very little endoplasmic reticulum and abundant ribosomes. The plasma membrane of sporonts is thickened by deposition of an electron dense surface coat. There is also more endoplasmic reticulum in sporonts. Sporonts acquire 2 diplokarya, then cytoplasmic fission separates 2 sporoblasts. Sporoblasts begin to get the organelles of the spore. They are often crinkled because they do not fix well. As they mature they become oval again and the wall layers are laid down. The spore wall consists of two layers: an outer amorphous electron-dense layer, the exospore and an inner electron transparent middle layer, the endospore. Within the wall the plasma membrane surrounds the cytoplasmic structures and nuclei. The polar tube is attached to the centre of the polar sac at the apex of the spore and then runs an oblique course backward and forms about 10 - 11 coils in the peripheral layers of the cytoplasm. The polaroplast surrounds the straight part of the polar tube. All the stages have diplokaryotic nuclei and the nuclear materials are homogenous.

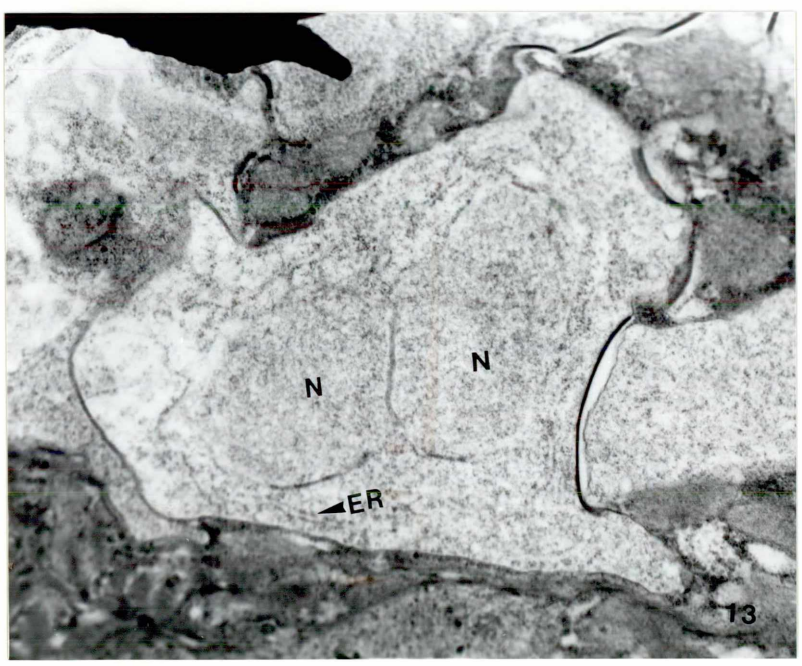
Figs. 12 - 17 Developmental stages of *N. bombycis* from midgut tissues of untreated *H. zea* larva.

Fig. 12 Meront with diplokaryon nucleus (N) and endoplasmic reticulum (ER). x27000

Fig. 13 Early sporont with diplokaryon nucleus and endoplasmic reticulum. x19500

Fig. 14 Dividing sporont. x16800


ER
N
N
12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ER
13

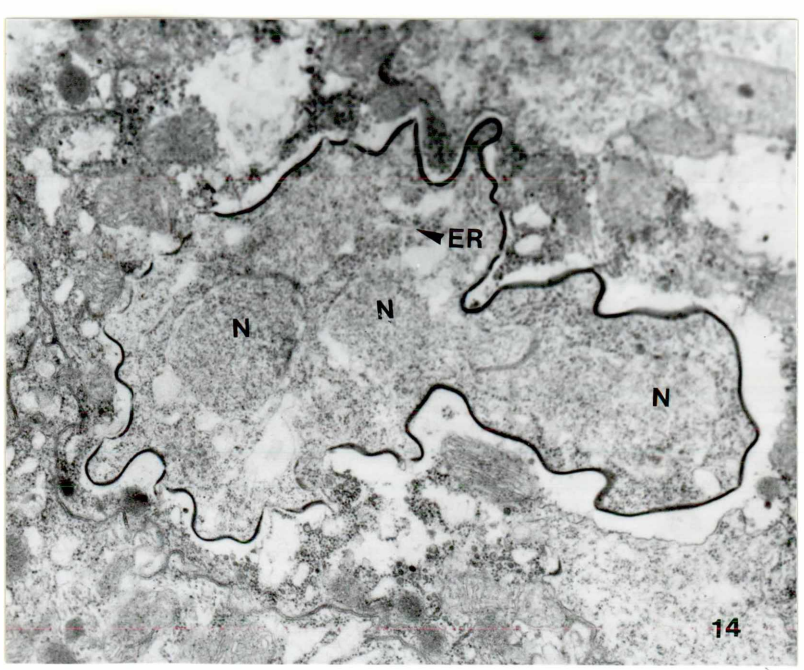
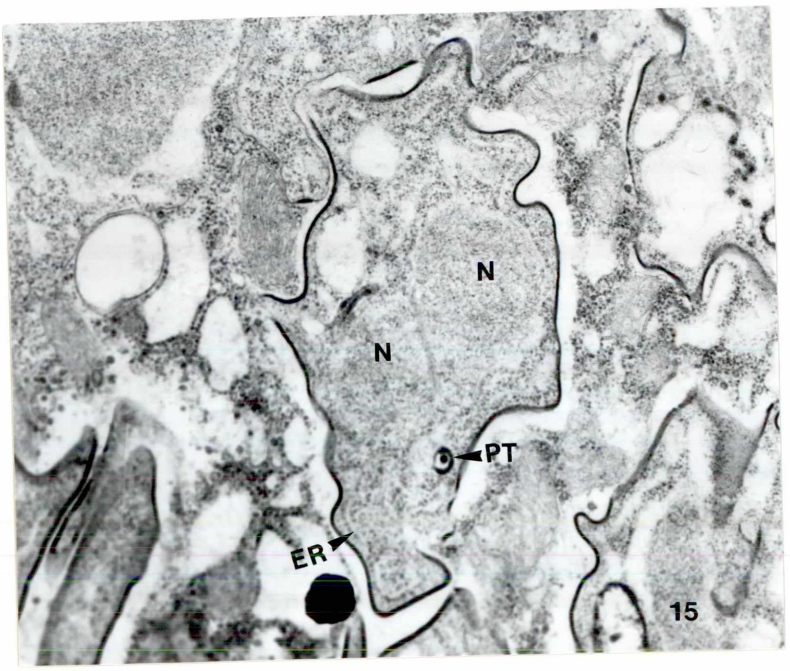
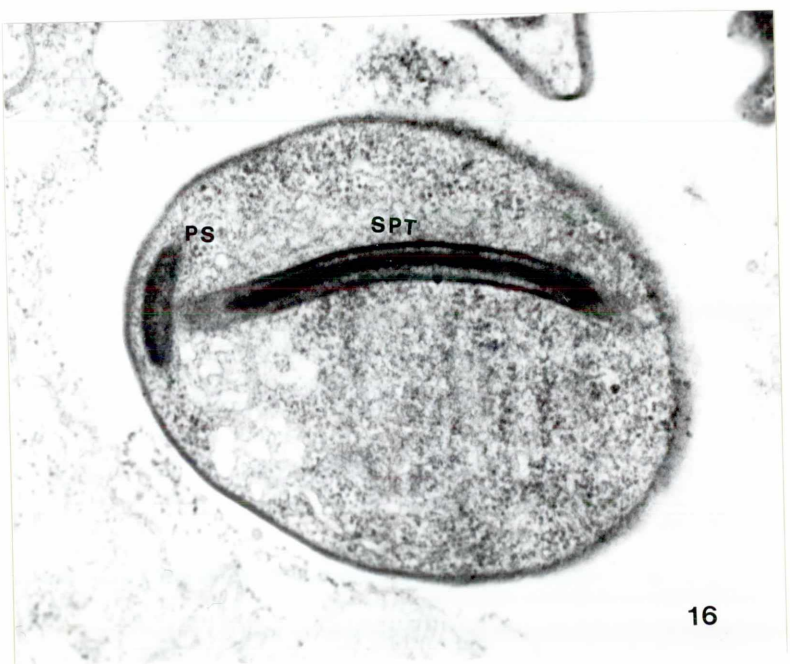

ER
N
N
N
14

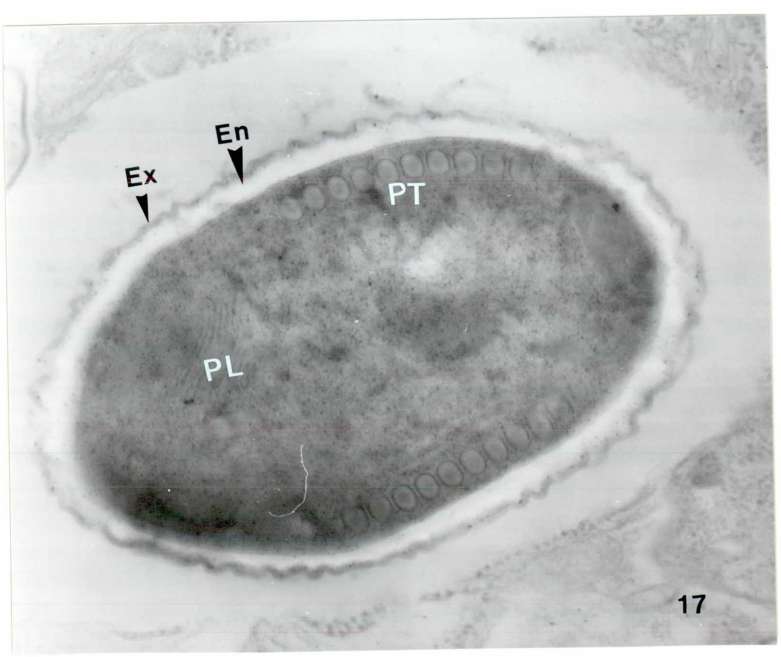
Fig. 15 Early sporoblast showing diplokaryon nucleus, endoplasmic reticulum and transverse section of polar tube (PT). x15100

Fig. 16 Late sporoblast showing part of polar sac (PS) and straight part of polar tube (SPT). x45200

Fig. 17 Spore illustrating membranes of polaroplast (PL), coiled region of polar tube (PT), exospore (Ex) and endospore (En). x49500


N
N
PT
ER
15


PS
SPT
16


Ex
En
PT
PL
17

4.3.2.2 Albendazole

The majority of meronts showed the presence of irregular masses of chromatin in the nuclei. The chromatin masses which looked like beads were usually located at opposite poles of the nuclei (Figs. 18, 19). In some cases, the nuclear membrane expanded adjacent to chromatin aggregates (Fig.18).

Although cytoplasmic organisation in meronts appeared normal, with abundant evenly-distributed ribosomes, there was almost total disorganisation in the later sporogonic stages. Nuclei were no longer visible. In sporoblasts the cytoplasm now contained irregular vesicles of different sizes and very few ribosomes (Fig. 20). Spores were similarly destroyed. They either appeared empty (Fig. 21) or packed with membrane whorls. There was no sign of the normal sporoplasm or extrusion apparatus and the wall consisted only of the exospore layer, sometimes with an incomplete endospore (Figs. 22, 23).

4.3.2.3 Benomyl

Benomyl treated meronts, and sporoblasts showed the presence of irregular masses of chromatin in the nuclei. In the meronts these masses resembled the condition brought about by albendazole, with a network of chromatin clumped near the nuclear envelope (Fig. 24). In sporoblasts the chromatin was clumped into a single mass (Figs. 25, 26). In sporoblasts there was evidence of the beginning of polar filament formation (Fig. 26) but the spores (Figs. 27, 28, 29) were entirely abnormal. The shape varied from oviod to semi-lunar. Within a partially developed wall the cytoplasm showed irregular vesicles and membrane whorls and often a large vacuole containing reticulate or tabular structures.

4.3.2.4 Sinefungin

Sinefungin treated meronts, and spores exhibited many deformities. The nuclei were no longer homogeneous but the contents now formed a network, with larger particles and narrower threads. The changes involved the entire volume of the nuclei not polarised as in the albendazole-treated parasites (Figs. 30, 31). The cytoplasm still contained abundant ribosomes but there were signs of development of clear spaces. Most of the spores were irregular in shape, either semi-lunar (Fig. 32) triangular (Fig. 33) or ovoid (Fig. 34). Internally the structure was completely disorganised with no sign of nuclei or spore organelles.

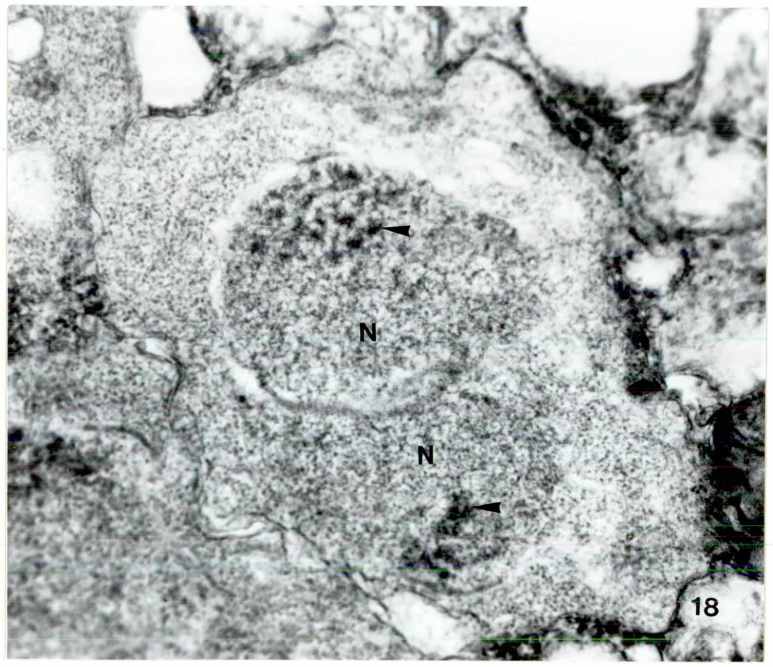
Figs. 18 - 23 Ultrastructural changes of *N. bombycis* induced by albendazole.

