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ORIGINAL PAPER

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In vitro studies on the effects of flubendazole against *Toxocara canis* and *Ascaris suum*

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Abstract Adult *Toxocara canis* and *Ascaris suum* were incubated in vitro in media containing 0.1, 1, 10 or 100 μg/ml flubendazole in order to study drug-derived effects. This incubation was done for 8 h and repeated (in some groups) after 24 h for another 8 h. The onset and intensity of flubendazole-derived effects were dosage-dependent and time-dependent, i.e. the same grade of damage was reached when incubating for a longer period at a low dosage or for a shorter period in medium containing a high amount (10 or 100 μg/ml) of flubendazole. A repeated incubation in drug-containing medium was superior to a single exposure. Flubendazole is apparently able to penetrate into the worm's interior via the cuticle. This became evident in worms with sealed orifices, which showed identical damage to worms which were not sealed. The type of tissue damage due to flubendazole was identical in both worm species when exposed to any of the drug dosages used. The principal mode of action of flubendazole was based on the complete reduction of microtubuli-polymerisation inside the parasite's cells. This apparently led to the complete destruction of the hypodermis, muscle layer and intestine. Flubendazole also stopped the formation of gametes. Summarising, even low concentrations of flubendazole (0.1 μg/ml) led to significant and irreversible damage in all worms studied.

Introduction

Helminths, and especially the numerous species of nematodes, are of considerable medical and economic importance because they threaten the health and life of both humans and animals (Mehlhorn 2001). Therefore, various anthelmintic drugs have been developed in order to control the diseases caused by helminths and to minimise the adverse effects of the worm burden. Since it is known that the efficacy of antiparasitic drugs may decrease within approximately 10 years of use depending on different factors (e.g. development of resistance; Prichard 1994; Sangster et al. 1985), it is necessary to determine the remaining activity from time to time and/or to restructure the pathways and modes of drug application. It is also worthwhile to examine the method of drug uptake and to study the mode of action of the drugs on the different tissues of the parasite, since this knowledge will give hints for the optimum timing of the treatment and will show the most effective dosages (including eventual repetition of treatment) to use. While the degree of general activity of a drug can only be tested using in vivo trials, the mode of uptake, the form of application, as well as the sites and range of activity can also be studied in vitro. The evaluation of such investigations may lead to findings which increase the efficacy of a drug.

Flubendazole is an anthelmintic compound (Denham et al. 1979) long known to be highly effective against nematodes such as the *Toxocara* species of carnivores. *Toxocara canis* worms were obtained from untreated dogs. Additionally, we used *Ascaris suum* worms obtained from pigs at the Düsseldorf slaughterhouse as a further model for gastrointestinal nematodes.

The in vitro experiments carried out in this study were aimed at clarifying the following questions:

1. How is flubendazole taken up by the worm (via mouth, via cuticle or both)?
2. What damage is caused by the drug in the different worm tissues and what is the drug's mode of action?
3. What is the influence of drug concentration on its efficacy?
4. What is the best choice – single or repeated drug application – in order to obtain an optimum curative effect (i.e. maximum parasite destruction)?

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Materials and methods

Parasites

Dogs that had been experimentally infected with embryonated eggs of *Toxocara canis* were killed and dissected 7 weeks later. The small intestines were opened longitudinally and a total of 70 adult *T. canis* were collected and transferred to body-warm modified Leibovitz 15 medium (L15). The infection and the dissection took place at the Institute of Parasitology, Tierärztliche Hochschule, Hannover. The harvested worms were kept body-warm within flasks containing modified L15 medium and transported to the Department of Parasitology in Düsseldorf. There, the in vitro experiments started immediately after arrival. The length of the adult female worms varied between 10 and 15 cm; the size of the adult male worms ranged between 5 and 8 cm.

A total of 33 adult *Ascaris suum* were obtained from the intestines of ten pigs at the slaughterhouse in Düsseldorf. After recovery the worms were washed and transported in body-warm Ringer solution (NaCl 130 mM, KCl 24 mM, $CaCl_2$ 6 mM, $MgCl_2$ 5 mM, 2-[*N*-morpholino]-ethanesulfonic acid 5 mM, pH 6.7) to the Düsseldorf institute. The length of the adult female worms varied between 25 and 35 cm. No male worms were used in these experiments since too few were recovered. However, in vitro experiments using levamisole, piperazine and emodepside (unpublished data) showed that male *A. suum* reacted nearly identically to the females.

In vitro experiments

All worms used were fully motile and in good condition at the beginning of the experiments. In 13 worms the mouth, anus, and sexual openings were sealed with Histoacryl (enbucrilate; Braun, Germany) in order to hinder the entrance of the drug containing incubation fluid via these orifices (unpublished data).

The incubation of the specimens was executed in modified L15 medium (Sigma, Deisenhofen) in the case of *T. canis* and in Ringer solution in the case of *A. suum*, both at 37°C and 5% CO_2. The concentrations of flubendazole (0.1, 1, 10, 100 μg/ml medium) were produced by dilution of a stock solution containing 50 mg/ml flubendazole dissolved in DMSO. DMSO was added to the media of treated groups and controls in order to obtain a final concentration of 0.2% during the periods of treatment. For details of the incubation see Table 1 (*T. canis*) and Table 2 (*A. suum*).

The worms were observed for the first hour of incubation. Thereafter, their activity was monitored at intervals of 8 h by observation and if necessary by mechanical excitation with forceps. The activity was evaluated according to the criteria described in the Results section.

Light and electron microscopic studies

Worms were taken at given intervals from the incubation media (see F in Tables 1 and 2) and transferred to the fixation fluid (5%

Table 1 Dosage and timing of treatments – *Toxocara canis*. *A* Addition of the compounds to the media in dosages as specified above; *At* time; *F* fixation of specimens for microscopic inspection; *h* hours after beginning the experiments; *M* optical control of motility, mechanical excitation with forceps, if necessary; *W* washing of the worms and transfer into fresh medium

	Concentration (μg/ml)	No. of group	No. of worms	At 0 h	At 8 h	At 24 h	At 32 h	At 48 h	At 56 h
Flubendazole	0.1	A1	3	A; M	M; F				
	0.1	A2	3	A; M	M; W	M	M; F		
	0.1	A3	3	A; M	M; W	M; A	M; F		
	0.1	A4	3	A; M	M; W	M; A	M; W	M	M; F
	1	A5	3	A; M	M; F				
	1	A6	3	A; M	M; W	M	M; F		
	1	A7	3	A; M	M; W	M; A	M; F		
	1	A8	3	A; M	M; W	M; A	M; W	M	M; F
	10	A9	3	A; M	M; F				
	10	A10	3	A; M	M; W	M	M; F		
	10	A11	3	A; M	M; W	M; A	M; F		
	10	A12	3	A; M	M; W	M; A	M; W	M	M; F
	100	A13	3	A; M	M; F				
	100	A14	3	A; M	M; W	M	M; F		
	100	A15	3	A; M	M; W	M; A	M; F		
	100	A16	3	A; M	M; W	M; A	M; W	M	M; F
	10, Sealed orifices	A11a	5	A; M	M; W	M; A	M; F		
	10, Sealed orifices	A12a	5	A; M	M; W	M; A	M; W	M	M; F
Controls	0	B1	3	A; M	M; F				
	0	B2	3	A; M	M; W	M	M; F		
	0	B3	3	A; M	M; W	M; A	M; W	M	M; F
	0, Sealed orifices	B4	3	A; M	M; W	M	M; F		

