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The Action of Polyether Ionophorous Antibiotics (Monensin, Salinomycin, Lasalocid) on Developmental Stages of *Eimeria tenella* (Coccidia, Sporozoa) in vivo and in vitro: Study by Light and Electron Microscopy

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Abstract. The effect of three polyether antibiotics (monensin, salinomycin, lasalocid) on developmental stages of *Eimeria tenella* (Coccidia, Sporozoa) was studied in vivo and in vitro by means of light and electron microscopy. It was found that these three drugs act against free merozoites, which are destroyed by bursting of the cell border (i.e. pellicle), endoplasmic reticulum and internal organelles even after very short exposure times (20 min) in media containing 1 ppm, 10 ppm or 100 ppm of these drugs. Sporozoites, however, survived these drug concentrations during an exposure time of 30 min (this would be sufficient to penetrate host cells and start development). Intracellular stages, which were situated in a parasitophorous vacuole within an intact host cell, were not attacked, apparently because these drugs are almost incapable of penetrating host cells. On the other hand, parasites (such as differentiated schizonts, gamonts) located within degenerating host cells showed slight disintegration, which did not necessarily led to their death. From these results it becomes clear why these polyether antibiotics have to be fed daily. Doses of 70 ppm salinomycin, 125 ppm monensin and 125 ppm lasalocid were found to bring about an equivalent protective effect against an infection with 40,000 *Eimeria tenella* oocysts.

Introduction

For several years polyether ionophorous antibiotics have been known to have significant anticoccidial effects especially against the Eimeria species of chicken (Lit. cf. McDougald 1982). Among these monensin (Haney and Hoehn 1967; Shumard and Callender 1967), lasalocid (Mitrovic and Schildknecht 1974, 1976), narasin (Berg and Hamill 1978), and salinomycin

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(Kinashi et al. 1973; Miyazaki et al. 1974; Danforth et al. 1977) are used commercially. However, the knowledge of the mode of action of these compounds is incomplete. Further information is also needed about which stages of the Eimerian life cycle are affected by these drugs. To help answer these questions the present cytological study was undertaken with stages of *Eimeria tenella* treated in vivo and in vitro.

Materials and Methods

A. In vivo Experiments

1. Experimental Animals. Eighty HNL-NICK-CHICK cockerels (Lohmann, Wallau), which were 3 (experiment I) or 5 days old (experiment II) before infection, were kept in cages in groups of four at 31° C and 65% relative room humidity.

2. Parasites. For all infections fresh, fully sporulated oocysts of a drug-sensitive strain of *Eimeria tenella* (isolate H) were used.

3. Drug Concentrations in Feed. a) Monensin 125 ppm (Elancoban); b) Lasalocid 125 ppm (Avatec); c) Salinomycin 70 ppm (Sacox); d) Unmedicated feed (controls).

4. Procedure of Infection and Treatment

a) Experiment I. Thirty-two 3-day-old birds were inoculated each with 40,000 sporulated oocysts on D0 (day of infection). Each group (2 birds) was fed ad lib medicated broiler starter for 24 h on the following days after infection (D+x).

1) Salinomycin:	from D+2 to D+3
	from D+3 to D+4
	from D+4 to D+5
	from D+5 to D+6
	from D+6 to D+7
	from D+7 to D+8
2) Monensin:	from D+3 to D+4
	from D+5 to D+6
3) Lasalocid:	from D+3 to D+4
	from D+5 to D+6
4) Non-medicated controls:	12 birds

In each group (a–c) one animal was killed 24 h and the other 48 h after terminating treatment. The non-medicated controls were killed successively from D+2 to D+10.

b) Experiment II. Four groups consisting of 12 3-day-old birds were fed ad lib from D-2 (2 days before infection) to D+7 with feed containing 125 ppm monensin, 125 ppm lasalocid, or 70 ppm salinomycin or unmedicated feed (controls). Each bird was inoculated with 40,000 sporulated oocysts of *Eimeria tenella* on D0. Two chicken of each group were killed on D+2, D+3, D+4, D+5, D+6 and D+7 and the intestine prepared for electron microscopy.

