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EVALUATION OF TOLTRAZURIL AS AN ANTIMICROSPORIDIAL DRUG

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The anticoccidial drug ' toltrazuril' was tested on the microsporidium *Nosema bombycis* in *Helicoverpa zea*, both by exposing larvae to drug after they had been infected with the microsporidia and by exposing uninfected larvae to the drug and microsporidian spores simultaneously. The drug at concentrations of 100, 200 and 400 µg/larva had no effect on the level of infection, estimated by the number of spores harvested from the larvae on day 8 post drug treatment. Midgut tissues from infected larvae, which had consumed 400µg of toltrazuril, still had massive infections of the parasite, and no cytological alterations of the parasite were observed when infected tissues were examined by light and electron microscopy. The pH of the gut of *H. zea* is alkaline and should not have affected the solubility of the drug nor its absorption into the midgut cells. It is concluded that toltrazuril, even at a dose of 400µg per larva, has no effect on *N. bombycis* in *H. zea*.

Key words. *Nosema bombycis, Helicoverpa zea* larvae, Toltrazuril inactivity.

Introduction

Microsporidia (Phylum microspora) are common parasites of invertebrates and vertebrates. Of many drugs which were tested prior to 1990 for antimicrosporidial activity, only fumagillin (Fumidil B) [1], benomyl [2] and itraconazole [3] caused significant reductions in infections in insects. Further experiments with fumagillin and benomyl using different microsporidia and host systems showed that infections were never completely eliminated [4-7]. Toxicity to the host, exemplified by increased larval period, larval mortality, reduced pupal weight and reduced adult fecundity and fertility, were evident at the concentrations required to affect the microsporidia and it was concluded that the toxic effects outweighed the benefits of microsporidial control [8-11]. Although ultrastructural changes in spores due to itraconazole were reported by Liu and Myrick [3], there appear to have been no follow up studies on its antimicrosporidial activity in insects.

Toltrazuril (Bayer), initially developed as an anticoccidial drug [12], was reported to give effective control for a wide range of protozoan parasites including microsporidia [13]. A concentration of 20 µg/ml in sugar solution, when fed to bees, wasps and flies infected with *Nosema* spp., killed the microsporidia and allowed the hosts to survive. Also, when fish infected with Glugea cysts were immersed in water containing the drug at 10 µg/ml, most of the parasites were killed within 2-4 hrs. The drug found to cause significant ultrastructural damage to developmental stages of *Glugea anomala* [14].

Effective control measures are needed for microsporidia, not only because they cause morbidity and mortality in beneficial insects, but also because they interfere with the establishment of colonies of insects for experimental work. Furthermore, the rapid expansion of aquaculture has provided environmental conditions which favour transmission of microsporidia among fish and crustaceans, while immunosuppressive diseases such as AIDS have uncovered serious microsporidial infections in man for which treatment is required [21,22]. Finally, arthropods which are mass reared for biological control of pests should be microsporidia free before being released into the environment, where they may spread to beneficial hosts. The reports of the antimicrosporidial activity of toltrazuril prompted the present study to evaluate its effect on *Nosema bombycis* in insects.

Materials and Methods

Rearing of host insects for drug treatment. The corn earworm, *Helicoverpa zea*, was used as an experimental host. Surface sterilized eggs were allowed to hatch in well-aerated sealed plastic boxes at 27°. The larvae were reared on semi-synthetic diet [15] modified according to C. F. Rivers (personal communication). The 1st and 2nd instars were reared *en masse* and the remaining instars were reared individually. The insects were kept in controlled chambers at 27° ± 1, 55% R. H. and 16 hrs light : 8 hrs dark. The frass was removed when necessary.

Assay of larvae for drug activity. Larvae were exposed to toltrazuril either after infection with *N. bombycis* (post exposure experiment) of concurrently with *N. bombycis* spores (concurrent experiment).

In the post-exposure experiment, late 2nd instar larvae were individually transferred to 4.0 x 1.5cm vials and starved for 6 hrs. Meanwhile, drops of suspension, each containing $1x10^5$ purified *N. bombycis* spores, were dispensed on to the

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surface of 4 mm^3 pieces of drug-free diet. The liquid was absorbed into 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 toltrazuril-treated or drug-free diet (controls). The toltrazuril was added to the liquid diet at 60° at concentrations of 0, 25, 50 and 100 µg/ml. Sixty five larvae were used for each of the drug concentrations and for the controls.