Table 2 Dosage and timing of treatments – *Ascaris suum*. For abbreviations see Table 1

	Concentration (μg/ml)	No. of group	No. of worms	At 0 h	At 8 h	At 24 h	At 32 h	At 48 h	At 56 h
Flubendazole	0.1	A1	3	A; M	M; W	M; A	M; W	M; A	M; F
	1	A2	3	A; M	M; W	M; A	M; W	M; A	M; F
	10	A3	3	A; M	M; F				
	10	A4	3	A; M	M; W	M	M; F		
	10	A5	3	A; M	M; W	M; A	M; F		
	10	A6	3	A; M	M; W	M; A	M; W	M	M; F
	100	A7	3	A; M	M; W	M; A	M; W	M; A	M; F
	100, Sealed orifices	A7a	3	A; M	M; W	M; A	M; W	M; A	M; F
Controls	0	B1	3	A; M	M; W	M; A	M; W	M; A	M; F
	0	B2	3	A; M	M; W	M; A	M; W	M; A	M; F
	0. Sealed orifices	B3	3	A; M	M; W	M; A	M; W	M; A	M; F

glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2). They were then cut into small, 4×4 mm pieces and immediately transferred to fresh 4°C cold fixation fluid. The fixation period (at 4°C) lasted at least 24 h. After this first fixation, the specimens were washed in the buffer and transferred for 2 h at 4°C into a solution containing 2% OsO_4 for postfixation and prestaining. After the pieces of worm had been washed in cold buffer solution, they were dehydrated in a series of graded acetone. Finally, specimens were embedded in epoxy resin of low viscosity (ERL) according to Spurr (1969). The final, hardened blocs were cut on Reichert OMU-3 ultramicrotomes to produce a series of semithin sections which were stained with methylene blue. These sections were studied and photographed using an Olympus BX50 microscope. In order to obtain the basis for a comparative analysis of drug-derived effects, the different tissues were classified into four groups: 0 = not affected; 1 = slight damage; 2 = medium damage; 3 = severe damage.

The blocs sectioned for light microscopy were then used for electron microscopical inspection by cutting ultrathin sections. These sections were stained with uranyl acetate and lead citrate according to the standard method of Reynolds (1963) and were studied using Zeiss TEM 902 and Zeiss TEM 9 S2 transmission electron microscopes. The damage to the different tissues was classified as described above for the light microscopic-sectioned material.

Results

Effects of flubendazole on the motility of *Toxocara canis* and *Ascaris suum*

Controls

The untreated *T. canis* reacted much more sensitively to changes in the environmental conditions than the untreated specimens of *A. suum*. *A. suum* from the control groups showed no loss of activity during the whole period of incubation (56 h) and were even fully active after 7 days, while specimens of *T. canis* showed an apparently increasing loss of fitness and a reduction of activity after a period of incubation of about 32–40 h.

The untreated worms of both species, the orifices of which had been sealed with Histoacryl, showed identical behaviour to those with open orifices.

Treated worms

T. canis treated with flubendazole seemed to suffer no apparent decrease in their motility. A single incubation of 8 h in medium containing 0.1 μg/ml flubendazole did not lead to any reduction in motility. In contrast to the control specimens, the flubendazole-treated worms were even more active to the extent that they appeared to be "hyperactive". In groups being incubated for 56 h with a double treatment of 0.1 μg/ml flubendazole paralysis occurred only in 50% of the specimens. This late effect, however, might be artificially produced, since the controls also showed a reduced activity after this period of incubation. In all other groups (i.e. those incubated in 1–100 μg/ml flubendazole) the worms remained for 24 h more or less as active as the controls and even appeared in some cases to be "hyperactive". These "hyperactive" worms showed uncoordinated movements. Beginning at 32 h after the first incubation, partial paralysis occurred in most treated worms. Full paralysis was restricted to those worms being incubated twice in the drug-containing media, at any of the drug concentrations used.

The specimens with sealed orifices showed the same behaviour as the worms with open orifices.

The observation of the motility of incubated *A. suum* confirmed the findings in *T. canis*. Since *A. suum* were less sensitive to changes in the living conditions during incubation, the untreated specimens showed no loss of vitality during the period of incubation. Thus, the partial paralysis of the specimens treated two or three times with 100 μg/ml flubendazole after 48 h is clearly a drug-derived effect.

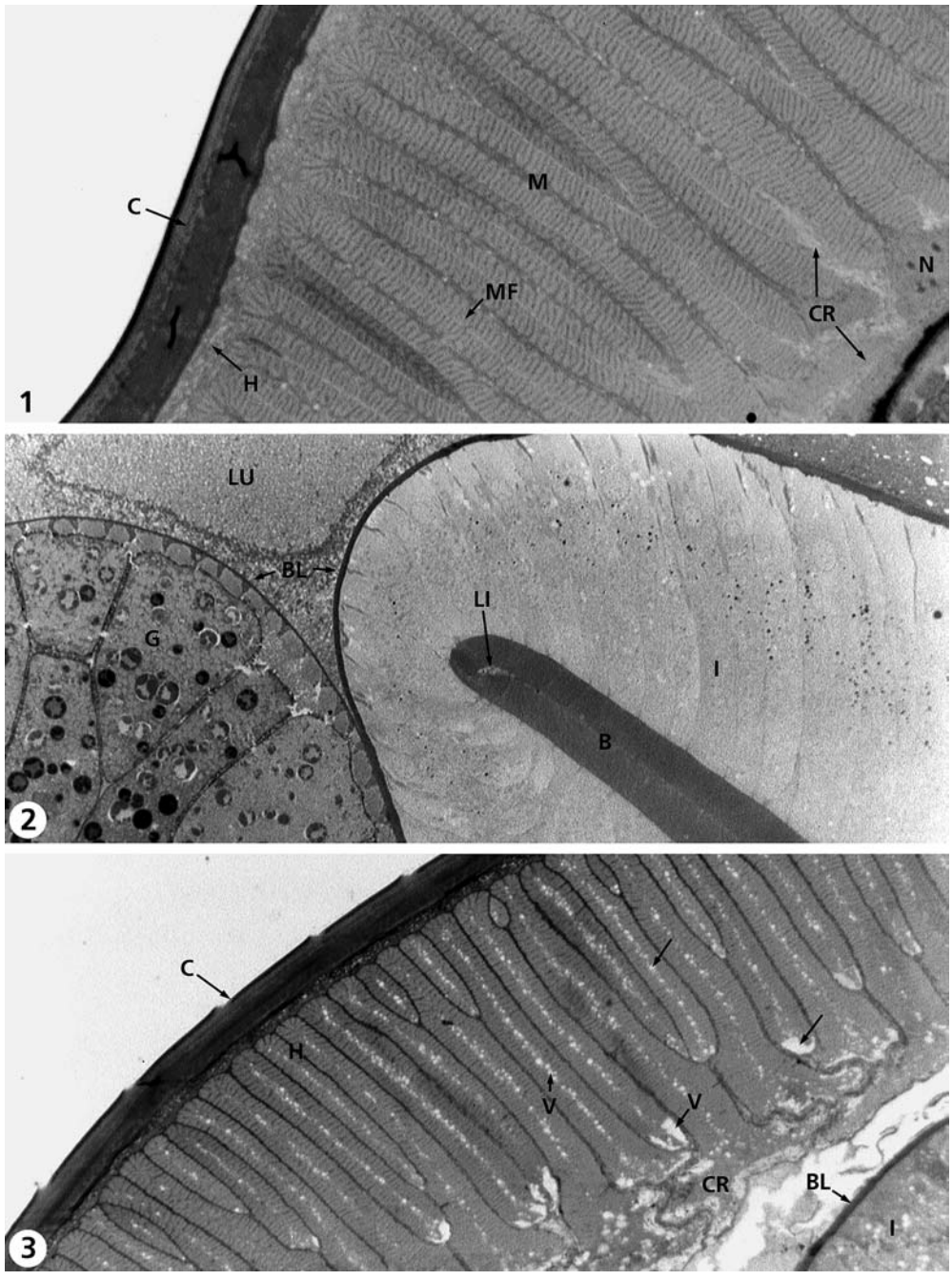
Sealing the orifices with Histoacryl caused no loss of motility in the controls. The worms treated two or three times with 100 μg/ml flubendazole showed a reduction of motility after 48 h corresponding to the specimens of the group with non-sealed orifices.