Figs. 18 & 19 Meronts with diplokaryon nucleus (N). Note polarised irregular masses of chromatin in the nuclei (arrow).

Fig. 18 x32300

Fig. 19 x28700

Fig. 20 Sporoblast. Note that the cytoplasm contains irregular vesicles and very few ribosomes. Nuclei not visible. x36300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N
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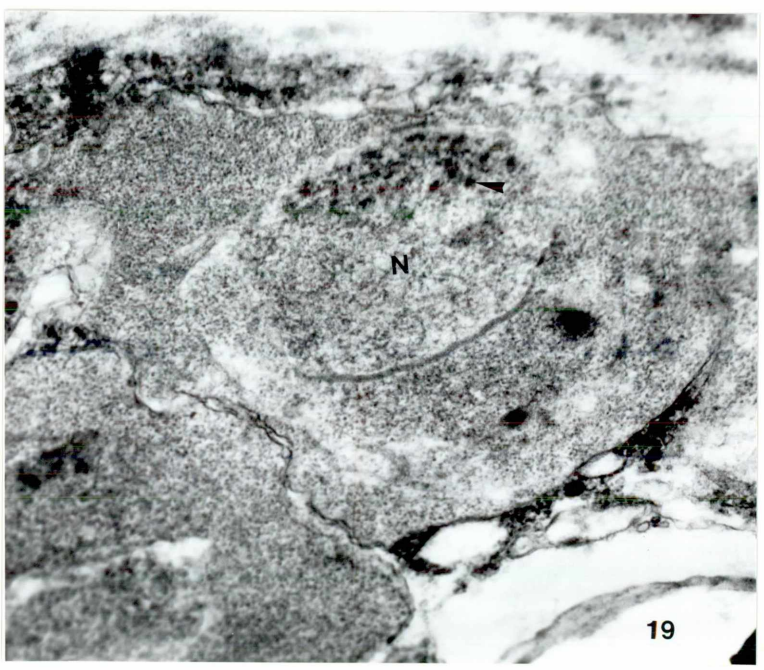
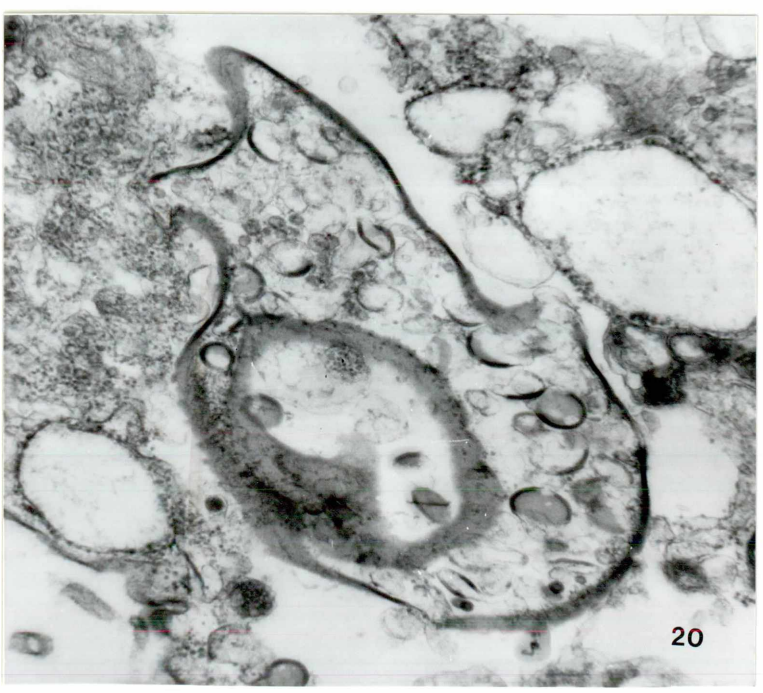

N
19

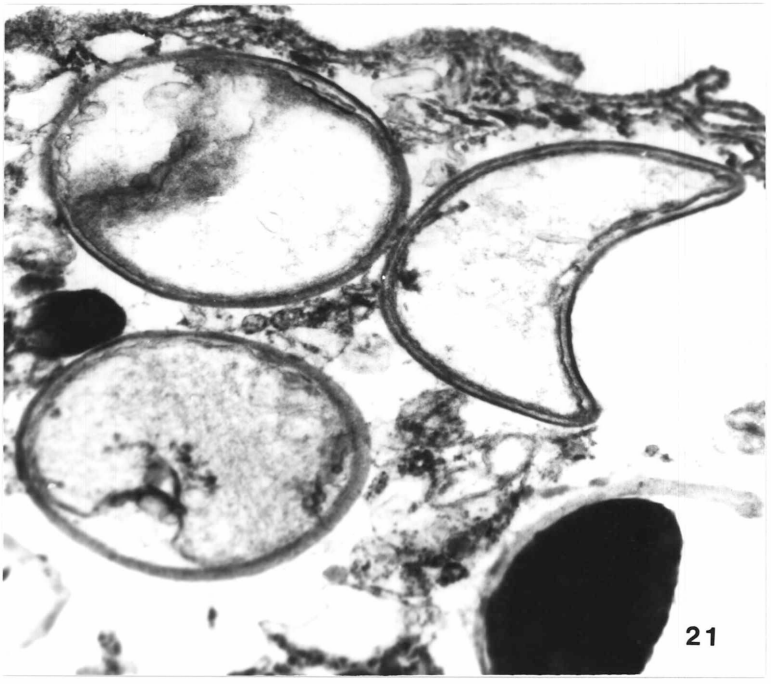

20

Figs. 21 - 23 Aberrant spores appeared either empty or packed with membrane whorls.

Fig.21 x33400

Fig.22 x38000

Fig.23 x59700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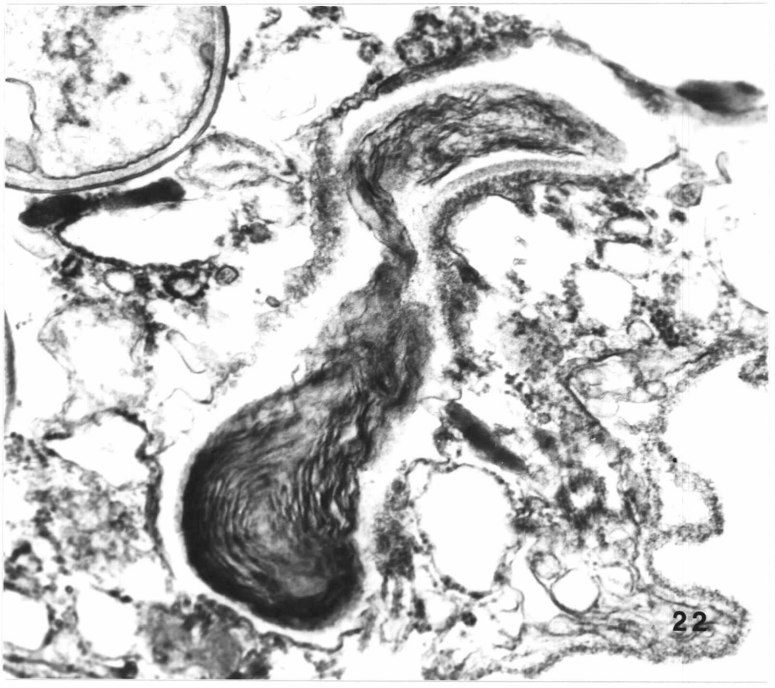
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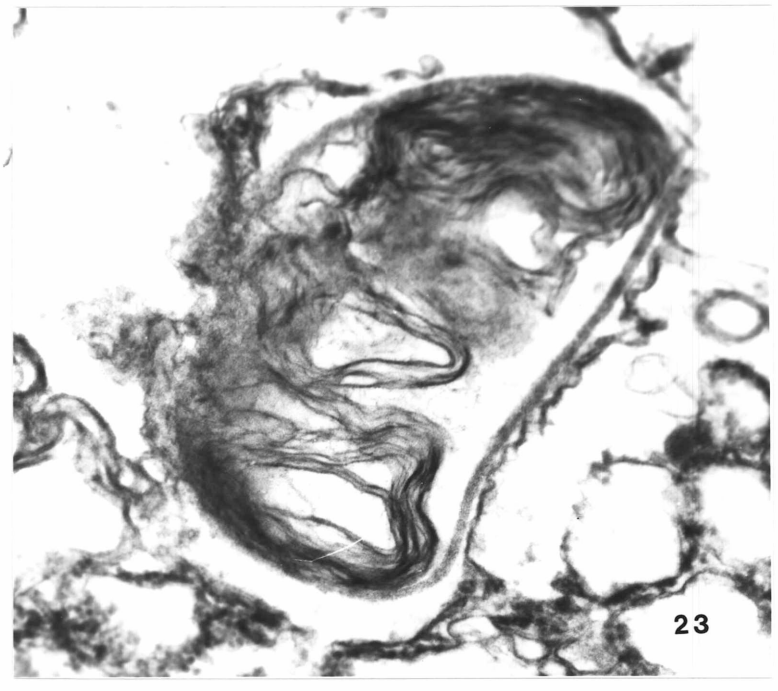

23

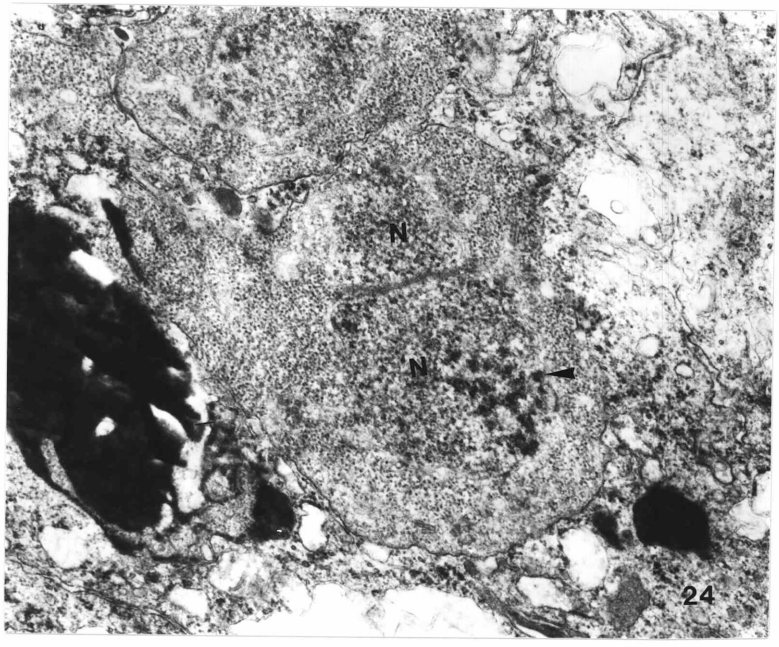
Figs. 24 - 29 Ultrastructural changes of *N. bombycis* induced by benomyl.

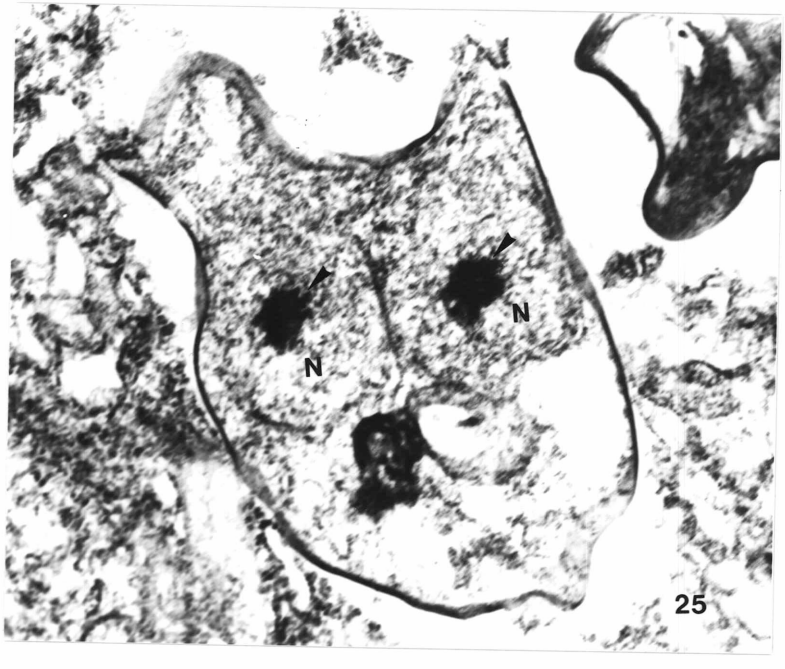
Fig. 24 Meront with diplokaryon nucleus (N). Note irregular masses of chromatin in the nuclei (arrow). x27100

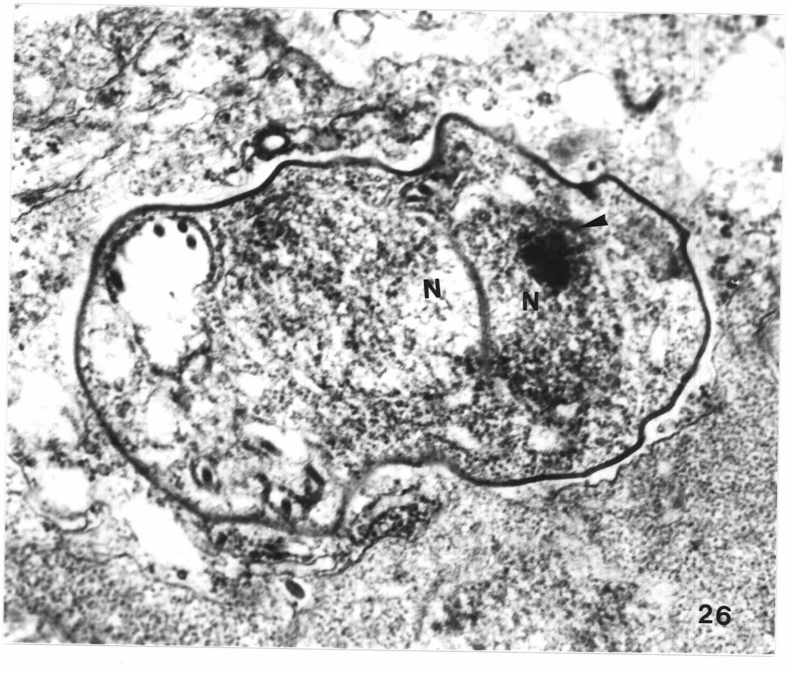
Figs. 25 & 26 Sporoblasts with diplokaryon nucleus (N). Note that chromatin is lumped into a single mass in the nuclei (arrow).