B. In vitro Experiments

1. Sporozoites Treated with Salinomycin, Monensin and Lasalocid (Experiment III)

a) *Preparation of Sporozoites.* Sporocysts (set free from their oocysts by homogenisation) were incubated at 39° C in an excystation medium (pH 7.2) consisting of:

a) 70 ml Ringer's solution for amoebae (i.e. 7.54 g NaCl; 0.225 g KCl; 0.18 g $NaHCO_3$; 0.27 g $CaCl_2 \times 2\ H_2O$; 1 litre distilled water);
b) 0.25% trypsin;
c) 4% taurodeoxycholat.
After about 2 h most of the sporozoites were set free and appeared to move intensively.

b) *Solution of Drugs and Incubation.* Crystalline salinomycin (potency 988 mg/g), monensin (potency 895 mg/g) and lasalocid (995 mg/g) were dissolved in 5% ethanol and then suspended in distilled water at 1,000 µg/ml. Tenfold dilutions were made in incubation medium containing 90% Ringer's solution; 9.5% distilled water; 0.5% ethanol; 3.6% taurodeoxycholat and 0.25% trypsin in order to test a range of dilutions of the drugs from 1,000 µg/ml to 1 µg/ml.

About one million free motile sporozoites were incubated in 1, 10, 100 or 1,000 µg/ml of each drug mentioned above at 39° C for 30 min. Sporozoites in non-medicated excystation medium with and without 0.5% ethanol served as controls. This experiment was repeated twice.

2. Merozoites Treated with Salinomycin, Monensin and Lasalocid (Experiment IV)

a) *Preparation of Merozoites.* The merozoites were obtained from the caeca of five infected birds on day 5 post-inoculation. The contents of the caeca were filtrated and diluted in 0.85 NaCl solutions.

b) *Solution of Drugs.* Crystalline salinomycin, monensin and lasalocid (potency see under 1 b) were dissolved in ethanol and then suspended in distilled water at 1,000 µg/ml. Tenfold dilutions were made 0.5% ethanol and 98.69% distilled water to test a range of dilutions of the drugs from 100 µg/ml to 1 µg/ml.

c) *Incubation.* Amounts of 9×10^6 merozoites were exposed at 39° C for 20 min and 1 h. Merozoites in non-medicated medium with and without 0.5% ethanol served as controls.