In contrast to specimens of the treated *T. canis* groups, none of the treated *A. suum* specimens became fully paralysed during the experiments, even after 56 h. Nevertheless, the flubendazole-treated *A. suum* showed the same type of "hyperactivity" that was noted in *T. canis*.

Effects of flubendazole on the tissues of *T. canis* and *A. suum*

The *T. canis* used in the experiments were, with respect to the degree of development of their gonads, mostly adults, since mature oocytes and spermatozoa were seen in light and electron micrographs. In the didelphic uterus system of many females, fertilised eggs or at least eggs with a strong and thick shell were found, indicating that copulation had occurred within the intestine of the experimentally infected dogs. In cross-sections – as seen in semithin sections – both the male and female worms appeared more or less spherical. Light and transmission electron micrographs of treated and untreated *T. canis* are shown in Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12.

Both sexes were covered by a rather dense non-cellular cuticle consisting of different layers and reaching diameters of about 30–60 μm (Figs. 1, 3). The cuticle is produced by the underlying, mostly smooth, about 8- to 15-μm-thick hypodermis (epidermis), which was composed of few, albeit large, nucleated cells. The nuclei were only found within the four cords that were produced by the hypodermis cells, contained numerous cristate mitochondria, layers of glycogen and a large number of small vacuoles (lipids?) that were even seen by light microscopy (Figs. 1, 3). The dorsal and the ventral chords were filled by nerve axons running longitudinally (Fig. 8), while the lateral cords contained the channels of the excretory system being surrounded by a series of apparently branching ductules which in electron micrographic cross-sections appeared as "vacuoles with



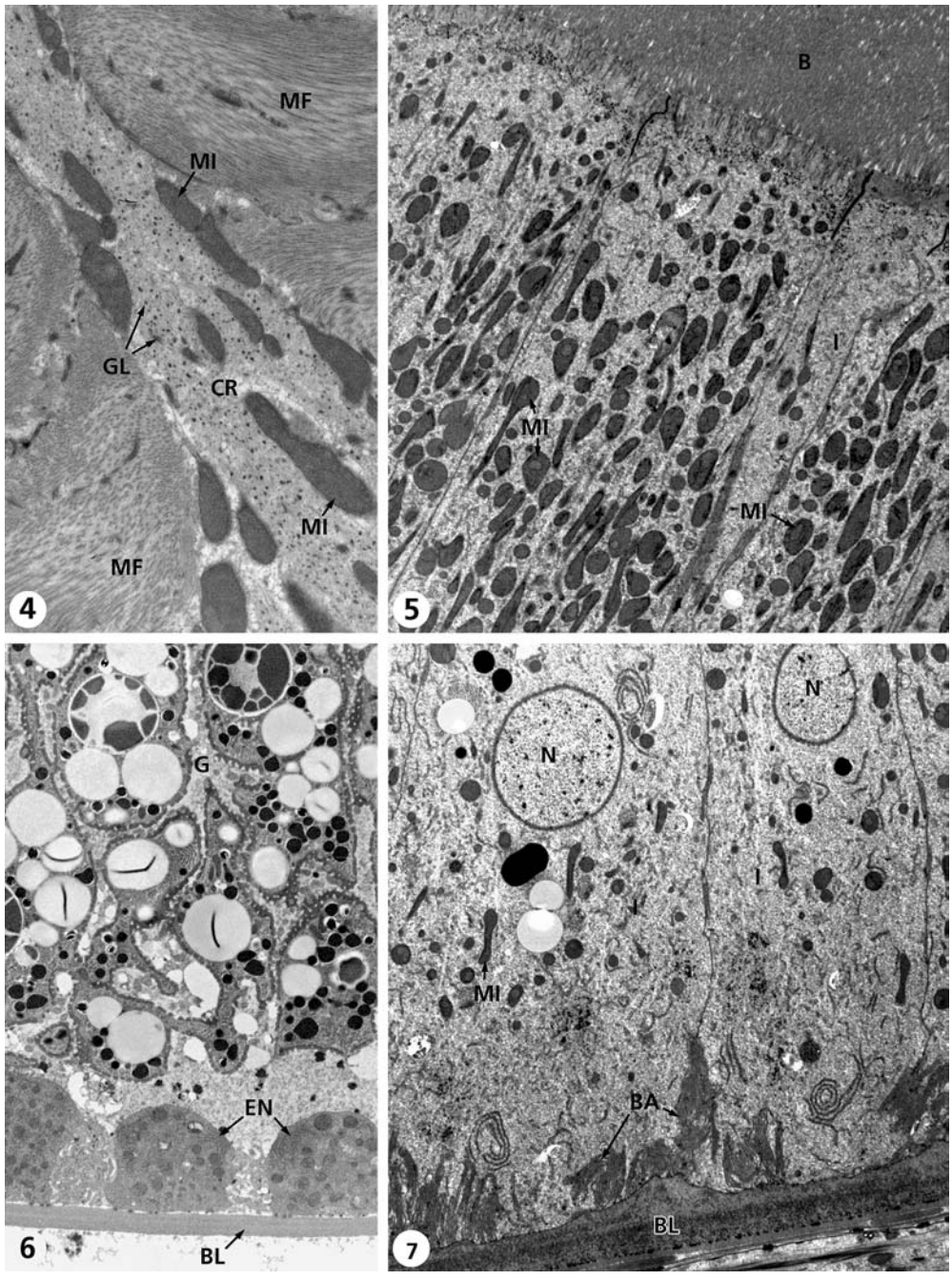
Figs. 1–3 Light micrographs of semithin sections of *Toxocara canis* worms

Fig. 1 Section through the periphery of an untreated worm showing an intact cuticle (*C*), hypodermis (*H*) and the layer of muscle cells (*M*). *CR* Cytoplasmic region of muscle cell, *MF* muscle filaments, *N* nucleus. ×670