Fig.25 x28100

Fig.26 x34600


N
N
24


N
N
25

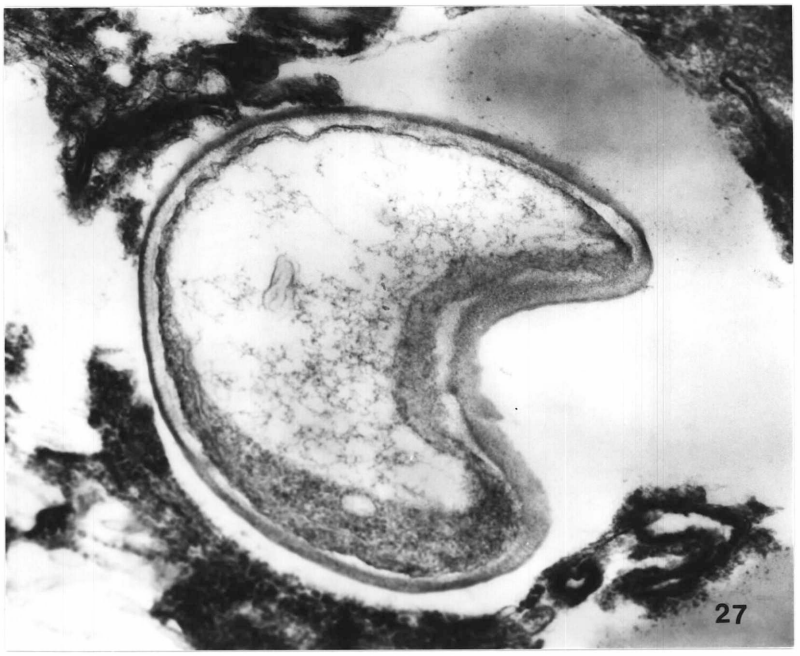

N
N
26

Figs. 27 - 29 Aberrant spores. Note the cytoplasm showed irregular vesicles and membrane whorls and often a large vacuole containing reticulate or tabular structures.

Fig.27 x33100

Fig.28 x40700

Fig.29 x35300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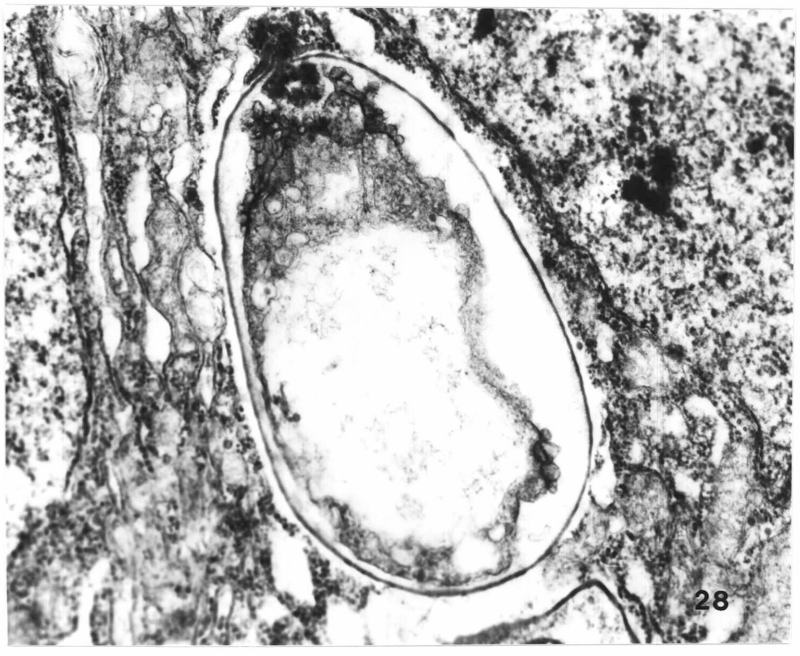
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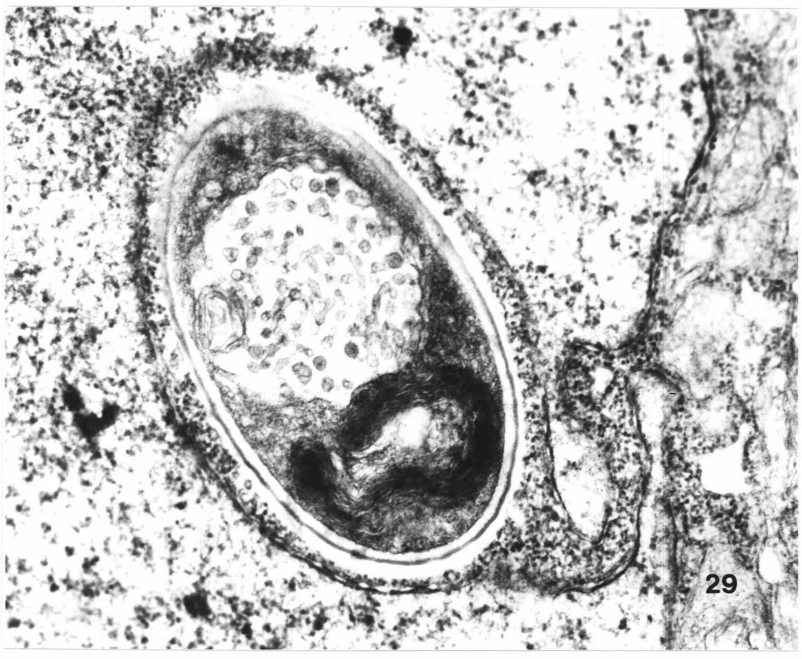
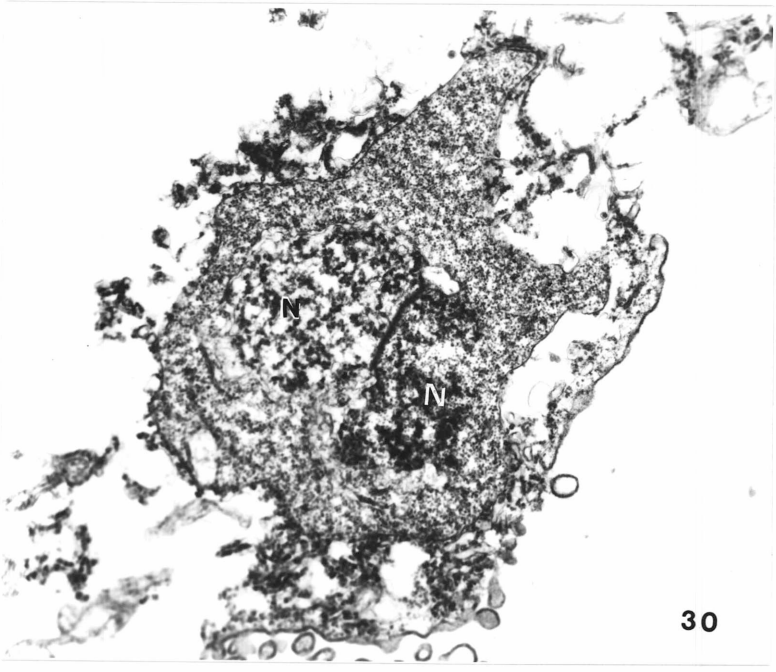

29

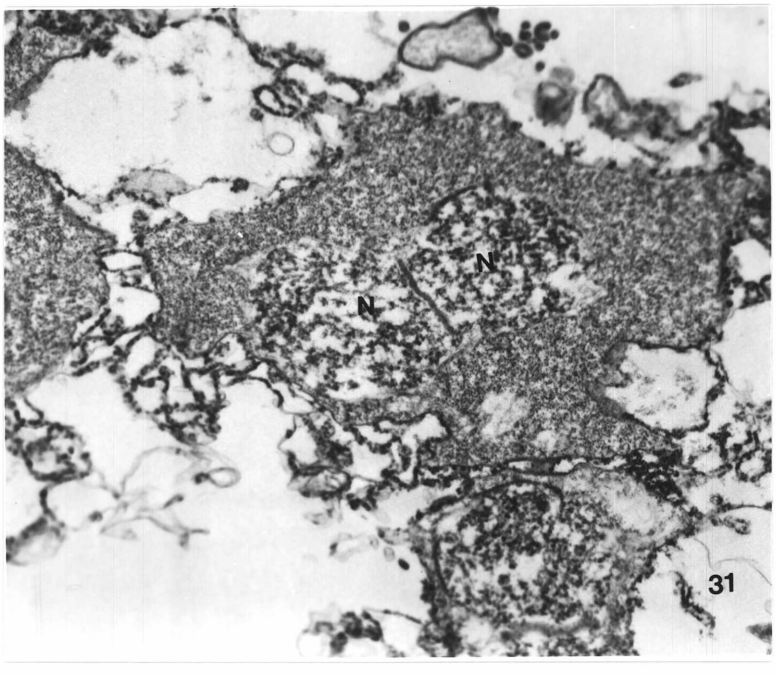
Figs. 30 - 34 Ultrastructural changes of *N. bombycis* induced by sinefungin.

Figs. 30 & 31 Meronts with diplokaryon nucleus (N). Note the nuclear material in the form of a network.

Fig.30 x19700

Fig.31 x17400


N
N
30

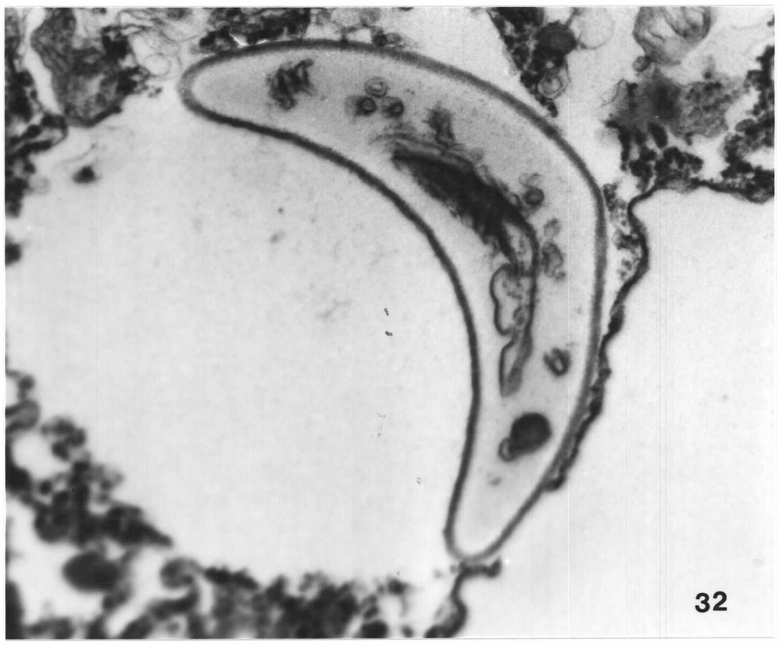

N
N
31

Figs. 32 - 34 Aberrant spores. Note that they are abnormal in shape and content.