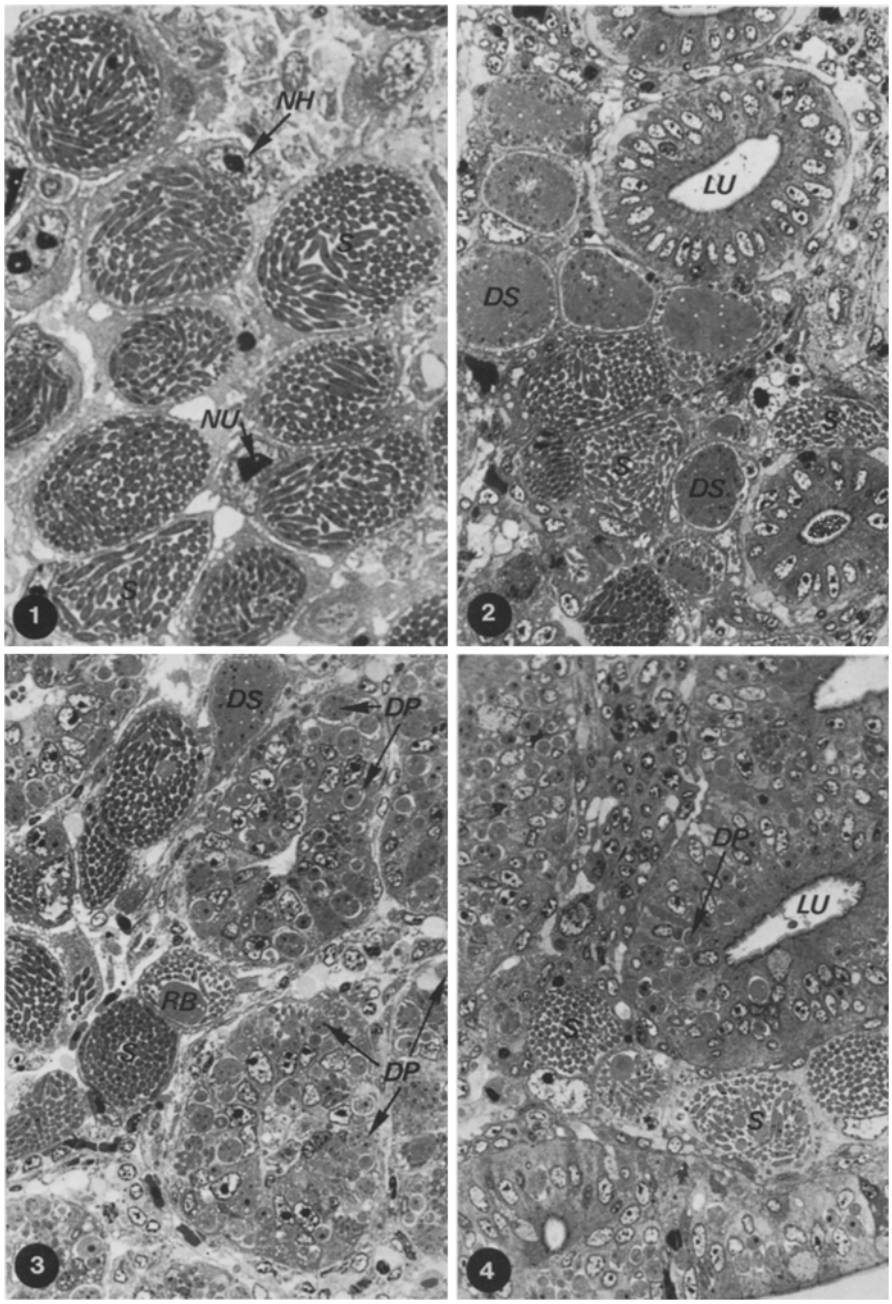
C. Electron Microscopy

Scrapings of the mucosa of the caeca (see experiments I, II) as well as free (sporozoites and merozoites, see experiments III, IV), were fixed for at least 48 h in 5% (v/v) glutaraldehyde buffered in 0.1 M Na-cacodylated (pH 7.3). The specimens were contrasted with 2% (w/v) OsO_4 for about 2 h, followed by washing, dehydration and embedding in Araldite (Ciba-Geigy). Ultra-thin sections were cut on a Reichert Ultracut-microtome, stained with an alcoholic solution of uranyl acetate for $^1/_2$ h and lead citrate (15 min) and finally studied with a Zeiss EM 9 S2.