Fig. 2 Section through the female gonad (*G*) and the intestine of an untreated control. Note that the brush border of the intestinal microvilli (*B*) does not leave a lumen. *BL* Basal lamina, *I* intestine, *LI* lumen of the intestine, *LU* lumen of the worm. ×560

Fig. 3 Section through the periphery of a worm incubated for 32 h in a medium containing 10 μg/ml flubendazole. Note the vacuolisation (*V*) in the centre and at the cytoplasmic base (*CR*) of the muscle cells (*arrows*) ×540

electron-lucent contents''. These four protruding cords subdivided the worm's periphery into four quadrants, all of which were filled with a large number of longitudinally arranged, striated muscle cells. These cells were composed of an outer peripheral region, which included the contractile elements (i.e. obliquely striated sarcomeres) (Figs. 1, 4), and of an inner electron-pale cytoplasmic portion which contained the nuclei. This inner portion of the muscle cell stretched out and formed a protrusion which was up to several millimetres long, connecting either the dorsal or the ventral nerve cord. These muscle cells were of the so called poly- and platymyarian types. The interior of the intestinal cells (Fig. 2) was closely filled with long stretching cristate mitochondria, numerous vacuoles with grey-appearing contents, many lacunae of the rough endoplasmic reticulum, as well as with free ribosomes and glycogen granules (Fig. 5). The nucleus, which was always situated close to the outer surface of the intestine, mostly appeared rather electron-pale and had a large nucleolus. The membrane of the intestinal cells formed a zone of deep invaginations adjacent to the basal lamina (Fig. 7). This so-called basal labyrinth increased the surface area considerably in order to enhance the transportation of food particles into the body cavity and from there to other organs.



Figs. 4–7 Transmission electron micrographs (TEM) of sections through untreated *Toxocara canis* worms (controls)

Fig. 4 Centre (cytoplasmic portion, *CR*) and periphery (contractile fibres, *MF*) of a muscle cell. *GL* Glycogen, *MI* mitochondria. ×12,000

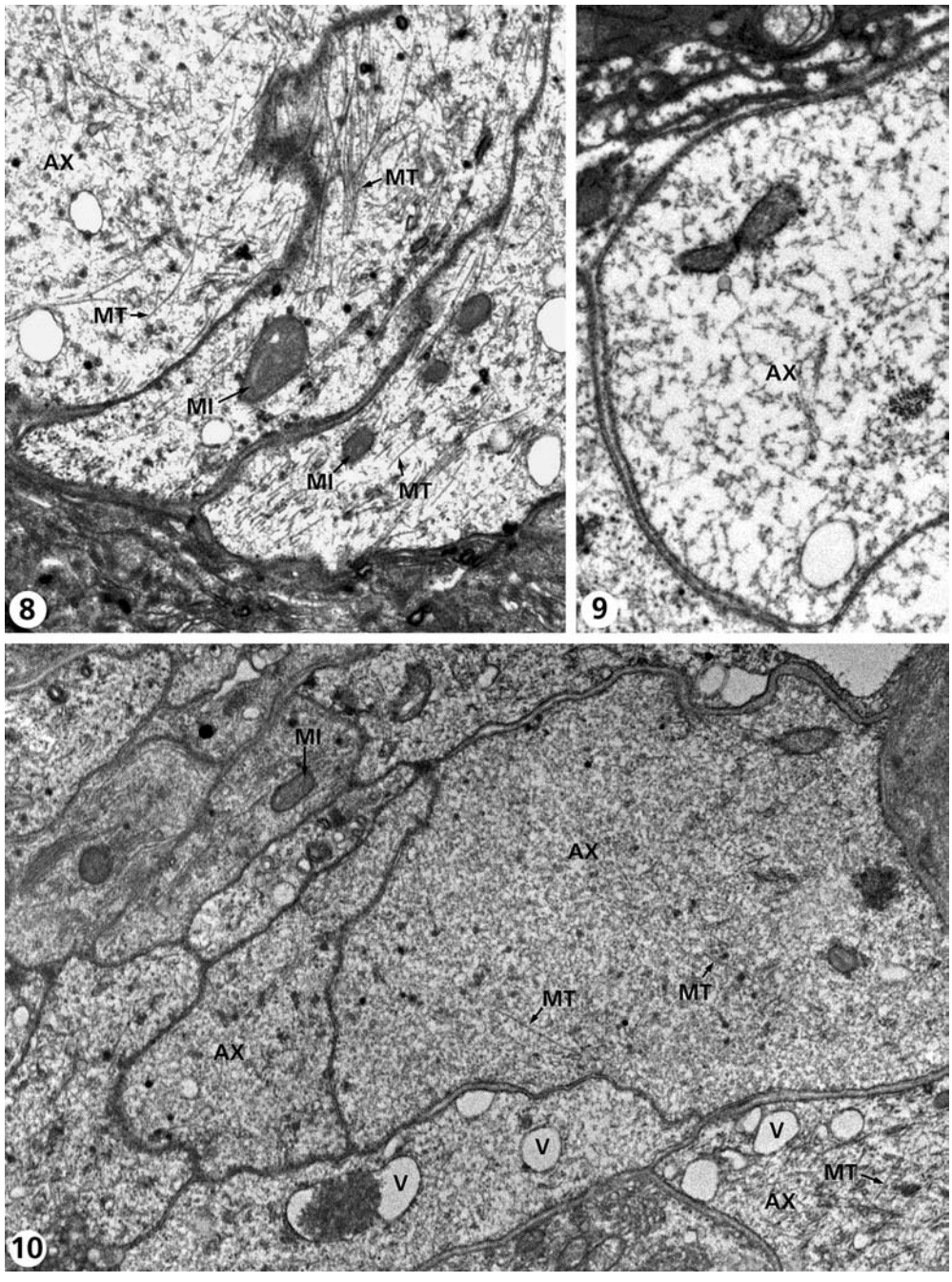
Fig. 5 Upper portion of intestinal cells (*I*). *B* Brush border of intestinal microvilli. ×5,400

Fig. 6 Periphery of female gonad (*G*). *BL* Basal lamina, *EN* endothelial cells in the gonad. ×4,800

Fig. 7 Basal (= outer region) of the intestine (*I*) showing the so-called basal labyrinth (*BA*). ×4,600

The body cavity in living worms is filled by the so-called body fluid which surrounds the intestinal tube and the convoluted sexual tubes (two in females, one in males). The function of this fluid, which was replaced in embedded specimens by hardening epoxy resin, is to stabilise the shape of the body and to act during movements as an antagonist to the body cover (cuticle, hypodermis and muscle layer). While in cross-sections through the worms the intestine is cut only once, the convoluted gonads appear several times. The male and the female sexual systems were surrounded by an outer, up to 0.4 μm thick, basal lamina and a single layer of endothelial cells (Figs. 2, 6). The developing gametes occurred inside of the tube. The eggs which were already formed, appeared artificially compressed in some sections, but in others preserved their regular, ovoid shape. Especially in the region of the so-called uterus-portion, the female gonads may reach a considerable diameter of up to 2 mm.