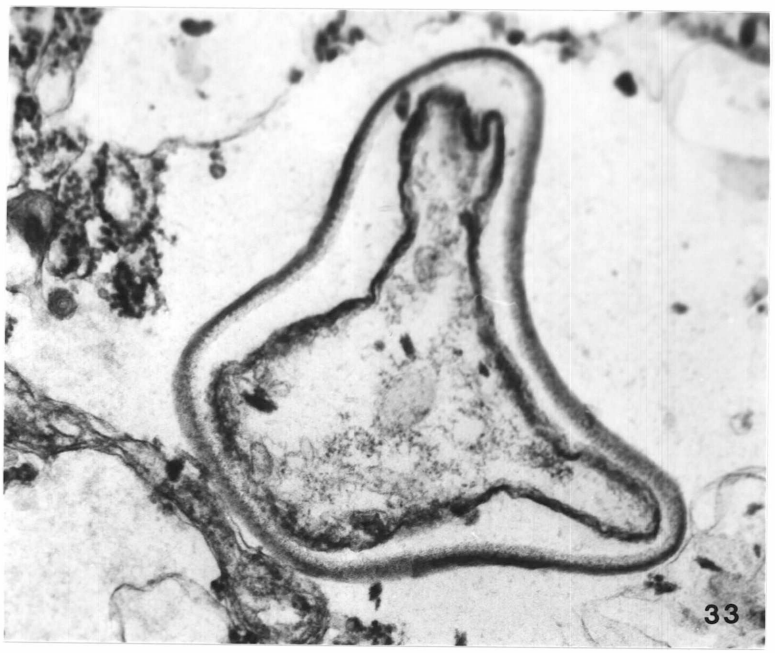
Fig.32 x41200

Fig.33 x48800

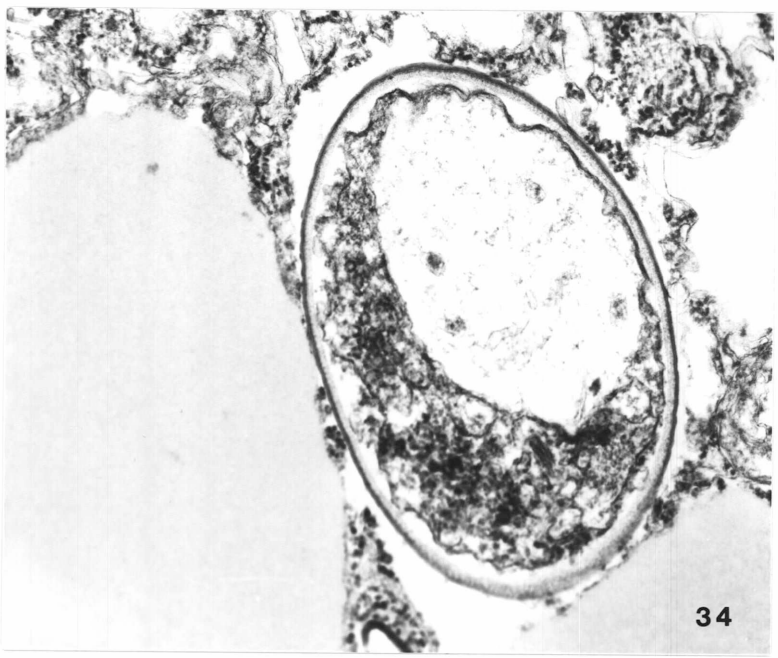
Fig.34 x65100



32



33



34

4.3.2.5 Fumagillin

Most of the stages present were sporonts, sporoblasts, and spores. The pre-spore stages showed signs of advanced disorganisation of nuclei and cytoplasm (Figs. 35, 36). The nuclei showed clumping of chromatin either as a single network or as several smaller networks distributed throughout the nucleoplasm. The nuclear envelope was not evident but as the membrane was not well preserved elsewhere, this might have been due to fixation. The cytoplasm contained abundant ribosomes but was abnormal in that it was permeated by irregular spaces. Spores were almost entirely abnormal in shape and content (Fig. 37).

4.3.2.6 Toltrazuril

Toltrazuril at 400µg/larva failed to alter the subcellular organisation of N. bombycis. No differences were observed between parasites in this group and the controls (Figs. 38, 39, 40). The stages observed were mainly meronts which were entering sporogony and the sporogonic stages. In pre-spore stages the cytoplasm contained abundant mono-ribosomes and cisternae of endoplasmic reticulum. The nuclei in diplokaryotic arrangement, appeared homogeneous with no sign of chromatin clumping. Mature spores did not fix well but in favourable sections of slightly immature spores the polar cap, polar filament with about 10 coils, nuclei and posterior vacuole were observed in an orderly arrangement. Cross sections of the polar tube indicated that the structure was normal (Fig. 40). These spores corresponded to the late stage spores described by Iwano and Ishihara (1989, 1991).

4.4 EFFECT OF DRUGS ON THE LARVAL AND PUPAL DEVELOPMENT OF Heliothis zea

4.4.1 Albendazole

The effects of albendazole on the larval and pupal developmental performances are presented in Table 35. The results revealed that albendazole even at 4mg/larva had no adverse effects on the larval and pupal development of H. zea. When uninfected larvae were fed albendazole treated diet and were compared with uninfected larvae fed on drug-free diet, there were no differences in larval period, development time, larval mortality, percent pupation, pupal weight and percent emergence.

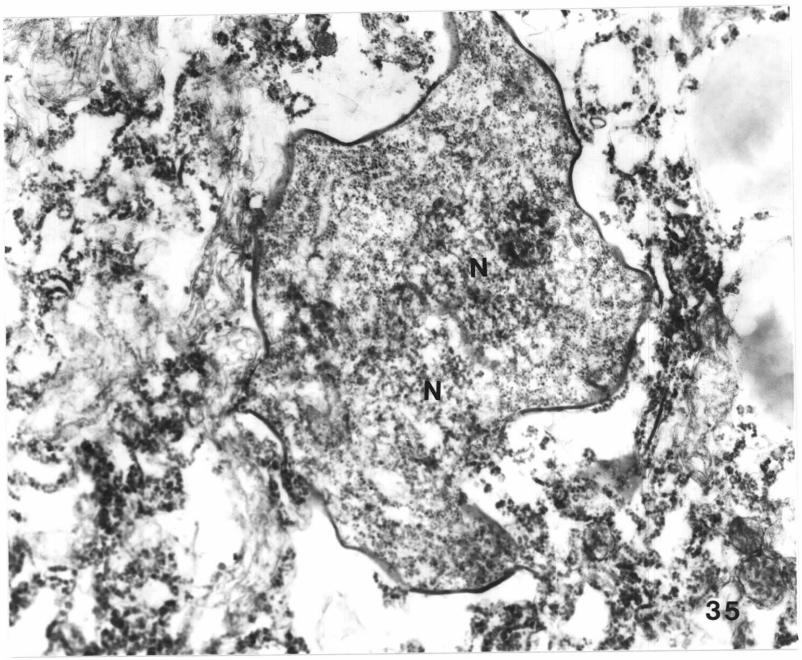
In the post-exposure experiment, as none of the larvae survived to pupation in infected-untreated group it was not possible to compare the

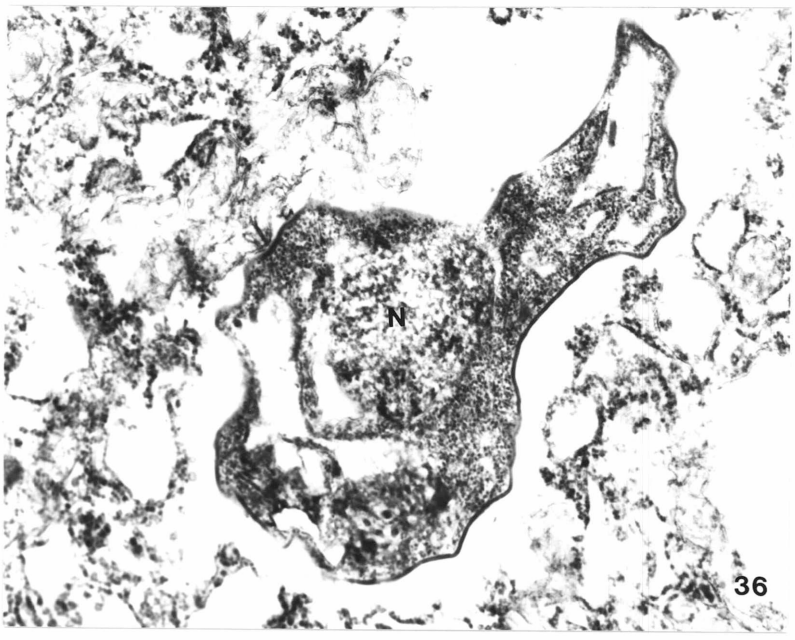
Figs. 35 - 37 Ultrastructural changes of *N. bombycis* induced by fumagillin.

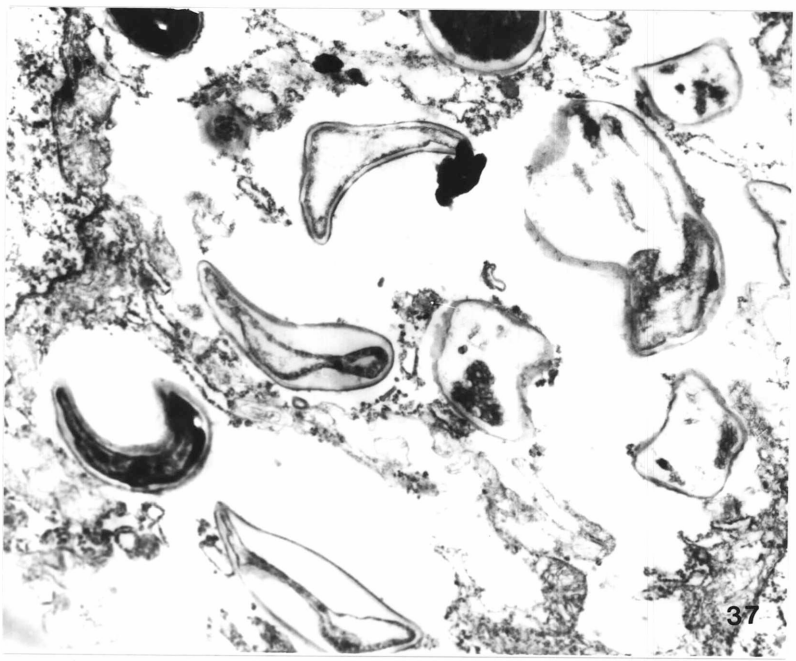
Fig. 35 Sporont with diplokaryon nucleus (N). Note the nuclear material in the form of a network. x23500

Fig. 36 Sporoblast. Note the nuclear material in the form of a network. x26800

Fig. 37 Aberrant spores. Note that they are abnormal in shape and content. x22500


N
N
35


N
36

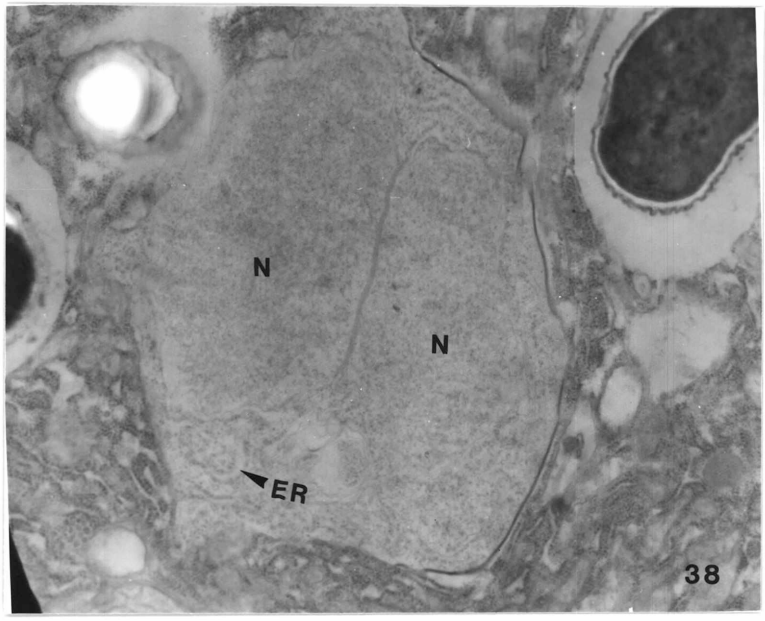

37

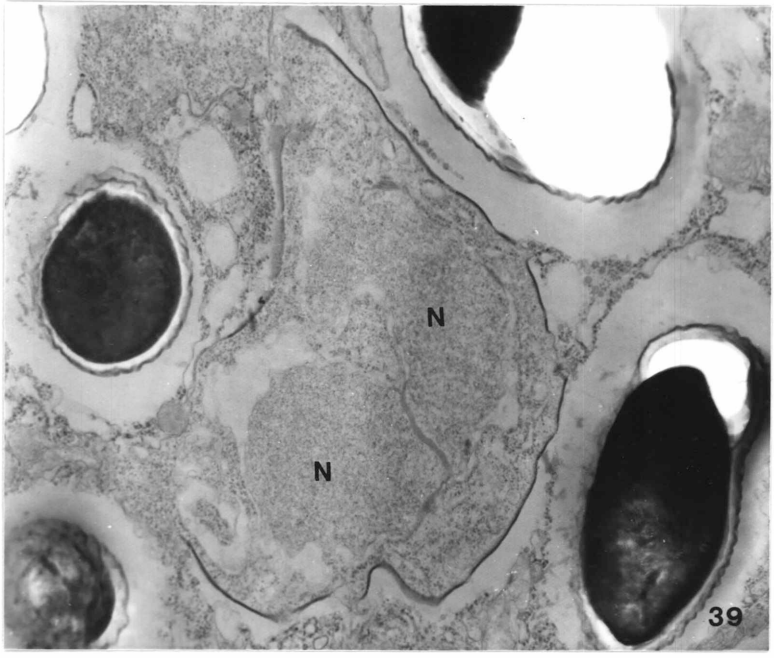
Figs. 38 - 40 Developmental stages of *N. bombycis* from midgut tissues of *H. zea* which consumed 400µg of toltrazuril.