In the concurrent experiment plastic cups containing 4ml of toltrazuril-treated or drug-free diet were prepared as in the post-exposure experiments. Spore suspension, containing $1x10^5$ spores in 25µl drops, were dispensed on to the surface of the food and spread as evenly as possible by quickly rotating the cup. The liquid was absorbed into the diet. Newly emerged 3rd instar larvae, starved for 6 hrs. were individually transferred to the cups and reared as before. Thirty five larvae were used for each drug concentration and for the controls.

On day 8 post-exposure to drug, when all the drug-treated diet had consumed, 5-10 larvae were randomly selected from the control and toltrazuril-treated cups to be checked for infection. Infections were determined by examination of fresh smears of the fat body and midgut tissues for spores by phase contrast microscopy. When it had been determined that all larvae were infected, another 10 larvae from the post-exposure experiment and 5 larvae from the concurrent experiment were randomly selected from each treatment and weighed before determination of spore burden, using the method of Cantwell [20]. Each larva was disrupted in 1ml distilled water in a tissue-grinder, the resulting suspension was diluted 1:10 and the spores counted using a haemocytometer. The number of spores was expressed as per mg larval body weight.

Histology. For light microscopy newly emerged 4th instar larvae, which had been infected with *N. bombycis* as for the assays, were exposed to 4ml of diet containing 100 µg of toltrazuril per ml of diet. When all the drug-treated food had been consumed, in 5-6 days, the larvae were dissected in phosphate buffered saline solution [pH 7.2]. The midgut was cut into pieces 10mm long, which were fixed in Carnoy's fluid, dehydrated in ethanol and embedded in paraffin wax. Five to six µm sections were stained by Giemsa/Colophonium. Smears of other tissues were examined fresh by phase-contrast microscopy.

For electron microscopy, $1mm^3$ pieces of midgut tissues from the same batch of larvae were fixed in Karnovsky's fixative in 0.1M cacodylate buffer for 10 mins at room temperature, then for 1 hr at 4°. Specimens were postfixed in 2.5% OsO_4 for 1 hr at 4°. Specimens were dehydrated through graded acetone solutions and embedded in Spurr's resin. Ultrathin sections were stained in uranyl acetate and lead citrate and examined at 80 KV. Tissues from larvae which had fed on diet without drug were processed similarly as controls.

Results and discussion

Effect of toltrazuril on N. bombycis in *H. zea.* The results on the effect of toltrazuril on *N. bombycis* in *H. zea* are presented in Table 1. The drug at 100, 200 and 400 µg/larva failed to eliminate *N. bombycis* from its experimental host, *H. zea*. There were no significant differences in the number of spores harvested from larvae between control and drug-treated groups in either the post-exposure or the concurrent experiments (Student's t-test).

Histology. Fresh smears of tissues from *N. bombycis* infected *H. zea* larvae, which had consumed 100-400 µg of toltrazuril, showed massive infections.

Light microscopic observations of midgut sections from *N. bombycis* infected *H. zea* larvae which had consumed 400 µg of toltrazuril also showed massive infections of the parasite (Fig. 1). In electron microscopy all stages of development of the parasite were recognised in midgut tissues. Compared with controls no ultrastructural changes were observed in

TABLE 1. NUMBERS OF *N. BOMBYCIS* SPORES PER mg BODY WEIGHT OF LARVAE OF *H. ZEA* AFTER ADDITION OF 100-400 µg TOLTRAZURIL TO THEIR DIET, COMPARED WITH LARVAE REARED WITHOUT DRUG.

Toltrazuril	Post-exposure	Concurrent-exposure
(µg/larva)	Mean(range) ±S.E./mg x 10^5	Mean(range)±S.E./mgx10^4
0	2.79 (1.46 - 4.22) ± 0.28	6.09 (3.73 - 9.91) ± 1.10
100	2.53 (1.20 - 5.50) ± 0.43	6.63 (4.54 - 7.75) ± 0.55
200	2.97 (1.08 - 4.88) ± 0.41	7.26 (6.22 - 8.03) ± 0.38
400	2.44 (1.49 - 3.59) ± 9.19	6.69 (4.09 - 9.78) ± 1.13