The single intestinal tube appeared depressed in fixed specimens and thus showed an ovoid appearance in cross-sections (Fig. 2). This tube ran from the anterior pole (lipped mouth) close to the posterior end, where it formed (as in most nematodes) a cloaca together with the single testis in males, but opened separately in females. The single sexual body opening (vagina) of the



Figs 8–10 TEM of sections through axons (*AX*) in *Toxocara canis* worms

Fig. 8 Untreated control. Note the occurrence of numerous long microtubules (*MT*). ×16,000

Fig. 9 Worm incubated in medium containing 10 μg/ml flubendazole for 8 h. Note the absence of microtubules and the degeneration of the ground substance of the axon (*AX*). ×20,000

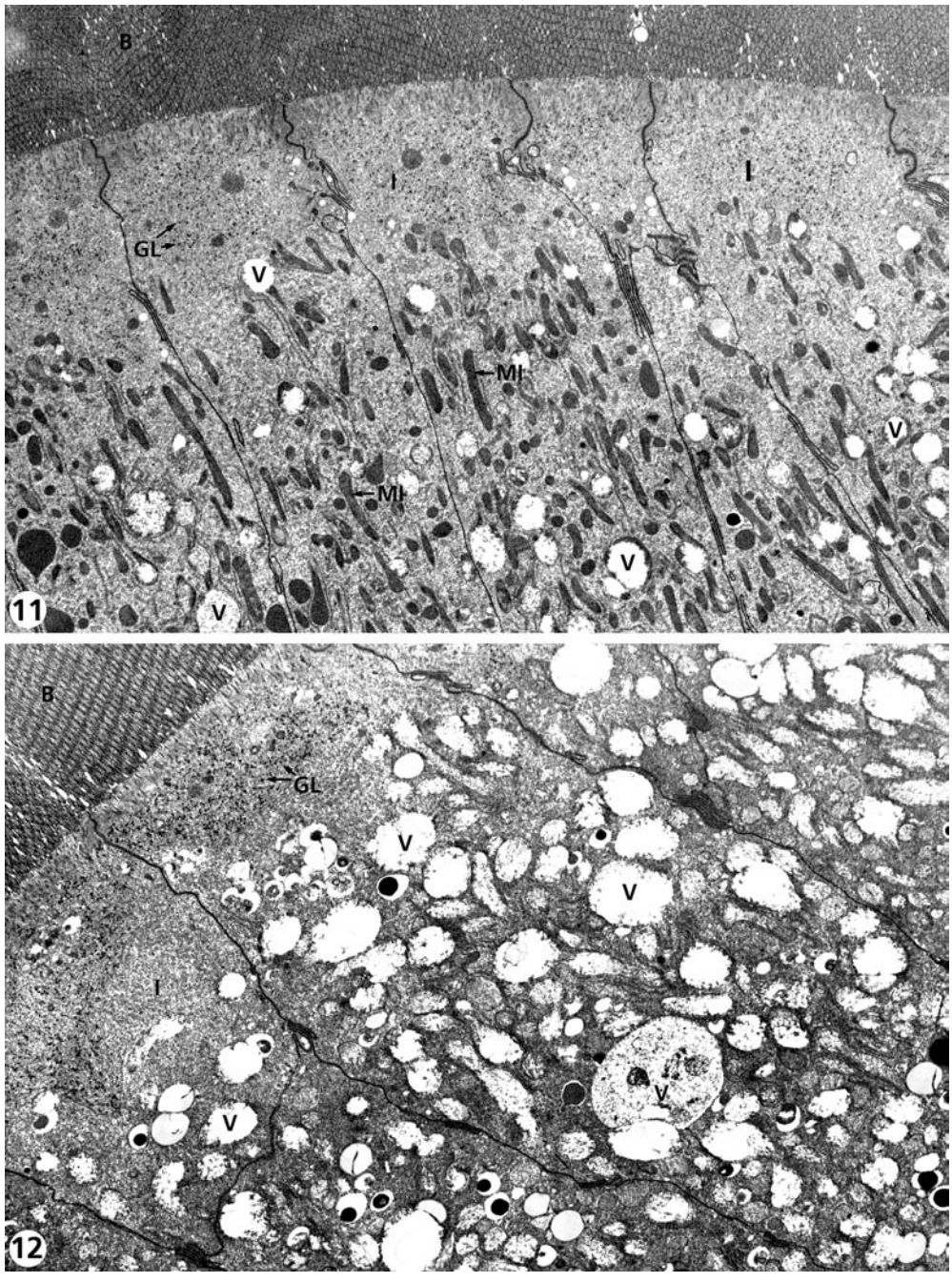
Fig. 10 Worm incubated for 8 h in medium containing 0.1 μg/ml flubendazole. Note the reduction in the number and length of microtubules (*MT*). *V* Vacuolisation. ×16,000

latter is found in the anterior third of the body (i.e. very close to the apical pole).

In light microscopy the cross-sections of the intestine appeared mostly without any lumen, although they reached diameters of up to 1 mm (Fig. 2). Only rarely did the brush border of microvilli give rise to a small intestinal lumen. In electron micrographs the intestine was surrounded by a 4 μm-thick basal layer. This surrounded a few flattened muscle cells of the smooth type and the single layer of epithelial cells, which usually appeared rather stretched, having a smaller diameter at the inner and a larger diameter at the outer surface. Directed to the intestinal lumen, these cells formed rather long microvilli of about 20 μm in length, arising from a very small electron-lucent zone (up to 1.7 μm in depth), which apparently represented the so-called terminal web (Fig. 5).

Figures 13, 14, 15, 16, 17, 18, 19, 20 show additional TEMs of both *T. canis* and *A. suum*.

The morphology of *A. suum* (Figs. 17, 18, 19, 20) was similar to that of *T. canis*. The most striking difference was the considerably larger size of the cells of *A. suum* in comparison to those of *T. canis*, corresponding to the bigger body of *A. suum*. Another difference was seen in the arrangement of the fibres in the muscle cells: in *A. suum* the muscle cells are of the coelomyarian type (Fig. 17).



Figs. 11–12 TEM of sections through intestines of treated adults of *T. canis*

Fig. 11 Worm incubated for 32 h in medium with 0.1 μg/ml flubendazole. Note the beginning of vacuolisation (*V*). *GL* Glycogen. ×5,300

Fig. 12 Worm incubated twice for 8 h in medium containing 10 μg/ml flubendazole. Note the intense vacuolisation (*V*). ×5,300

Some of the untreated *T. canis* which were incubated for 56 h showed reactions and destruction, especially at the level of the hypodermis, in the cytoplasmic region of the muscle cells and inside the intestinal cells. In general, however, the morphological features of all control worms of both species remained intact during the whole period of the experiments. Thus the damage seen after the incubation of the worms in media containing different dosages of flubendazole, as described below, was drug-derived.