Fig. 38 Meront (late) with diplokaryon nucleus (N) and endoplasmic reticulum (ER). x21700

Fig. 39 Sporont with diplokaryon nucleus (N). x21700

Fig. 40 Spore illustrating membranes of polaroplast (PL), coiled region of polar tube (PT), straight part of polar tube (SPT), part of polar sac (PS), exospore (Ex) and endospore (En). x29000


N
N
ER
38


N
N
39

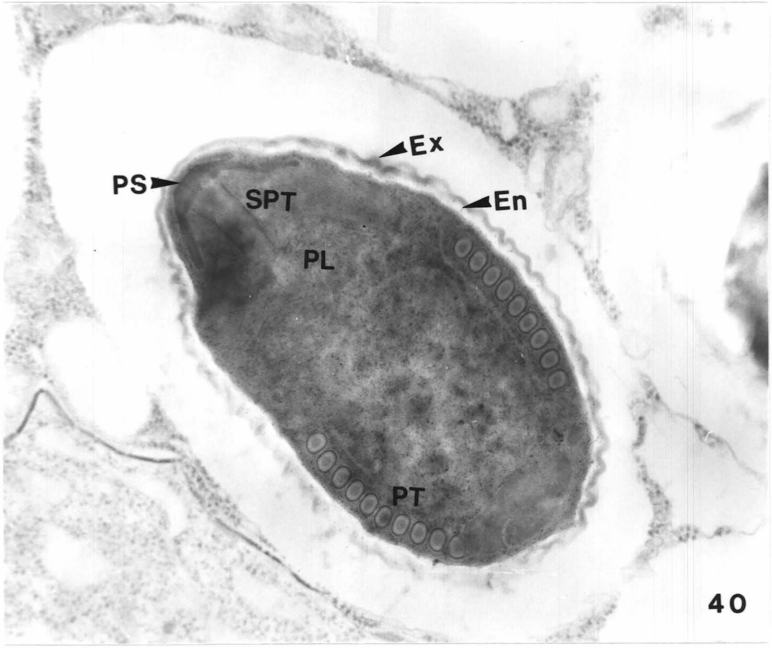

Ex
PS
SPT
En
PL
PT
40

Table 35 Effect of albendazole on the larval and pupal development of *H. zea*

Observations	Uninfected		Post-exposure experiment				Concurrent experiment			
			Infected				Infected			
	Untreated	Treated (4mg/L)	Untreated	Treated (1mg/L)	Treated (2mg/L)	Treated (4mg/L)	Untreated	Treated (1mg/L)	Treated (2mg/L)	Treated (4mg/L)
Larval periods (days)	16.2±0.1 (n=20)	16.4±0.1 (n=20)	-	19.7±0.2 (n=26)	19.6±0.1 (n=29)	19.4±0.1 (n=28)	20.1±0.3 (n=15)	17.6±0.2** (n=15)	17.2±0.1** (n=15)	17.0±0** (n=15)
Development time (days) (from hatch to emergence)	28.1±0.3 (n=19)	27.5±0.2 (n=20)	-	32.5±0.3 (n=14)	31.8±0.2 (n=17)	32.0±0.2 (n=17)	32.9±0.2 (n=9)	30.4±0.2** (n=9)	30.4±0.2** (n=9)	28.3±0.2** (n=9)
Percent larval mortality	0 (n=20)	0 (n=20)	13.3 (n=30)	13.3 (n=30)	3.3 (n=30)	6.7 (n=30)	0 (n=15)	0 (n=15)	0 (n=15)	0 (n=15)
Percent pupation	100 (n=20)	100 (n=20)	0 (n=24)	96.2 (n=26)	100 (n=29)	100 (n=28)	93.3 (n=15)	100 (n=15)	100 (n=15)	100 (n=15)
Pupal weight (mg)	419.2 ±9.1 (n=20)	416.1 ±10.2 (n=20)	-	431.5 ±6.3 (n=25)	427.0 ±5.4 (n=29)	425.3 ±6.5 (n=28)	373.4 ±10.8 (n=14)	411.0* ±8.9 (n=14)	410.6* ±12.3 (n=14)	422.3** ±5.7 (n=14)
Percent emergence	95 (n=20)	100 (n=20)	-	100 (n=14)	94.4 (n=18)	100 (n=17)	100 (n=9)	100 (n=9)	100 (n=9)	100 (n=9)

* Significant at the 5% level in comparison to infected-untreated group
** Significant at the 1% level in comparison to infected-untreated group

observations of infected-treated groups with infected-untreated groups. Compared to the uninfected-untreated group the larval period and development time were significantly longer in all infected-treated groups ($P<0.01$) (Student t-test). In the concurrent experiment the larval period and development time in all infected-treated groups were significantly shorter ($P<0.01$) than infected-untreated groups but were significantly longer ($P<0.01$) than uninfected-untreated groups except the development time at the highest dose which was not significantly different from uninfected-untreated groups (Student t-test). The pupal weight, in the concurrent experiment was significantly greater in the infected-treated groups than in the infected-untreated group (Student t-test). The difference at the highest dose was significant at 1% level and at the other two doses at 5% level. Compared to the uninfected-untreated group, the pupal weights in all infected-treated groups were not significantly different, either in the post-exposure or the concurrent experiments (Student t-test). There were no significant differences in percentage mortality, percent pupation and percent emergence between infected-treated groups and the uninfected-untreated group (Chi-square test).

4.4.2 Benomyl

The effects of benomyl on the larval and pupal developmental performances are presented in Table 36. In the post-exposure experiment the percentage larval mortality was significantly higher at the highest dose of benomyl (Chi-square test) ($P<0.01$) and no mortality was recorded at 2mg/larva treatment. In the concurrent experiment 26.7% larvae died at the highest dose of benomyl and none of the larvae died in other treatments.

All the larvae pupated when exposed to the lower 2 doses (1.0mg/larva and 2.0mg/larva) of benomyl in the concurrent experiment and 92.9% and 90% pupated with the corresponding doses in the post-exposure experiment. In the concurrent experiment 63.4% pupated when exposed to the 4mg/larva dose and in the controls 80% pupated: these figures were both significantly lower than the figures for the lower doses of drug (Chi-square test) ($P<0.01$). In the post exposure experiment a surprisingly low number of larvae pupated in the untreated (control) group, i.e. 39.3%. Consequently the pupation rate in groups exposed to all levels of drug were significantly higher (Chi-square test) ($P<0.01$).

Table 36 Effect of benomyl on the larval and pupal development of *H. zea*

Observations	Post-exposure experiment				Concurrent experiment			
	Infected				Infected			
	Untreated	Treated (1mg/L)	Treated (2mg/L)	Treated (4mg/L)	Untreated	Treated (1mg/L)	Treated (2mg/L)	Treated (4mg/L)
Percent larval mortality	6.7 (n=30)	6.7 (n=30)	0 (n=30)	36.7** (n=30)	0 (n=15)	0 (n=15)	0 (n=15)	26.7 (n=15)
Percent pupation	39.3 (n=28)	92.9** (n=28)	90.0** (n=30)	89.5** (n=19)	80 (n=15)	100** (n=15)	100** (n=15)	63.4 (n=11)
Percent deformed pupae	17.9 (n=28)	7.1 (n=28)	10 (n=30)	5.3 (n=19)	0 (n=15)	0 (n=15)	0 (n=15)	18.2 (n=11)
Pupal weight (mg)	386.2±12.3 (n=10)	419.3±12.9 (n=10)	422.8±13.7 (n=10)	385.5±6.8 (n=10)	364.8±10.8* (n=10)	395.4±8.9 (n=10)	399.1±8.9 (n=10)	313.3±15.5 (n=7)
Percent emergence	-	87.5 (n=16)	100 (n=17)	71.4 (n=7)	100 (n=7)	100 (n=10)	100 (n=10)	-

* Significant at the 5% level
** Significant at the 1% level

With regard to deformities of pupae, in the concurrent experiment 18.2% deformities were recorded at the 4.0mg/larva drug treatment but none at the lower doses or in the controls. In the post-exposure experiment 5.3% of pupae were deformed with 4mg/larva, 10% with 2mg/larva and 7.1% with 1mg/larva, none of which were significantly less than in the control (17.9%) (Chi-square test).

With regard to pupal weights, in the post-exposure experiment the weights in the 1mg/larva and 2mg/larva drug-treated groups were higher than the controls and the weights of those in the 4mg/larva group were about the same as the controls but the differences between groups were not significant (Student t-test). In the concurrent experiment in comparison to control the weights were significantly higher in the lower drug-treated groups ($P<0.05$) but significantly lower ($P<0.05$) in the high drug-treated group (Student t-test).

All the pupae emerged in the control group and groups receiving 1mg/larva and 2mg/larva in the concurrent experiment. At the 4mg/larva dose only 2 pupae remained and both emerged as adults (data not shown). In the post exposure experiment 100% of pupae emerged as adults after treatment with 2mg/larva and the figures for 1 mg/larva and 4 mg/larva treatments were 87.5% and 71.4% respectively. These figures did not differ significantly from one another (Chi-square test). In the untreated groups the single pupae that remained failed to emerge (data not shown).

5. DISCUSSION

5.1 SOLUBILITY OF DRUGS

The main problem of testing drugs *in vitro* is their insolubility in culture medium. Usually the pH of culture medium varies from slightly acid to slightly alkaline. The culture medium used in this study was TC-100, which is adjusted to pH 6.2. Some drugs have very narrow solubility pH ranges. Again there are some drugs which are insoluble in water but can be dissolved in chemical solvents. Sometimes the drug-solvent solution precipitates out again when added to culture medium or the amount of solvent needed to dissolve the drug is toxic to cell cultures.

In the present study, out of 20 drugs considered for testing against *N. bombycis*, 11 drugs namely, toltrazuril, albendazole, itraconazole, compound 566C80, rimocidin sulphate, novobiocin, streptolydigin, benomyl (Benlate), cerulenin, sulphathiazole and sulphapyridine are water insoluble. All except cerulenin, sulphathiazole and sulphapyridine are soluble in Dimethyl sulfoxide (DMSO). Only rimocidin sulphate remained in solution when the drug-solvent solution was added to culture medium. The other drugs are practically insoluble under acidic conditions.

Haberkorn and Stoltefuss (1987) reported that the drug toltrazuril is normally water insoluble, but a water soluble form can be made in alkaline pH. Bogan and Marriner (1984) reported that the limiting factor in the use of albendazole is its low water solubility, a maximum concentration of 0.2µg/ml can be achieved using phosphate buffer at pH 7.4. In the present study small amounts of toltrazuril, albendazole, itraconazole and compound 566C80 were dissolved in culture medium after sonication. The highest concentrations achieved in culture medium were 29.94, 5.3, 5.9 and 11.88µg/ml for toltrazuril, albendazole, itraconazole and compound 566C80, respectively. However when novobiocin, streptolydigin and benomyl precipitated out of the solvent on addition to culture medium it was not possible to redissolve them even after sonication. Windholz (1976) cited that streptolydigin is water insoluble and novobiocin only soluble in alkaline conditions, above pH 7.5. Sohi and Wilson (1979) tested benomyl *in vitro* against *N. disstriae* in *M. disstria* haemocyte cell cultures. They used foetal bovine serum to dissolve the drug. In this study Benlate, the commercial preparation of benomyl was used and this was not soluble in foetal calf serum. The manufacturer of Benlate (DuPONT) noted that this fungicide is water insoluble.

The drug sulphathiazole is soluble in hydrochloric acid (HCL) but the amount of HCL needed to dissolve the drug was found to be toxic to the cell cultures. Sulphapyridine and cerulenin are soluble in 2N NaOH and absolute alcohol, respectively. The amount of these solvents needed to dissolve the drugs are not toxic to cell cultures and the drugs did not precipitate out of solvent when added to culture medium. The drugs benzimidazole, pentostam and thimerosal are water soluble and when tested remained in solution over a wide pH range, from acidic to highly alkaline conditions.

5.2 EFFECT OF DRUGS ON Nosema bombycis

5.2.1 Albendazole

Albendazole is a broad spectrum anti-parasitic agent effective against protozoa (Dieckmann-Schuppert and Franklin, 1989; Meloni et al., 1990), cestodes (Horton, 1989) and nematodes (Cline et al., 1984; Maisonneuve et al., 1985). In the present study albendazole also showed potent anti-microsporidial activity against N. bombycis both in vitro and in vivo. Albendazole at a concentration of 5.3µg/ml reduced the level of infection of the parasite in S. frugiperda cell cultures by 62% within 7 days and kept the infection level static throughout the remaining 14 days of the experimental period. However, replacement of drug-treated medium with drug-free medium resulted in an increase of the level of infection.

In H. zea, albendazole at 2mg and 4mg/larva in the post-exposure experiments and at 1mg and 2mg/larva in concurrent experiments almost completely eliminated the parasite from the mature larvae. However, low level infections were recorded in the pupal stage suggesting that a few parasites persisting in the larval stage could multiply after feeding on diet containing drug ceased. The drug at 4mg/larva in concurrent experiments completely eliminated the parasite from the mature larvae as well as from the pupae. Thus, it would appear that the drug was more effective when given concurrently with the infection than when the infection was well established before exposure to drug. N. bombycis infections begin in the gut cells and spread to other tissues of the insect-host. Thus drug absorbed into the gut epithelial cells could prevent the development of sporoplasms when inoculated into gut epithelial cells. Bogan and Marriner (1984) reported that gastrointestinal parasites were more easily killed by albendazole than were parasites with a predilection for sites in the systemic compartment. Therefore, the drug is

likely to be more effective against these microsporidia when infections are limited to the intestinal tract. Recently, the effectiveness of albendazole against human microsporidia, Enterocytozoon bieneusi in patients with AIDS was reported at the Westminster Hospital, London (Dr. C. Blanshard, personal communication).

The primary mode of action of benzimidazole compounds including albendazole, is to bind to the colchicine-sensitive sites of tubulin, inhibiting its polymerisation in to microtubules (Lacey, 1990), thus interfering with nutrient uptake, cell division and maintenance of cell shape. The ultrastructure studies of albendazole treated N. bombycis revealed the presence of irregular masses of chromatin in the nuclei of meronts. Possible explanations are:

(1) the chromatin had condensed for nuclear division but in the absence of spindle fibre, failed to align in metaphase or separate at anaphase;

(2) chromosome separation and nuclear division may have taken place before the drug was effective but the chromosomes were unable to decondense in the nucleoplasm.

The aggregation of chromosomes into irregular masses due to failure of proper nuclear division has been reported in benomyl and carbendazim treated fungi (Davidse, 1973; Richmond and Phillips, 1975). These drugs are also benzimidazole compounds. Davidse (1973) suggested that carbendazim might inhibit mitosis by interfering with spindle formation, while Richmond and Phillips (1975) suggested that this fungicide might induce chromosome stickiness. As cell division in microsporidia takes place both in merogony and sporogony, the drug could inhibit proliferation of numbers and interfere with the further development into spores of the stages committed to sporogony.