Results

1. Experiment I

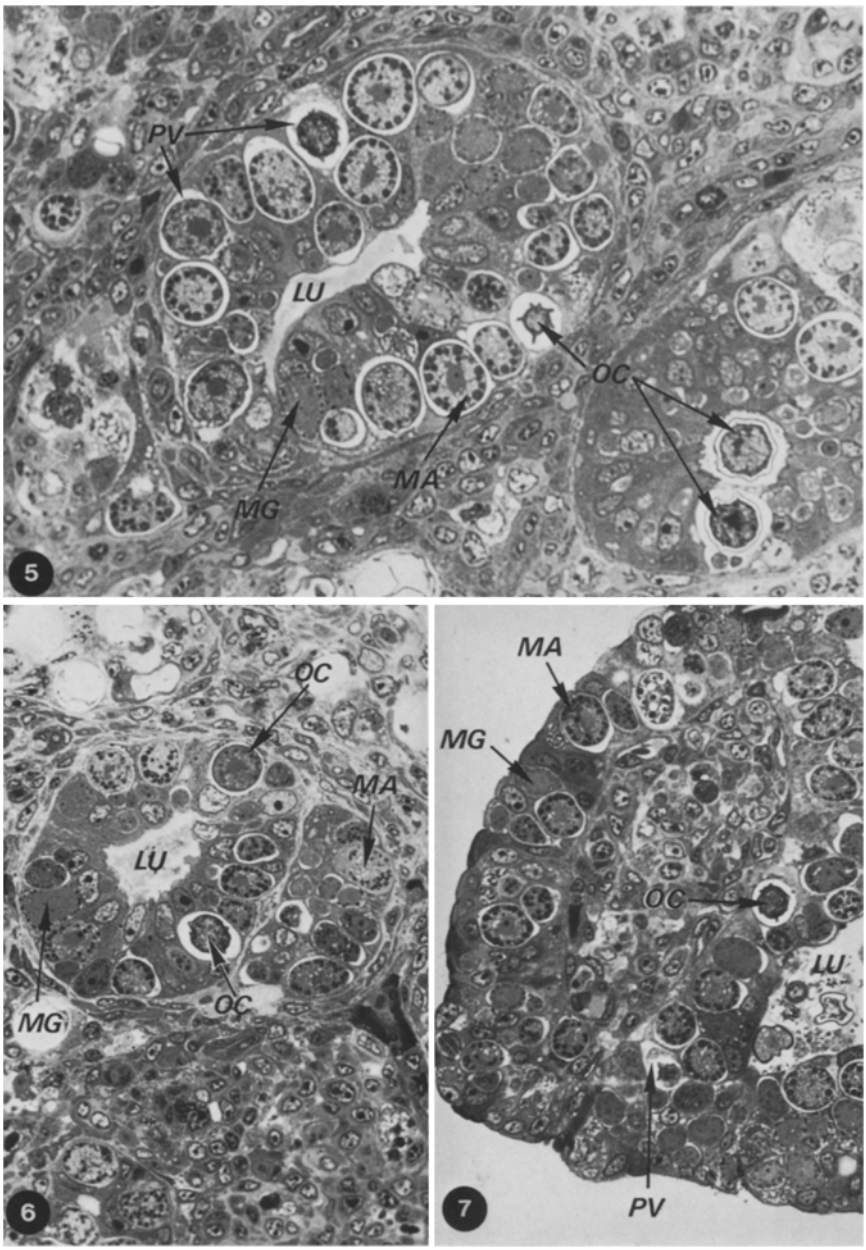
During the first experiment chickens infected with *Eimeria tenella* were treated with the three drugs for only 1 day (see Materials and Methods) and killed 1 or 2 days later. No significant effect was found after this treatment: the number of intracellular developmental stages (schizonts, gamonts etc.) in drug-treated animals appeared to be identical with those in the controls (Figs. 1–4). This was also the case in caeca sections of those animals, which were killed 2 days after exposure to drug treatment (Figs. 1–7). The fine structure of the drug-treated intracellular parasites did not differ from controls (Figs. 8, 10). It must be concluded that all three drugs have no significant effect on intracellular stages (schizonts or sexual stages) within their parasitophorous vacuoles.



Figs. 1–19. *Eimeria tenella:* developmental stages within cells of the caeca of experimentally infected chicken.

1–7 Light micrographs of semi-thin sections. **8–19** Transmission electron micrographs.

Figs. 1–4. 1 Untreated control of D+4 (4 days after infection). ×1,225. **2** Treatment was carried out with monensin on D+3 and the bird killed on D+4. ×600. **3** Treatment was carried out with salinomycin on D+3 and the bird killed on D+4. ×600. **4** Treatment was carried out with lasalocid on D+3 and the bird killed on D+5. ×600. Note no decrease in the number of parasites nor any damage to the parasites



Figs. 5–7. Gamonts, gametes and oocysts. **5** Untreated control on D+6. ×750. **6** Treatment was carried out with monensin on D+5 and the bird killed on D+6. ×600. **7** Treatment was carried out with salinomycin on D+6. ×600. Note no decrease in the number of parasites nor any damage to the parasites