The evaluation of the light microscopical and electron microscopical sections of *T. canis* led to almost identical results, although some damage was too small to be seen by light microscopy. Taking into consideration the type and site of the effects and their onset and intensity, several conclusions can be drawn:

1. The compound is probably taken up via both the cuticle and the intestine.
   The fact that the intensity of the damage seen in the tissues was identical both in worms with sealed mouthparts and in ones with unsealed mouthparts when these were incubated for the same time in media containing the same amount (i.e. 10 μg/ml flubendazole) clearly indicates that the uptake of the drug occurs via the cuticle. The general observation that

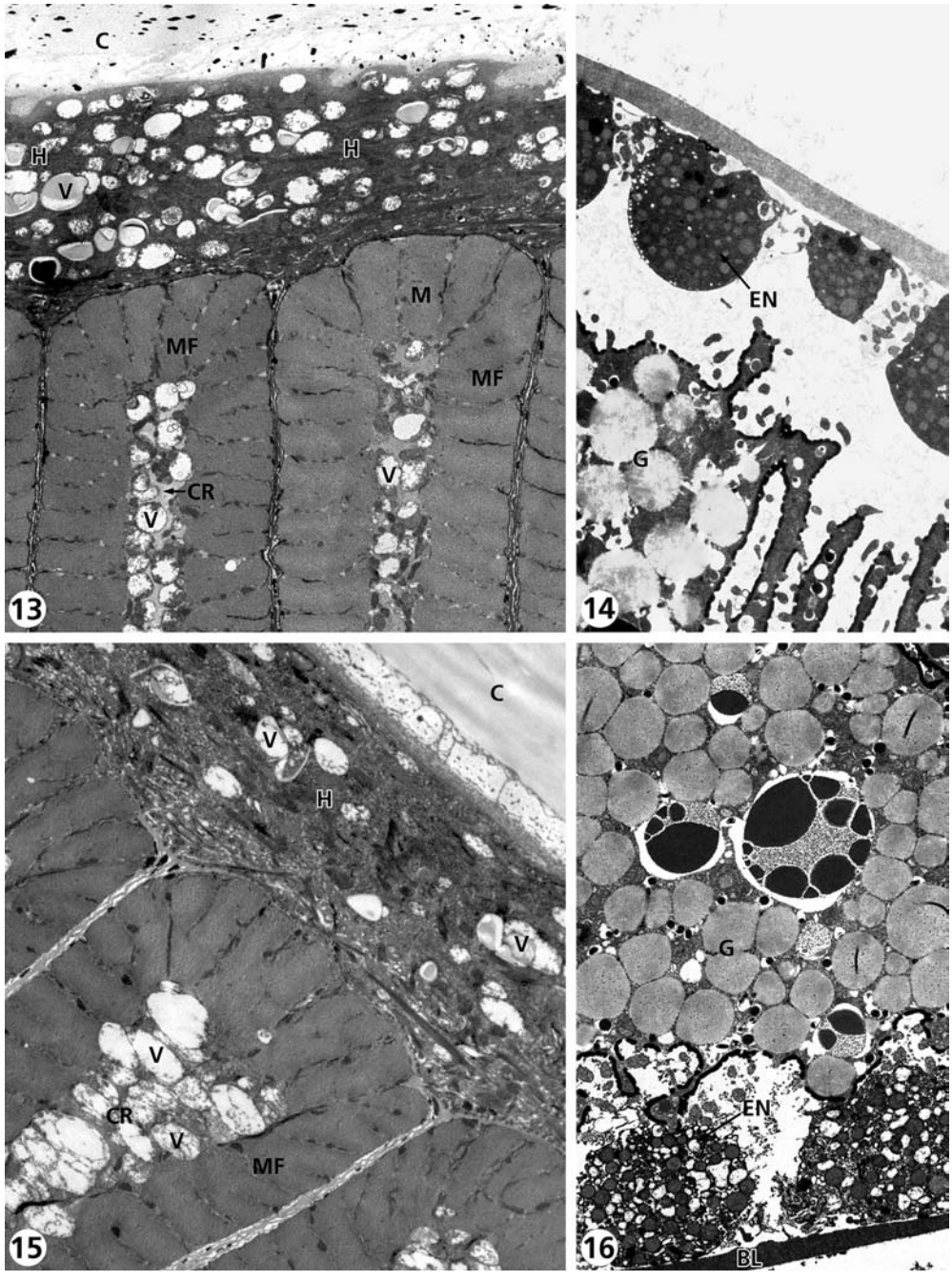
Figs. 13–16 TEM of sections through the muscle cells (Figs. 13, 15) and female gonads (Figs. 14, 16) of treated *Toxocara canis*

Fig. 13 Worm incubated twice for 8 h in medium containing 0.1 µg/ml flubendazole. Note the vacuolisation (*V*) in the hypodermis (*H*) and in the cytoplasmic portion of the muscle cell (*CR*). *C* Cuticle, *M* muscle cell, *MF* muscle filaments. ×4,600

Fig. 14 Female gonad (*G*) of a worm incubated twice for 8 h in a medium containing 100 µg/ml flubendazole. Note the degeneration of the endothelial cells (*EN*) and that occurring along the sexual cells. ×4,700

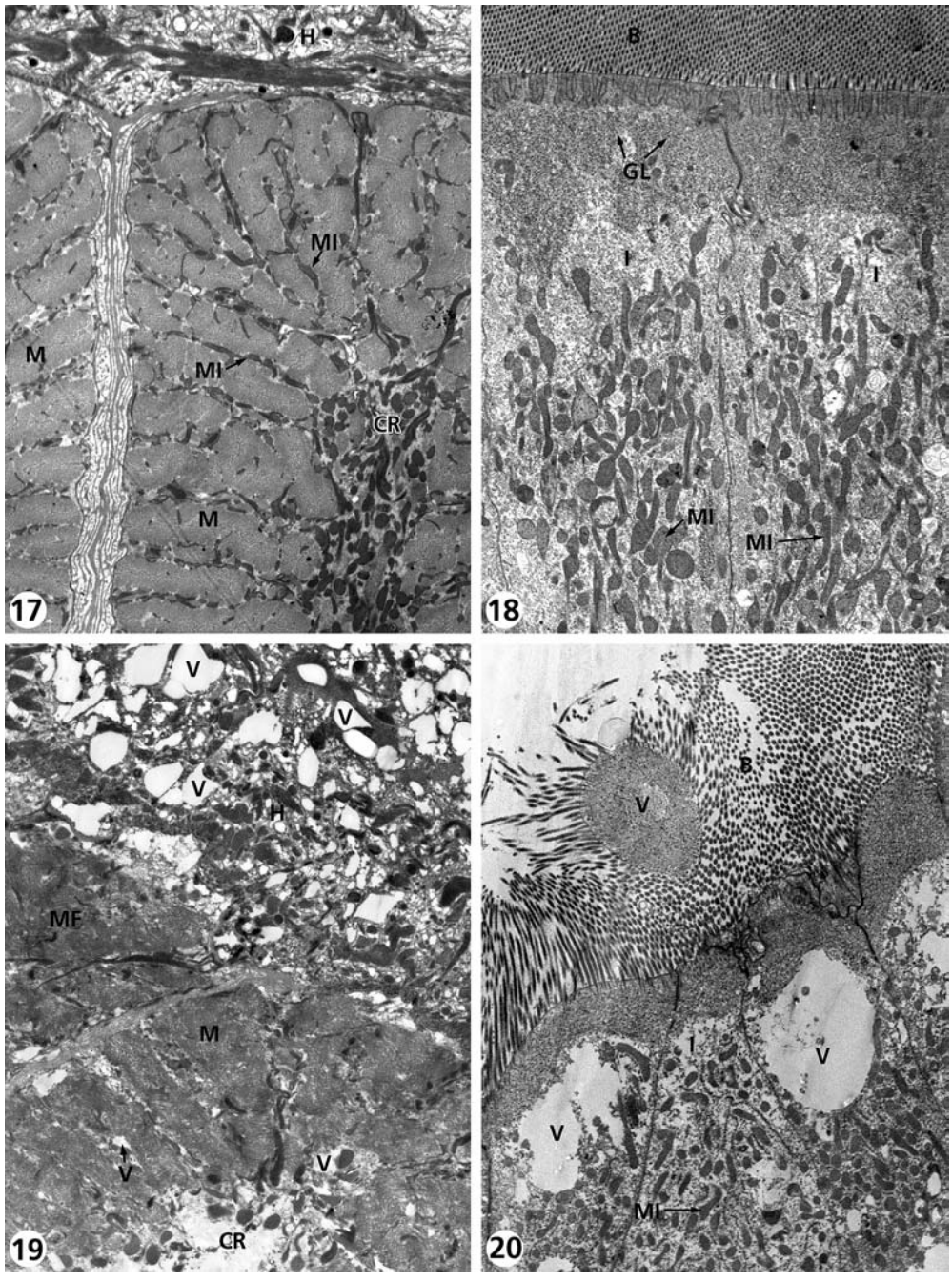
Fig. 15 Muscle cell (*M*) of a worm incubated twice for 8 h in a medium containing 10 µg/ml flubendazole. Note vacuoles (*V*) within the hypodermis (*H*) and in the cytoplasmic portion of the muscle cells (*CR*). ×5,000