5.2.2 Benomyl

Hsiao and Hsiao (1973) were the first authors to report on the anti-microsporidial activity of benomyl, when they found that it completely eliminated a Nosema sp. from larvae of the alfalfa weevil Hypera postica. However, the possible reappearance of the microsporidia in pupae and adults was not mentioned. Since then several workers have confirmed that benomyl will suppress microsporidial infections e.g. Armstrong (1976). Brooks et al. (1978) reported that infection of N. heliothidis was completely eliminated

from mature larvae of H. zea after continuously exposing infected larvae to benomyl treated diet but low level of infections were recorded during pupal and adult stages. Harvey and Gaudet (1977) and Briese and Milner (1986) also reported that benomyl suppressed microsporidian infections for as long as treatment was given but the microsporidian resurged during the non-feeding pupal stage.

In the present study, benomyl at all the doses tested, almost completely eliminated the infection of N. bombycis in mature H. zea larvae. However, examination of pupae revealed recrudescence of the microsporidiosis. A contrasting result was obtained by Mussen and Furgala (1975) who did not find benomyl efficaceous in controlling Nosema disease in honey bees.

Carbendazim, a breakdown product of benomyl is reported to be the compound mainly responsible for the fungitoxicity of benomyl (Clemons and Sisler, 1969; Sims et al., 1969; Fuchs et al., 1972; 1974). On further examination Clemons and Sisler (1971) observed that benomyl interferes with DNA synthesis or with some closely related process such as nuclear or cell division in fungi. Davidse (1973) found that the chromatin occurred as irregular masses in fungi after treatment with carbendazim and concluded that carbendazim inhibits mitosis by interfering with spindle formation. Richmond and Phillips (1975) also observed similar chromatin aggregates in fungi after treatment with carbendazim or benomyl and suggested that these fungicides inhibit mitosis, probably by inducing chromosome stickiness.

A clue to the mode of action of benomyl in microsporidia was provided by Brooks et al. (1978) who found nuclear aberrations in meronts and aberrant spores which were visible by light microscopy of Giemsa stained and fresh prepartions of N. heliothidis in H. zea after benomyl treatment. In the present study of the effects of benomyl, electron microscopy revealed in N. bombycis the same kind of chromatin formations in the nuclei of dividing stages, as previously observed in fungi. The chromatin aggregates were similar to those seen in N. bombycis after albendazole treatment.

It is possible in microsporidia that benomyl acts on tubules in the same way as albendazole. Thus, the principle effect will be on nuclear division, probably by inhibiting spindle microtubule formation. It is not clear what was meant by "stickiness" of chromosomes as proposed by Richmond and Phillips (1975).

5.2.3 Sinefungin

Sinefungin, isolated from cultures of Streptomyces griseolus (Hamill and Hoehn, 1973) is a potent antifungal (Gordee and Butler, 1973) and antiviral agent (Pugh et al., 1978; Pugh and Borchardt, 1982). Recently, the antibiotic was shown to display antiprotozoal activity, being effective against Plasmodium falciparum (Trager et al., 1980; Messika et al., 1990), Leishmania spp. (Bachrach et al., 1980; Neal et al., 1985, 1989; Avila et al., 1990), Trypanosoma spp. (Nadler et al., 1982; Dube et al., 1983; Zweygarth et al., 1986; Zweygarth and Röttcher, 1989), Toxoplasma gondii (Ferrante et al., 1988) and amoebae (Ferrante et al., 1984, 1987; Gupta et al., 1986, 1987).

In the present study the antibiotic also showed activity against N. bombycis both in cell cultures and in insects. In cell culture, the antibiotic at 100µg/ml in medium prevented spread of infection by maintaining the infection at the initial level throughout the experiment. At concentrations of 250 and 500µg/ml in medium it reduced the infection from the initial level by the 3rd day post-exposure and kept the infection static throughout the rest of the experimental periods. In H. zea, sinefungin at 5, 10 and 25µg/larva significantly reduced the production of N. bombycis spores compared to the controls.

Vávra et al. (1991) found that sinefungin at 100µM in 50% sugar solution blocked completely the development of N. apis in Apis mellifera and led to its elimination from the bees. Unfortunately they found that at this concentration the drug was toxic to the bees, particularly to newly emerged bees. In the present study, sinefungin also exhibited toxicity against H. zea larva when used at 25µg/larva and above. However, the toxicity might have been exacerbated by the method of administration of the drug to the insect. Larvae were exposed to a very small piece of drug-treated food which was consumed by the larva within a few hours. Intake of large amounts of drug over a short period might enhance toxicity. This could be minimized by exposing larvae to lower doses of drug-treated food for longer periods and this might also help to increase the effectiveness of the drug against the parasite.

In studies on the mode of action of sinefungin, it was found that it inhibited mRNA methylation in viruses (Pugh et al., 1978; Pugh and Borchardt, 1982) and both protein and tRNA methylation in eukaryotic cells (Vedel et al., 1978; Borchardt et al., 1979; Vedel and Robert-Gero, 1981). However, Paolantonacci et al. (1985, 1986) found no such inhibition in Leishmania

species. Lawrence and Robert-Gero (1986) reported that it was DNA synthesis in Leishmania that was inhibited and Paolantonacci et al. (1987) emphasised that it was particularly nuclear DNA synthesis. Messika et al. (1990) also found that sinefungin inhibited DNA synthesis in studies on P. falciparum. In the present study electron microscopy revealed entire nuclear disorganisation in meronts which clearly indicated that sinefungin inhibits DNA synthesis in N. bombycis. Such inhibition led to the production of aberrant spores.

Messika et al. (1990) also found that inhibition of DNA synthesis in P. falciparum was not due to a decrease in the amount of DNA polymerase, but to the depletion of polyamines which were required for DNA synthesis. Nolan (1987) found no inhibitory activity of sinefungin on DNA polymerase in Leishmania. Knutson and Morris (1978) reported that the rate of DNA synthesis in isolated lymphocyte nuclei was reduced by polyamine depletion. Thus, it is clear that sinefungin inhibits DNA synthesis in protozoan parasites by interfering with polyamines biosynthesis.

The main routes of polyamine biosynthesis in animals and micro-organisms originate from ornithine and methionine. Ornithine is decarboxylated by ornithine decarboxylase to yeild putrescine. Methionine is phosphorylated and combined with adenosine to form S-adenosylmethionine (SAM). Decarboxylated SAM, generated through SAM decarboxylase, provides aminopropyl groups for spermidine and spermine synthesis. The latter are formed by the addition of one or two aminopropyl groups to putrescine, the reaction being catalyzed by spermidine and spermine synthase, respectively. There are two possible ways for sinefungin to interfere with polyamine biosynthesis:

(1) the antibiotic might interfere with the SAM decarboxylase enzyme so that the phosphorylated product of methionine fails to provide aminopropyl groups for spermidine and spermine synthesis;

(2) the antibiotic might interfere with spermidine and spermine synthase, two important enzymes in polyamine biosynthesis, so that putrescine fails to synthesise spermidine and spermine.

As Gupta et al. (1987) and Messika et al. (1990) found no inhibitory effect of sinefungin on SAM decarboxylase, the inhibition of polyamine biosynthesis must be due to its interference with the activity of the two main enzymes, spermidine and spermine synthase. Hibasami et al. (1980) confirmed that sinefungin acts directly on spermidine and spermine synthases.

Still there remains the question of the presence of polyamine in microsporidia. There are no records so far of the existence of polyamine in microsporidia. However, their existence would not be unexpected, as polyamines have already been reported from several protozoan parasites (Bacchi, 1981; Messika et al., 1990).

5.2.4 Fumagillin

Fumagillin showed marked anti-microsporidial activities against N. bombycis both in vitro and in vivo. The antibiotic as low as 5µg/ml reduced the level of infection of the parasite in S. frugiperda cell cultures by 86.4% within 7 days. However, the dose failed to eliminate the parasite completely, even when the cultures were exposed to drug for up to 5 weeks. There was no further decrease in infection from the level achieved at 2 weeks exposure to drug although observations were made up to 5 weeks. When drug-treated medium was replaced with drug-free medium, the level of infection sharply rose again. Kurtti and Brooks (1976, 1977) reported that fumagillin suppressed the development of N. disstriae in H. zea cell cultures as long as the antibiotic remained in the culture medium but resurgence of infection took place after replacement with antibiotic-free medium. Similar observations were made by Shadduck (1980) using the drug against Encephalitozoon cuniculi in rabbit kidney or canine embryo cell cultures. Bayne et al. (1975) also failed to eliminate the Pleistophora-like microsporidian infections from Biomphalaria glabrata cell cultures, even when the cultures were exposed for 15 weeks to 50 µg/ml fumagillin in culture medium. In contrast, Sohi and Wilson (1979) obtained complete elimination of N. disstriae from M. disstria haemocyte cultures within 5 weeks with fumagillin as low as 2µg/ml of culture medium.

In H. zea larvae, fumagillin showed anti-microsporidial activities against N. bombycis in a dose-dependent fashion. The antibiotic at the higher two doses tested significantly reduced the production of spores of the parasite in comparison with the controls. Due to unavailability, the drug could not be tested by exposing larvae to the drug-treated diet throughout their larval period as had been done with other drugs. This was unfortunate because it would be anticipated that the drug would be more efficient if given for long periods.

Numerous experiments have been conducted using fumagillin for controlling Nosema disease of bees (e.g. Bailey, 1953b, 1955; Katznelson et al., 1955; Mommers, 1957; Furgala and Boch, 1970). The results in every case

have indicated that the antibiotic showed potent anti-Nosema activity but failed to eliminate the disease completely from bee colonies. The suppressive effects of the antibiotic against microsporidian infections in other insect host-parasite relationships have also been reported by several workers (Lewis and Lynch, 1970; Lewis et al., 1971; Lynch and Lewis, 1971; Flint et al., 1972; Wilson, 1974; Armstrong, 1976; Thomas, 1977).

Jaronski (1972) demonstrated by cytochemically that RNA had almost completely disappeared from the cytoplasm of sporonts and sporoblasts of N. apis after treatment with fumagillin, while the DNA content remained unchanged. Nuclei in both treated and untreated parasites appeared the same with all the staining protocols. The result of the present study is somewhat contradictory with these findings. Electron microscopy clearly revealed complete nuclear disorganisation in the fumagillin treated sporonts and sporoblasts of N. bombycis. If fumagillin acts on RNA content, this should be visible at the ultrastructural level by ribosome depletion but this was not observed. The cytoplasm appeared normal which suggests that the drug was also not acting at the level of translation at the ribosomes. Thus, the mechanism of action of fumagillin appeared to be at the DNA level which concurs with the result of Hartwig and Przelecka (1971) who cytochemically and autoradiographically proved that fumagillin arrested DNA replication in N. apis. Development of abnormal spores, in the present study, might be due to the inhibition of DNA transcription leading to complete failure of the cell to synthesise spore organelles. Liu (1973) reported that Fumidil-B might inhibit lipid metabolism and thus interfere with spore membrane development in N. apis. Although the spore wall is not known to have a lipid component, there are numerous membrane systems in the spore in its extrusion apparatus - polaroplast, polar sac and polar tube. If lipid metabolism is inhibited, formation of these membranes would be disrupted. However, investigation of the mode of action of this drug, whether on DNA synthesis or transcription or on lipid metabolism, was beyond the scope of this study.

5.2.5 Toltrazuril

The results of the present study indicate that toltrazuril has no effect on N. bombycis. The drug at a concentration of 29.94µg/ml failed to affect the development of N. bombycis in the cell line even after exposure of

the cultures to the drug for up to one week. Toltrazuril at 100, 200 and 400 µg/larva also failed to eliminate the parasite from its experimental host, H. zea. Toltrazuril is water soluble at alkaline pH and the gut of the lepidopteran insect H. zea is also alkaline, pH 8.0 (Swingle, 1931, 1938). Although the drug precipitated in culture medium it should have remained in solution in the insect gut and be available for absorption into the midgut epithelial cells. Mehlhorn et al. (1984) and Schmahl and Mehlhorn (1989) reported that the drug toltrazuril could pass through host cell membranes and affect the development of intracellular parasites.

Mehlhorn et al . (1988) in a short communication reported that toltrazuril at 20µg/ml in sugar solution prevented death of honey bees (Apis mellifera), wasps (Pimpla turionellae), and flies (Drosophila melanogaster) infected with a species of Nosema. The gut of these insects was acidic, that of the honey bee for example, varying in different regions from pH 5.4 to 6.3 (Hoskins and Harrison, 1934). Under these circumstances the drug would normally precipitate from solution and not enter the gut epithelial cells to inhibit the development of Nosema. It is unlikely that toltrazuril controlled these infections by killing the extracellular stages in the gut lumen because these are spores with a highly resistant wall. It is now generally accepted that microsporidia infect new hosts after ingestion of spores by the discharge of the polar tube into gut walls and inoculation of the sporoplasm directly into host cell cytoplasm. There are, thus, no free stages in the gut lumen. Also the infections in bees were naturally occurring so it would have been necessary for the drug to enter the midgut epithelial cells to the already established intracellular stages.

In the present study, toltrazuril was tested against N. bombycis in H. zea by exposing already infected larvae to the drug, and by exposing uninfected larvae to the drug and spores concurrently. In both cases toltrazuril failed to reduce the parasite burden from its host.