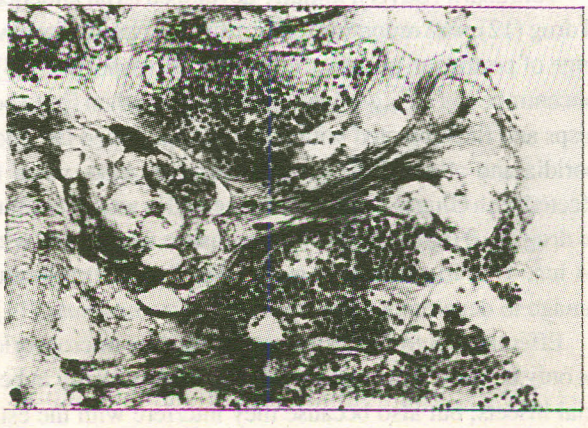

Fig.1. Section of midgut tissues from *N. bombycis* infected *H. zea* larvae after exposure to 400 µg toltrazuril per larva, showing massive infections of the parasite (light microscopy).

stages of merogony or sporogony present in midgut cells. Meronts and sporonts with abundant ribosomes in the cytoplasm and with diplokaryotic nuclei of ten exhibited signs of division (Figs 2-4). Mature spores did not fix well but in favourable sections of slightly immature spores (Fig. 5) the arrangements of organelles was orderly with about 10 coils of the polar tube corresponding to the late-stage spores described by Iwano and Ishihara [16].

Fig.2. Meront with unthickened memberane and diplokaryotic nuclei (n).

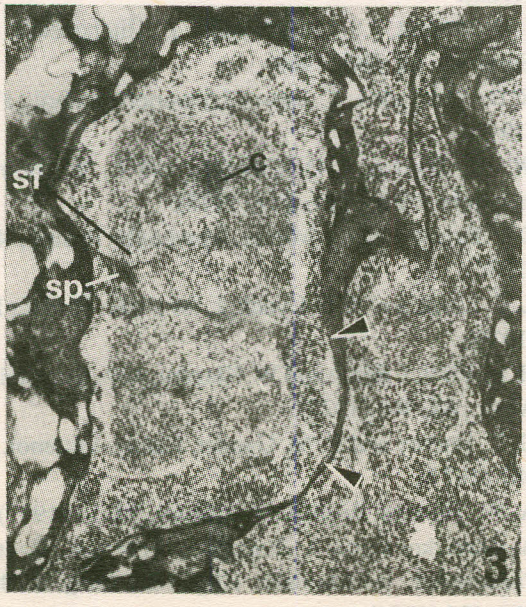

Fig.3.Meront with unthickened membrane (arrowheads) and nuclei in division showing spindle plaque (sp), spindle fibres (sf) and chromosomes(c).

Toltrazuril, which is only soluble at alkaline pH, was tested against *N. bombyc*is in *H. zea* at concentrations of 100, 200 and 400 µg/larva. The drug was administered to previously infected larvae and by exposing unifected larvae to the drug and spores concurrently. In both cases toltrazuril failed to reduce the parasite burden of its host. A lepidopteran insect, *H. zea*, was chosen as the experimental host because the midgut pH of 8.0 would allow toltrazuril to remain in solution and be absorbed by the midgut cells. Mehlhorn *et al.* [17] found that toltrazuril (Bay V19142) caused severe degeneration of intracellular stages of coccidia when administered to

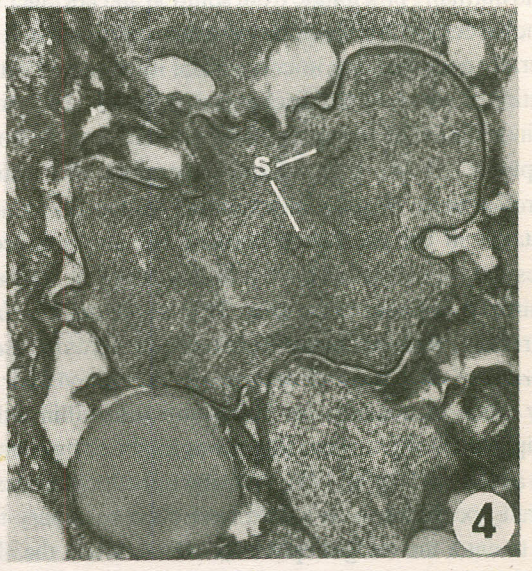

Fig.4. Sporont with thickened membrane and diplokaryotic nuclei with spindles (s) in mitotic anaphase.