Fig. 16 Female gonad (*G*) of a worm incubated twice for 8 h in a medium containing 100 µg/ml flubendazole. Note the complete disruption of the endothelial cells (*EN*). ×4,600



the hypodermis in the higher dosage groups showed the quickest and most intensive damage further fortifies the conclusion that the drug is able to penetrate through the rather thick cuticle, which itself, however, was not changed at all. On the other hand, the observation that the intestine also showed very severe damage in all cases indicates that the drug is also taken up via the mouth. This conclusion is underlined by the observation that in worms with sealed mouthparts the intestinal damage was slightly less severe than in worms with unsealed orifices but which were incubated in the same medium for the same time.

2. Double incubation in flubendazole-containing media is superior to single incubation.
   This conclusion can be easily drawn since in all dosages the use of a double treatment significantly increased the intensity of the tissue damage.
3. The onset and intensity of the drug effects are dosage-dependent.
   As can be seen in worms which were incubated for 8 or 32 h in media containing only 0.1 µg/ml flubendazole, it usually takes 32 h to reach at least some severe reactions, while incubation for only 8 h in the same medium introduced only very slight effects. Even those worms being incubated for 8 h in media



Figs. 17–20 TEM of *Ascaris suum*-worms

Fig. 17 Muscle cells (*M*) of controls. ×4,900

Fig. 18 Intestinal cells (*I*) of controls. ×5,300

Fig. 19 Worm incubated twice for 8 h in a medium containing 10 μg/ml flubendazole. Note the enlargement of the muscle cell (*M*), the degeneration of its central cytoplasmic region (*CR*) and the degeneration of the hypodermis (*H*). *V* Vacuolisation.×5,100

Fig. 20 Worm incubated twice for 8 h in a medium containing 10 μg/ml flubendazole. Note the apical vacuolisation (*V*) of the intestinal cells (*I*). (×6,000)

containing 1 μg/ml flubendazole showed only slight effects, while the organs of worms incubated for 8 h in 100 μg/ml flubendazole were heavily damaged. Thus, in worms incubated only once, the initiation of severe effects began between 10 μg/ml and 100 μg/ml flubendazole.

4. The drug effects are clearly time-dependent.
   In worms which were incubated only once in flubendazole-containing media, tissue damage was usually more severe after 32 h, when compared with worms after 8 h of incubation. An exception occurred in the highest dosage-groups (100 μg/ml flubendazole), for which – apparently due to the initially very high dosage – the onset of the adverse effects was as rapid in the 8 h-group as in the 32 h-groups. The strongest drug effects were already reached after 32 h and these were only rarely less severe than after 56 h.
5. The type of tissue damage is identical at all dosages. The damage seen in the different tissues is as follows:

   a. **cuticle:** none (Figs. 3, 13, 15)
   b. **hypodermis**: increasing vacuolisation (Figs. 3, 13, 15)
   c. **longitudinal muscles:** (1) initially an enlargement of the distance between the ''muscle packages'' occurred (Figs. 3, 13), (2) vacuolisation occurred

close to the hypodermis (Fig. 15), (3) enlargement and finally complete disruption of the mitochondria occurred within the cytoplasmic portion, (4) disruption of the nucleus occurred

d. **nerves:** (1) shortening and reduction in number of microtubuli (Figs. 9, 10), (2) enlargement of the diameter of the axons (Fig. 9), (3) vacuolisation of axons and of surrounding tissues (Fig. 10)
e. **genitalia:** the same effects occurred (if at all) in both male and female genitalia. The endothelial cells may be damaged and the membranes of the developing oocytes and spermatozoa may become disrupted (Figs. 14, 16). The depression (= flattening of fertilised/walled) eggs is probably due to the fixation and embedding procedures, since this looks similar to controls
f. **intestine:** the least damage is seen in the occurrence of a few vacuoles close to the basal layer of the intestine (Fig. 11), followed by an overall occurrence of such vacuoles (Fig. 12), and a final complete disruption of the intestinal cells. This process was supported by the swelling and final disruption of the endoplasmic reticulum.

6. The damage to tissues is multi-effective and thus lethal.
   The drug-derived damage compromises – even if it is low-grade – essential functional systems of the worms. The failure of the function of the hypodermis and of the intestine disturb the uptake of important nutrients, while the destruction or the induction of a malfunction (in groups with low grade damage) in the nerve and muscle cells will make it difficult for the worms to persist inside the intestine of the host.

Since such intestinal worms are always obliged to act against the peristaltic movement of the host gut, any disturbance of the worm's motile system will have a fatal outcome for the parasite and end in its expulsion.

The effects of flubendazole on the ultrastructure of *A. suum* were rather similar to those seen in *T. canis*:

1. Since the group with sealed orifices showed the same damage as the group with open orifices treated in the same way (three times 100 μg/ml flubendazole medium for 56 h), it can be concluded that flubendazole can be taken up via the cuticle.
2. While a double treatment with flubendazole showed a marked increase in the grade of damage in the tissues compared to a single treatment, a third treatment caused an only slightly more intense destruction than the double treatment at a given concentration and incubation time.
3. The drug-dosage needed to produce severe drug effects seemed to be a somewhat higher for *A. suum* than for *T. canis*. A single incubation with 10 μg/ml flubendazole for 8 h caused only slight, but nevertheless clearly discernible, effects.
4. The time- and dosage dependency of the drug was clearly visible.
5. The type of damage was very similar to that observed in the *T. canis* specimens (Figs. 19, 20).
6. As was the case for *T. canis*, the damage to the tissues of *A. suum* treated with flubendazole was multi-effective and thus had a lethal outcome.