Mehlhorn et al. (1988) also reported that naturally occurring Glugea cysts were cleared from sticklebacks (Gasterosteus aculeatus) within 2 - 4 h of incubating the fish in aerated aquaria containing toltrazuril at 10µg/ml. Glugea cysts are greatly hypertrophied host cells (xenomas) which develop subcutaneously. There are 2 possible routes by which the drug could reach the parasites in subcutaneous host cells: the drug may be absorbed through the skin of the host and then pass to the target cell, or the drug may be absorbed

through the intestinal tract and then pass to the target cell. In either case, after reaching the site of the xenomas, the drug has to pass through a "cyst wall" composed of up to 60 layers of surface coat sloughed off from the host cell. This wall may be 10µm thick. Nevertheless the wall is not impenetrable to small molecules because pinocytotic activity is noticeable at the true surface of the cell beneath the "cyst wall" showing that nutrients are absorbed for the continued growth of the xenoma. Whether toltrazuril can cross the xenoma wall may depend on the size of the molecule. Mature Glugea xenomas naturally drop off from the surface of infected fish. Although this could possibly account for the apparent effect of the drug, Schmahl and Mehlhorn (1989) reported that there were ultrastructural changes in the parasite with cysts on toltrazuril-treated fish.

In the present study, histological observations of the midgut tissues from the larvae which consumed 400µg of toltrazuril showed massive infections of the parasite. Electron microscopic observations of the midgut tissues from drug-treated larvae showed no alterations in host cell or parasite organisation. Haberkorn and Stoltefuss (1987) reported that toltrazuril is stable for 14 days at 70°C. Thus, addition of the drug to the diet at 60°C would not account for its lack of activity against N. bombycis. Lack of activity of toltrazuril against Nosema disease of honey bees was also found by Dr.J.Vávra (personal communication). The present results are in agreement with those of Dr. Vávra.

Harder and Haberkorn (1989) investigating the mode of action of toltrazuril in vertebrates reported that it primarily affects the respiratory chain and secondarily, some enzymes involved in pryimidine synthesis. As toltrazuril is effective as an anticoccidial drug, it would appear that these metabolic pathways are also affected in coccidia and possibly the metabolic pathways in microsporidia differ from those in coccidia and are unaffected by toltrazuril.

5.2.6 Thimerosal

Liu (1988) reported that thimerosal, the active principle of Nosemack caused ultrastructural changes in N. apis after treating the infected excised bee midgut with 5.0µg/ml of thimerosal in culture medium for 24 h in vitro. He noted that nuclei lacked chromatin and that the cytoplasm was depleted of ribosomes, even on the membranes of endoplasmic reticulum. He suggested that

thimerosal, which is a complex organic mercury compound, acted on the pre-spore stages of N. apis via the nucleic acids. There were, however, no effects on the spores. In the present study, thimerosal also exhibited some anti-microsporidial properties against N. bombycis in S. frugiperda cell cultures. Although the drug did not prevent an overall increase in the percentage of infected cells, concentrations of 2.0 and 5.0µg/ml significantly reduced the increase relative to controls during the first few days (3 days for the 2.0 µg concentration and 2 days for the 5.0 µg concentration). However, these concentrations were toxic to the cell cultures when they were exposed for longer periods to the drug. Thimerosal was also toxic to H. zea larvae at 100µg/larva and above. A concentration of 20µg/larva was not toxic but had no effect on N. bombycis.

The in vitro results of thimerosal against N. bombycis supported the in vivo findings of Mommers (1957), who found that Nosemack significantly prevented the increase of Nosema infection in Dutch bee colonies. Furgala and Boch (1970) found that Nosemack at the lower of three doses tested (100, 200, 400mg/l) had no effect on N. apis infection and that the highest dose suppressed the infection only slightly. However, at these doses thimerosal was sufficiently toxic to bees that the toxicity outweighted the benefits.

The present results support the findings of Furgala and Boch (1970) that drug toxicity outweighs benefit in reducing microsporidial infections.

5.2.7 Benzimidazole

Benzimidazole, an anti-helmintic agent showed marked anti-microsporidial activities against N. bombycis in S. frugiperda cell cultures. The drug at a concentration of 250µg/ml prevented further increase in the level of infection and at 500µg/ml reduced the infection from the initial level within 7 days. The drug, as low as 50µg/ml, also significantly reduced the microsporidian infection compared to controls. In contrast to its activity in vitro the drug at 1, 2, 4, and 10mg/larva failed to inhibit the development of the parasite in H. zea. Diet containing the highest dose tested was unpalatable to the larvae but the other doses were consumed but had no effect. Possible explanations for inefficacy of benzimidazole in vivo are that benzimidazole might have anti-microsporidial activities in acidic conditions but no in alkaline conditions, that benzimidazole might not be taken up into the midgut cells or that the temperature at which the drug was added to diet might have resulted in its loss of activity.

The cell culture medium used in this study is slightly acidic, having pH 6.2 and becomes progressively more acid as the cells grow. On the other hand, the gut of H. zea larvae is alkaline, pH 8.0. Solubility tests showed that the drug remained in solution over a wide pH range, from acidic to highly alkaline, pH 6 - 15. It is unlikely therefore that the drug precipitated in the insect midgut preventing its uptake into midgut cells.

Benzimidazole was tested both by exposing infected larvae to the drug and by exposing healthy larvae to the drug and parasites simultaneously. The drug would have to be taken up into the cells to exert an effect because it is unlikely to penetrate the resistant wall of spores which are the only stages present in the gut lumen. The most likely of the three explanations is that the temperature of 60°C, at which the drug was added to the diet affected its activity. However, the drug deserves to be tested against microsporidia under circumstances where it is not subjected to high temperature. This is one of a group of drugs with similar molecular structure and is related to albendazole which has also shown activity in this study. It would be advantageous to test other drugs in this group.

5.2.8 Pentostam

Pentostam, a potent anti-leishmanial drug showed some anti-microsporidial properties against N. bombycis in S. frugiperda cell cultures. The drug at 500 - 1,500µg/ml significantly reduced the percentage infection of the parasite in comparison with controls. The drug at 2,000µg/ml prevented further spread of infection of the parasite. However in larvae of H. zea, the drug at 4 and 8mg/larva had no effect on the development of the parasite.

As pentostam remains soluble over a wide pH range, from highly acidic to highly alkaline, no explanation can be given for the lack of activity in the alkaline gut conditions of H. zea larvae. As with the other drugs, pentostam was tested by exposing infected larvae to the drug and by exposing healthy larvae to the drug and parasites simultaneously. As with benzimidazole a possible explanation for the difference in activity in vitro and in vivo is that drug activity is altered at the temperature (60°C) at which the drug was added to insect diet.

Chulay et al (1985) reported that the drug altered the ultrastructure of amastigotes of Leishmania donovani causing a reduction in average size,

greater irregularity of the cell outline, and a moderate increase in the electron density of the cytoplasm associated with a greater concentration of ribosomes. They suggested that the drug affects active transport functions or permeability of the plasma membrane. Berman et al. (1985) found that when amastigotes of Leishmania mexicana were exposed to pentostam, there was a decrease in viability and in synthesis of RNA, DNA and protein. In addition, there was a decrease in net ATP formation but an increase in net AMP and ADP formation. They postulated that the anti-leishmanial mechanism of pentostam might be associate with the inability of the amastigotes to phosphorylate ADP. They concluded that the inability of the amastigotes to phosphorylate ADP to ATP might be due to the inhibition of glycolysis and the citric acid cycle. Later (1987) they confirmed that glucose catabolism via glycolytic enzymes and fatty acid β-oxidation, but not glucose metabolism via the hexosemonophosphate shunt or the citric acid cycle, was specifically inhibited in pentostam-exposed L. mexicana amastigotes.

No ultrastructural studies were carried out on the in vitro drug-treated N. bombycis and it is therefore not possible to relate drug activity to structural changes in the nucleus or cytoplasm. It is possible that inhibition of growth in the microsporidia by pentostam is also due to the inhibition of enzymes of the glycolytic pathway but as nothing is known about metabolism in microsporidia, no conclusions can be drawn.

5.2.9 Ciprofloxacin

Fluoroquinolones are potent antibacterial agents. Ciprofloxacin is one of this group of compounds. The mode of action of these drugs is to inhibit DNA synthesis during bacterial replication by interfering with topoisomarase II (DNA gyrase). Wang (1985) suggested that topoisomerases may be good targets for drugs in rapidly proliferating cells. This is the case for protozoan parasites. Thus, ciprofloxacin has a marked effect at achievable serum concentrations on Plasmodium falciparum in vitro (Divo et al., 1988; Krishna et al., 1988; Midgley et al., 1988) and in vivo on Leishmania donovani (Raether et al., 1989) and Plasmodium yoelii (Salmon et al., 1991).

In the present study ciprofloxacin also exhibited some anti-microsporidial properties against N. bombycis in vitro. The drug at 100 μg/ml in medium significantly reduced the percentage of infected cells compared with the controls although there was an overall increase from the initial level.

Concentrations of 250 and 500µg/ml prevented further spread of the parasite and gradually reduced the infection from the initial level during the period of observation. The concentrations required to inhibit development of N. bombycis in vitro were very high compared to achievable serum concentration. The peak serum concentration in man following a single 750mg oral dose of ciprofloxacin is 2.5 - 4.3µg/ml and levels do not increase on repeated dosage (North West Thames Regional Health Authority, Pharmacy Information Service, Westminster Hospital, London; personal communication). These concentrations had no effect on N. bombycis in vitro and thus preclude its use in vivo.

5.2.10 Imidocarb dipropionate

Imidocarb dipropionate has been effectively used for the treatment of babesiosis, anaplasmosis and theileriosis. Göbel and Dennig (1976) and Simpson and Neal (1980) reported that imidocarb affected the intraerythrocytic stages of Babesia. Neal and Croft (1984) also reported that imidocarb dipropionate has some effect on the intracellular amastigote form of L. donovani in vitro. Nathan et al. (1979) found that imidocarb completely eliminated Trypanosoma brucei infections from mice.

In this study imidocarb dipropionate was tested against N. bombycis in S. frugiperda cell cultures. The drug at 500µg/ml and over proved toxic to cell cultures and at 100 and 250µg/ml it had no effect on the development of N. bombycis.

5.2.11 Itraconazole

Itraconazole, one of the imidazole group of compounds has been reported as an effective antifungal agent (Espinel-Ingroff et al., 1984; Van Cutsem et al., 1984; Perfect et al., 1986; Denning et al., 1989; Viviani et al., 1989). The imidazole's mechanism of action against fungal pathogens is via a blockage of ergosterol synthesis and causes membrane damage (Sud and Feingold, 1981; Ansehn and Nilsson, 1984; Saag and Dismukes, 1988).

In microsporidia, Liu and Myrick (1989) reported that itraconazole caused ultrastructural changes in the spores of N. apis when infected bees were treated with itraconazole at 0.01µg/ml in sugar solution. They found that the drug inhibited the development of the polar filament, interfered with lipid metabolism and disrupted the integrity of the plasma membrane. Recently Yee et al. (1991) reported that a microsporidial keratopathy in an AIDS patient caused by Encephalitozoon hellem resolved after a few weeks of

treatment of the patient with 200mg itraconazole given orally twice daily. The patient had been treated previously for the eye infection with several antibiotics applied topically without success and the itraconazole was administered fortuitously for cryptococcal meningitis. However, they believed that debulking of the infection by corneal scraping may have contributed to the success by removing potentially drug-resistant spores.

In the present study itraconazole, which was tested *in vitro* at the maximum achievable concentration by sonication of 5.9µg/ml of culture medium, had no effect on *N. bombycis* in cell cultures. It remains for future work to determine whether the resolution that *E. hellem* infection was primarily due to itraconazole or to corneal scraping.

5.2.12 Metronidazole

Metronidazole is another of the imidazole group of drugs which has broad spectrum activity against bacterial anaerobes and against anaerobic protozoa, especially *Trichomonas*, *Giardia* and intestinal amoebae. Metronidazole inhibits DNA synthesis in *Trichomonas vaginalis* (Ings and Constable, 1975) and inhibits the synthesis of and degrades existing DNA in *Clostridium bifermentans* (Plant and Edwards, 1976). Knight *et al.* (1978) reported that reduced metronidazole caused a loss of DNA helix content, strand breakage, and a concomitant impairment of its function as an enzyme template. Recently Eeftinck Shattenkerk *et al.* (1991) reported that metronidazole was effective in controlling diarrhoea caused by the microsporidium *Enterocytozoon bieneusi* in human patients infected with HIV-1. Although in most of the patients there was improvement or even disappearance of the diarrhoea after metronidazole treatment, the drug did not eliminate the parasite which appeared normal in repeat biopsies.

In the present study metronidazole was tested at different concentrations against *N. bombycis* in *S. frugiperda* cell cultures. The drug, which is water soluble was used at concentrations up to 2mg/ml in culture medium but had no effect on the development of *N. bombycis* after exposure of the infected cultures to the drug for 7 days. Beyond this dose the drug was toxic to cell cultures. Atabekova (1981) found that metronidazole was ineffective in the treatment of pebrine disease of *B. mori* larvae caused by *N. bombycis*. Inefficacy of metronidazole against *Nosema* disease of honey bees was also reported by Moffett *et al.* (1969) and Hitchcock (1972) and the present study substantiates these results.

5.2.13 Novobiocin

The antimicrobial agent novobiocin ihibits DNA synthesis both in prokaryotic and eukaryotic cells. Smith and Davis (1967) reported that the primary effect of novobiocin in bacterial cell was on DNA synthesis. Gellert (1981) also reported that the antibiotic inhibited DNA synthesis in bacteria by interfering with topoisomerase II (DNA gyrase). Edenberg (1980) reported that novobiocin inhibited simian virus 40 DNA synthesis by antagonising DNA polymeraseα. Nakayama and Sugino (1980) found that novobiocin was antagonistic to DNA polymerase I and II and topoisomerase I in yeast. Recently, novobiocin was shown to inhibit the growth of the protozoan parasite, Trypanosoma cruzi (Pate et al., 1986) by inhibiting DNA synthesis probably by interfering with topoisomerase II (Kerschmann et al., 1989).