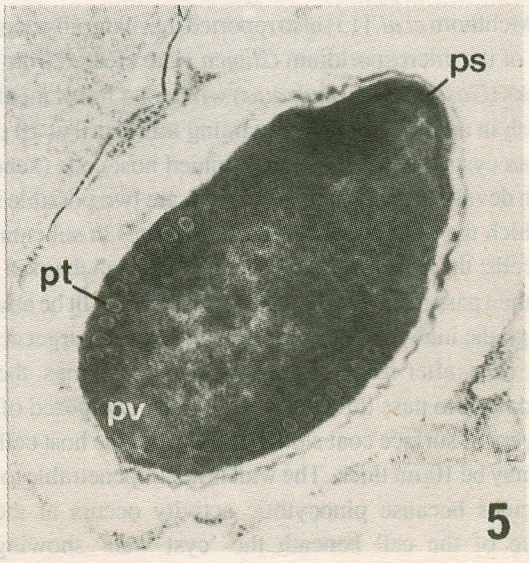

Fig.5. Immature spore showing posterior vacuole (pv), 10 coils of the polar tube (pt) and anterior insertion of polar tube into polar sac (sp).

[Fig. 2-5 Electron micrographs of stages of *N. bombycis* in midgut cells of *H. zea* after exposure to 400 µg toltrazuril per larva.]

chickens is drinking water and concluded that the drug could pass through host cell membranes [13]. The pH of avian intestine is also alkaline.

In experiments on the effect of toltrazuril on microsporidia, Mehlhorn *et al.* [13] provided toltrazuril, at 20 µg/ml in sugar solution, to honey bees (*Apis mellifera*), wasps (*Pimpla turionellae*) and flies (*Drosophila melanogaster*) infected with species of *Nosema* and reported that the drug prevented mortality of the insects. The gut of these insects is acidic, that of the honey bee for example, varying in different regions from pH 5.4 to 6.3 [18]. 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 now generally accepted that microsporidia infect new hosts after injestion of spores by the discharge of polar tube into gut walls and inoculation of the sporoplasm directly into the host cell cytoplasm [23-24]. It is unlikely, therefore, that toltrazuril controlled these *Nosema* infections by killing the extracellular stages in the gut lumen because the only extracellular stages are spores which have a highly resistant wall almost certainly impervious to the drug. This is supported by *in vitro* experiments with the mammalian microsporidium, *Encephalitozoon cuniculi,* in which mature spores retained their infectivity when incubated with 500 µg/ml fumagillin although multiplication of pre-spore stages was inhibited by concentrations as low as 5 µg/ml [25]. Also the infections in bees were naturally occurring so it would have been necessary for the drug to enter the midgut epithelial cells to affect the already established intracellular stages. This would imply that the particulate drug was absorbed in these insects by a mechanism which does not operate in the gut of *H. zea.*

Mehlhorn *et al.* [13] also reported that naturally occurring cysts of the microsporidium *Glugea* were cleared from sticklebacks (*Gasterosteus aculeatus*) within 2-4 hrs of incubating the fish in aerated aquaria containing toltrazuril at 10 µg/ml. *Glugea* cysts are greatly hypertrophied host cells (xenomas) which develop subcutaneously. There are two possible routes by which the drug could reach the parasites in subcutaneous host cells: the drug might be absorbed through the skin of the host then pass to the target cells or the drug might be absorbed through the intestinal tract and then pass to the target cells. In either case, after reaching the site of the xenomas, the drug would have 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. The wall is not impenetrable to small molecules because pinocytotic activity occurs 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 et el. [14] reported that there were ultrastructural changes in the parasite within cysts on toltrazuril-treated fish.

In the present study, histological observations of the midgut tissues from the larvae which had 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 compared with drug-free infected larvae. Haberkorn *et al.* [19] reported that toltrazuril is stable for 14 days at 70°. Thus addition of the drug to the diet at 60° would not account for its lack of activity against *N. bombycis*. Lack of activity of toltrazuril against microsporidia was also found by Dr. J. Vavra working with *Nosema* disease of honey bees (personal communication). The present results are in agreement with those of Dr. Vavra.

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