Discussion

Motility tests

The experiments and the observations of the present study point out that flubendazole does not lead to the sudden immobilisation or death of the worms. Such a complete stop of motility occurs, for example, in cestodes which come into contact with praziquantel (Becker et al. 1981). During the whole period of the present experiments, none of the treated *Ascaris* or *Toxocara* worms died immediately. Initially, the flubendazole-incubated worms showed an even higher rate of activity and more intense movement than the untreated controls. However, flubendazole introduced a significant partial paralysis of *T. canis* when they were incubated for 32 h in medium containing at least 1 μg/ml flubendazole. Furthermore the type of movement was rather uncontrolled in the treated groups. This reduction and change of motility should be sufficient – apart from the drug-derived destruction of organs – in vivo to expel the worms from the host's intestine, as has been shown for other drugs (Mehlhorn and Harder 1997).

The observation that the untreated controls of *T. canis* also showed partial or complete paralysis after an incubation period of 48–56 h should not lead to doubts with respect to the significance of the comparisons after 8, 16, 24 or even 32 h.

Since *A. suum* from the control groups showed no loss of motility during the whole incubation-period (56 h), the partial paralysis, after 48 h, of the specimens treated twice or three times with 100 μg/ml flubendazole medium was definitely drug-derived.

Sealing the mouth and other body orifices did not bring about any changes in the motility, either in the controls or in flubendazole-treated worms of both species. This confirms that both nutrients and the drug are able to penetrate through the cuticle of these worm species. Flubendazole is not the only active anthelmintic compound which can be taken up via the cuticle of nematodes. For example, Mackenstedt et al. (1993) proved by autoradiographic studies that the preadults (but not the adults) of *T.canis* absorb pyrantel pamoate, mainly through the whole body surface.

Drug-derived effects in worm tissues

The evaluation of drug-derived effects is rather difficult. The main problem is that the effects/destruction of cells

do not occur at all sites of an organ at the same time and in the same intensity. Furthermore many of the effects are so small that they remain invisible under light microscopy, where only severe damage can be registered. Thus our evaluation and grading of the damage are mainly based on electron micrographs and some repeated semi-thin sections of different regions of the incubated worms.

The morphological destruction seen after incubation of the worms in media containing different amounts of flubendazole is most prominent in the hypodermis, intestine and muscle cells. This corresponds to the effects seen in nematodes after treatment with other drugs. The most severe damage in the tissues of *T. canis* specimens treated with pyrantel pamoate was observed in the same body-regions (Mackenstedt et al. 1993). Another different type of drug, emodepside, also causes damage predominantly in these tissues (unpublished data). The drug-derived destruction of the hypodermis and lysis of the intestine resulted in the starvation of the nematode, finally leading to complete immobilisation due to lack of nutrients. The effect of increasing paralysis was supported by the ongoing disintegration of the muscle cells. This chain of events, which was most clearly visible by electron microscopy, is based on the inhibition of intracellular microtubuli polymerisation (Koehler and Bachmann 1981; Barrowman et al.1984; Lubega and Prichard 1990), inducing as a secondary effect the described failure of all functions, thus leading to the final destruction of the organelles. The effects of flubendazole remained within the range of other benzimidazoles (Zintz and Frank 1982; Magambo 1996; Raether 2001). The rather slow onset of the cellular reactions and the increase of effects after repeating the exposure of the worms also corresponded to the effects seen in other benzimidazoles. The present experiments clearly point out that, in the case of a double treatment, even low dosages (e.g. 1 μg/ml) were sufficient to produce irreversible damage in the most important organs of the worms.

That the genitalia of the flubendazole-treated worms appeared relatively unchanged does not mean that these sexual systems remain functional. The complete blocking of microtubuli polymerisation due to the action of the benzimidazole also induced the total stop of any cellular division (mitosis, meiosis) and thus interrupted the reproductive function of the gonads (Osman et al. 1994). The organelles of the different cells of these systems, however, became affected later than, for example, the mitochondria of the intestine (Bughio et al. 1994). This leads to the impression of ''lower reactivity'' of the gonads to flubendazole and other benzimidazoles.

Since flubendazole reacted rather slowly, a double treatment was superior to a single treatment. In vivo studies with flubendazole confirmed the superiority of repeated treatments (Vanparijs et al. 1985; Sukontason et al. 2000). The destruction of the tissues was obviously additive and a double treatment led to irreversible and lethal damage. In the case of double treatment/incubations even the lowest dosage (0.1 μg/ml flubendazole) was sufficient to reach the goal of lethality. In the case of a single treatment, only the application of 100 μg/ml flubendazole had the same efficacy as the 0.1 μg-dosage in a double-incubation trial.

These results were underlined by the findings in the experiments on *A. suum*. The drug-derived damage in the low dosage groups (0.1 and 1 μg/mlflubendazole) was somewhat less intense compared to the *T. canis* specimens of comparable groups.

Flubendazole led principally to the same effects as those seen after incubation with other benzimidazoles. Similar studies to those described in this paper were carried out with oxfendazole (unpublished data). *A. suum* and *T. canis* treated with oxfendazole showed the same drug-derived effects as those treated with flubendazole. Comparing the results of the single-treatment series in both drugs, oxfendazole led to a quicker onset of the deleterious effect than flubendazole.

Similar studies were also done with pyrantel (unpublished data). This compound induced paralysis more quickly in the worms and led to more severe tissue-damage than flubendazole or oxfendazole.

Although the principal mode of action of pyrantel – occupying the acetylcholine receptors on the muscle membranes (Austin et al. 1966; Aubry et al. 1970) – is different from the initial effects of the benzimidazoles (prohibition of microtubuli polymerisation) the resulting, morphologically visible destruction was very similar in all three drugs, since the death of the worms occurred due to the intensive blocking of the energy metabolism. This phenomenon led to the disruption of the mitochondria and the final disintegration of all cells.

Conclusions

1. Flubendazole had a clear effect under in vitro conditions on adult *T. canis* and *A. suum*.
2. The reduction of the motility corresponded clearly with the increase in tissue damage.
3. Flubendazole was taken up both via the mouth and cuticle by *T. canis* and *A. suum*.
4. Double incubation of worms in flubendazole-containing media was significantly superior to a single incubation, as is the case in all benzimidazoles (Raether 2001).
5. The onset and intensity of flubendazole-derived effects are dosage- and time-dependent.
6. The type of tissue damage caused by flubendazole was identical for both species at all dosages. This means that the damage occurred at the level of the hypodermis, muscle and intestinal cells and that the same phenomena (e.g. disruption of mitochondria, vacuolisation of the cell-interior) always occurred independently of the amount of the drug.

7. The principal mode of action of flubendazole was based on the blocking of microtubuli polymerisation inside the cells and thus the disappearance of microtubuli. This effect had a lethal outcome for the worms due to the resulting complete destruction of the cells.
8. Flubendazole also had significant effects on the sexual reproduction of the worms by stopping all meiotic and mitotic processes.
9. The results of the different experiments in the present in vitro study (motility test, study of drug uptake, dose-dependence, time dependence, site and mode of action and comparison with other drugs) has increased our knowledge and will make it easier to propose the optimum application (dosage and timing) of flubendazole.

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