In the present study, novobiocin was tested at 4mg and 10mg/larva against N. bombycis in H. zea larvae but no effects were observed. The antibiotic is only soluble in alkaline conditions above pH 7.5 (Windholz, 1976). The gut of H. zea larvae is alkaline, pH 8.0 so the drug should have remained in solution and been taken up into midgut epithelial cells. This implies that some pathways in DNA synthesis of the microsporidia differ from those in organisms which are susceptible to the drug. However, nothing is known about DNA synthesis in microsporidia. The temperature ($60^{o}C$) at which the antibiotic was added to the diet might also have affected the efficacy of the drug.

5.2.14 Streptolydigin

Streptolydigin is a potent anti-bacterial antibiotic which affects RNA synthesis by interfering with RNA polymerase (Schleif, 1969; Cassani et al., 1971). Ishihara and Hayashi (1968) reported the presence of ribosomes with bacterial (prokaryotic) properties in N. bombycis. The ribosomes had sedimentation coefficients of 70S with subunits of 50S and 30S. Curgy et al. (1980) confirmed findings for Thelohania maenadis and Inidosporus sp. and added the information that the large and small subunits each have a single rRNA of 23S and 16S respectively. Vossbrinck and Woese (1986) showed that the nucleotide sequence of the 5.8S rRNA of eukaryotes is incorporated into the 23S rRNA of the microsporidium Vairimorpha necatrix as it is in prokaryotes and Vossbrinck et al. (1987) showed that the 16S rRNA has little sequence homology with eukaryotes.

As streptolydigin inhibits RNA synthesis in bacteria, it was anticipated that it might inhibit RNA synthesis in microsporidia but the result of the present study indicates that the antibiotic has no effect on N. bombycis when tested in H. zea larvae. Possible explanations are: RNA polymerase in microsporidia may differ from that of bacteria or the temperature (60°C) at which the antibiotic was added to the diet affected its activity.

5.2.15 Sulphadimidine

Sanyal et al. (1985) reported that the coccidiostat sulphadimidine was effective against schizonts and early gamonts of Eimeria bareillyi. In the present study the anti-coccidial drug was tested at 100, 300, 600, and 900 µg/ml against N. bombycis in S. frugiperda cell cultures. The drug up to 600 µg/ml failed to show any effect on the development of the parasite and at 900 µg/ml was toxic to the cell cultures.

5.2.16 Sulphapyridine

The anti-coccidial drug sulphapyridine was tested at 50, 100, 250, 500 and 1,000µg/ml against N. bombycis in S. frugiperda cell cultures. The drug at all the doses tested had no effect on the development of N. bombycis up to 7 days post-exposure.

5.2.17 Sulphathiazole

Razbitskii et al. (1990) reported that sulphathiazole exhibited coccidiocidal effects probably by interfering with protein biosynthesis. An attempt was made to test sulphathiazole against N. bombycis in vitro. Tested doses were 50, 100, 250, 500 and 1,000µg/ml of culture medium. Hydrochloric acid (HCl) was used as the solvent. As only one control was incorporated into the test, i.e. 5% HCl, this concentration of acid was used with all the concentrations of drug. This was found to toxic to the cell cultures. In order to test whether the lowest dose of drug (50µg/ml), would be effective if dissolved in less HCl, it was tested in 2 wells containing 0.25% HCl (the minimum amount of acid which could be used to solubilise the drug) but even this concentration of acid was toxic to the cultures.

5.2.18 Rimocidin sulphate

The antibiotic rimocidin is derived from Streptomyces rimosus and has very low bactericidal properties but is actively fungicidal against most of the pathogenic fungi. The drug is known to interfere with membrane function (Gottlieb and Shaw, 1967). It also has in vitro activity against certain protozoa as reported by Seneca et al (1952) who found that it was active against Entamoeba histolytica, Trypanosoma cruzi, Leishmania donovani and Leishmania tropica. For this reason it was tested against N. bombycis in cell cultures but the results showed that it had no effect.

5.2.19 Cerulenin

Nomura et al. (1972) reported that cerulenin inhibited the growth of fungi by affecting lipid metabolism and evidence has been provided that other fungicides affect lipid metabolism in microsporidia as well. Thus, lipid metabolism of N. apis was impaired by fumagillin (Vandermeer and Gochnauer, 1971; Liu, 1973) and by itraconazole (Liu and Myrick, 1989). For this reason cerulenin was added to the list of drugs tested against N. bombycis in cell cultures. However, it was found to be toxic to the host cells at high concentrations and lower concentraions had no effect on the development of the parasite.

5.2.20 Compound 566C80

Recently, this compound (566C80) was shown to be effective against malarial parasites (Wellcome Foundation, personal communication). The results of this drug against N. bombycis in vitro indicated that the drug has no anti-microsporidial properties. The drug is thought to act against mitochondria in eukaryotic cells (Dr. W.E. Gutteridge, personal communication) and since microsporidia have no mitochondria the results is as expected.

5.2.21 Effect of combined drugs

This experiment was carried out to see whether there existed any synergistic effects between two drugs. Sinefungin, benzimidazole, albendazole and fumagillin were considered for combined treatment because these drugs had previously been shown to have marked anti-microsporidial activities in vitro. The lowest dose of each drug which produced reductions in the percentage of infected cells which were not significantly different from those produced by the highest dose were selected for the test with combined drugs. However, with albendazole a concentration of 2.5µg/ml was selected although this dose had

given no significant reduction when used on its own. This was the maximum concentration which could be achieved in medium after dissolving in DMSO and mixing with medium containing the second drug.

No additive or synergistic effects were seen except with albendazole and sinefungin which were additive when used up to 7 days. As this combination showed some toxicity on the 7th day post-exposure and sinefungin alone was toxic to H. zea larvae at lower doses, the practicality of using the combined drugs in vivo for long periods is questionable.

5.3 EFFECT OF DRUGS ON INSECT HOST

5.3.1 Albendazole

The results of tests with albendazole on larval and pupal development of H. zea indicate that the drug has no adverse effects on the insect host. No differences were recorded in all the larval and pupal parameters studied between uninfected insects fed with diet containing the highest dose and those fed diet without drug. In the infected drug-treated groups the larval period and total development time were slightly longer than those of the uninfected-untreated counterparts and these differences were significant. This increase was probably due, not to the effect of the drug but to the effects of the parasite. Increased developmental times of H. zea, due to microsporidian infections have been reported by several workers (Gaugler and Brooks, 1985; Teakle, 1977; Darwish, 1985).

In the concurrent experiment using the highest drug dose, the total development time was not significantly different from the uninfected-untreated group. It should be recalled that this dose completely eliminated the parasite from the insect host. There were no significant differences between infected-treated groups and uninfected-untreated groups in respect of larval mortality, pupation, pupal weight and adult emergence.

The fecundity of adult insects particularly of lepidopteran insects is largely dependent on the reserves of nutrients which are accumulated in the larvae. As there were no differences between the uninfected-untreated and uninfected-treated groups in all larval and pupal characters studied, no efforts were made to study the effects of the drug on the fecundity of the adults.

5.3.2 Benomyl

The results revealed that only the highest dose (4mg/larva) of the drug exerted adverse effects on the larval and pupal development of the host insect. The other two doses of benomyl (1mg and 2mg/larva) had no adverse effects. Even in comparison with the uninfected-untreated group (Table 35), both the doses did not induce significantly different effects with respect to percentage larval mortality, percentage pupation, pupal weight and percentage adult emergence.

Armstrong (1976) reported that although benomyl at 250 - 500mg/litre caused some adverse effects on Drosophila willistoni flies, these were acceptable because of the advantageous effects of the drug on microsporidian parasites. However, at concentrations of 1,000mg/litre and above the drug was lethal for the flies. Harvey and Gaudet (1977) reported that benomyl at 75 ppm and above reduced the growth and fertility of C. fumiferana. In contrast, Hsiao and Hsiao (1973) reported that benomyl at a level of 250 ppm had no harmful effects on the alfalfa weevil. Briese and Milner (1986) also failed to observe significant adverse effects of benomyl even at 400 ppm on the biology of Anaitis efformata. Brooks et al. (1978) found no obvious detrimental effects of benomyl when used up to 1,000 ppm in H. zea, after exposing the larvae throughout their larval periods to drug-treated diet. However, they did not present the full data.

In the present study benomyl at 4mg/larva (1000µg/ml of diet) exerted some adverse effects on the larval and pupal development of H. zea but, as the drug used at 1mg/larva (250µg/ml of diet) almost completely eliminated the parasite from mature H. zea larvae, the drug is clearly safe to use in this host at doses which will control microsporidial disease.

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APPENDIX 1

Semi-synthetic diet for rearing *Heliothis zea*
(Modification of Hoffman's Tobacco Hornworm diet; C.F.Rivers, pers. comm.).

1. Mix together in a blender:

Agar	18.75g
Casein	33g
Wheat germ	72g
Wesson's salts	9.38g
Yeast powder	14.25g
Sugar	29.25g
Cholesterol	0.94g
Sorbic acid	1.50g

2. Add and mix thoroughly:

Water	815ml
Linseed oil	1.88ml

3. Autoclave mixture in covered vessel for 20 min at 20 lbs per sq.in. Cool to 60 - 70^{o}C.

4. Add and mix thoroughly:

Vitamin and antibiotic mixture	5.63g
Choline chloride	0.94g

5. The diet must be poured into containers quickly before the agar sets and stored in a refrigerator.

Wesson's salts

$CaCO_3$	120g
K_2HPO_4	129g
$CaHPO_4$ $2H_2O$	30g
$MgSO_4$ $7H_2O$	40.8g
NaCl	67g
$FeC_6H_5O_2$ $5H_2O$	11g
KI	0.32g
$MnSO_4$ $4H_2O$	2g
ZnCl	0.1g
$CuSO_4$ $5H_2O$	0.12g

Vitamin and antibiotic mixture:

Nicotinic acid	5.0g
Calcium pantothenate	5.0g
Riboflabin	2.50g
Aneurine hydrochloride	1.25g
Pyridoxine hydrochloride	1.25g
Folic acid	1.25g
Biotin	0.10g
Cyanocobalamine	0.01g

For every gram of this mixture, add:

Streptomycin sulphate	2g
Ascorbic acid	40g

Mix well and store in a refrigerator.

APPENDIX 2

Preparation of specimens for light microscopy

Carnoy's fluid

Absolute alcohol	60ml
Chloroform	30ml
Acetic acid	10ml

1.	Carnoy's fluid	1 h
2.	90% alcohol	1 h
3.	Absolute alcohol (two changes)	1 h
4.	Cedarwood oil	overnight
5.	Xylene (two changes)	30 min
6.	Paraplast wax (three changes)	30 min
7.	Embedded in paraffin wax.	

Sections (5 - 6 µm)

Staining

1.	Xylene (two changes)	2 min
2.	95% alcohol	2 min
3.	90% alcohol	2 min
4.	80% alcohol	2 min
5.	70% alcohol	2 min
6.	60% alcohol	2 min
7.	50% alcohol	2 min
8.	40% alcohol	2 min
9.	Giemsa stain	1 h
10.	Differentiate with 15% Colophonium in acetone	
11.	Wash in acetone	
12.	Wash in mixture of 70% acetone + 30% xylene	
13.	Clean in xylene (two changes)	
14.	Mount in green euparal	

Giemsa stain

100 ml Buffer

10 ml Acetone

10 ml Methanol

Mix well and add 10ml Giemsa stain to 90ml of the above.

Buffer solution (pH 7.2)

Potassium dihydrogen orthophosphate	2.1g
Di-sodium hydrogen orthophosphate	5.0g

Dissolve in 1000ml de-ionized distilled water.

APPENDIX 3

Preparation of specimens for transmission electron microscopy

Karnovsky's fluid

Mix:	Paraformaldehyde	2g
	$CaCl_2$	25mg
	Distilled water	20ml

Heat to 65°C, add 2 drops IM NaOH
Stir until clear, cool to room temperature
Add 10ml of 25% gluteraldehyde and make up to
50ml with 0.2M sodium cacodylate buffer, pH 7.4

1. Karnovsky's fluid for 10 min at room temperature
2. Karnovsky's fluid for 1 h at 4°C
3. 0.12M sodium cacodylate (two changes) for 15 min at 4°C
4. 2.5% (w/v) OsO_4 in 0.1M sodium cacodylate, 1 h at 4°C
5. 0.1M sodium cacodylate (two changes), for 10 min at 4°C on rotator
6. 0.25% (w/v) uranyl acetate in water for 1 h at 4°C
7. 0.1M sodium acetate (two changes) for 10 min at 4°C on rotator
8. 35% acetone for 5 min at 4°C
9. 50% acetone for 5 min at 4°C
10. 70% acetone with 1% uranyl acetate and 1% phosphotungstic acid, overnight at 4°C
11. 90% acetone (three changes) for 15 min at room temperature
12. 100% acetone (three changes) for 15 min at room temperature
13. 50/50 acetone/Spurr's resin, 6 h at room temperature on rotator
14. Spurr's resin, 24 h at room temperature on rotator
15. Embed in fresh Spurr's resin and polymerize in 60°C for 24 h

Post-staining of sections

Reynold's lead citrate

Lead nitrate	1.32g
Sodium citrate	1.76g

Dissolve compounds separately, then mix together in a 50ml volumetric flask and make up to 30ml.

Add 6ml of 1N NaOH and dilute total to 50ml.

1. Float grids, section-side downwards, on drops of 2% aqueous uranyl acetate on dental wax for 7 min.
2. Pick up grids and wash with clean distilled water.
3. Dry grids on filter paper.
4. Place grids, section-side downwards, on drops of Reynold's lead citrate on dental wax in a closed petri dish with some pellets of NaOH. Leave 10 mins.
5. Pick up grids and wash with 0.02M NaOH.
6. Dry grids on filter paper.
7. Wash grids with boiled distilled water.
8. Dry grids on filter paper and examine.