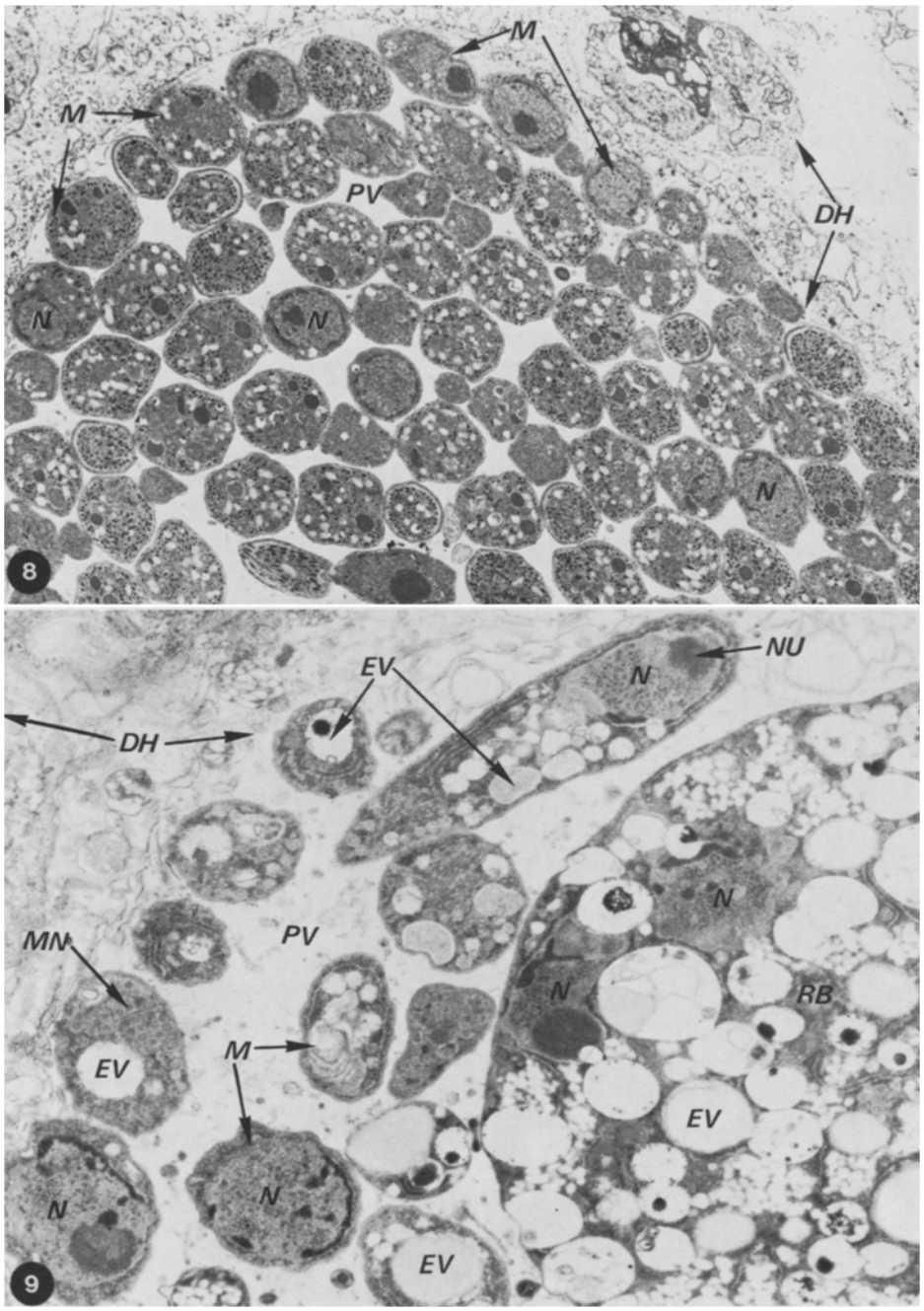
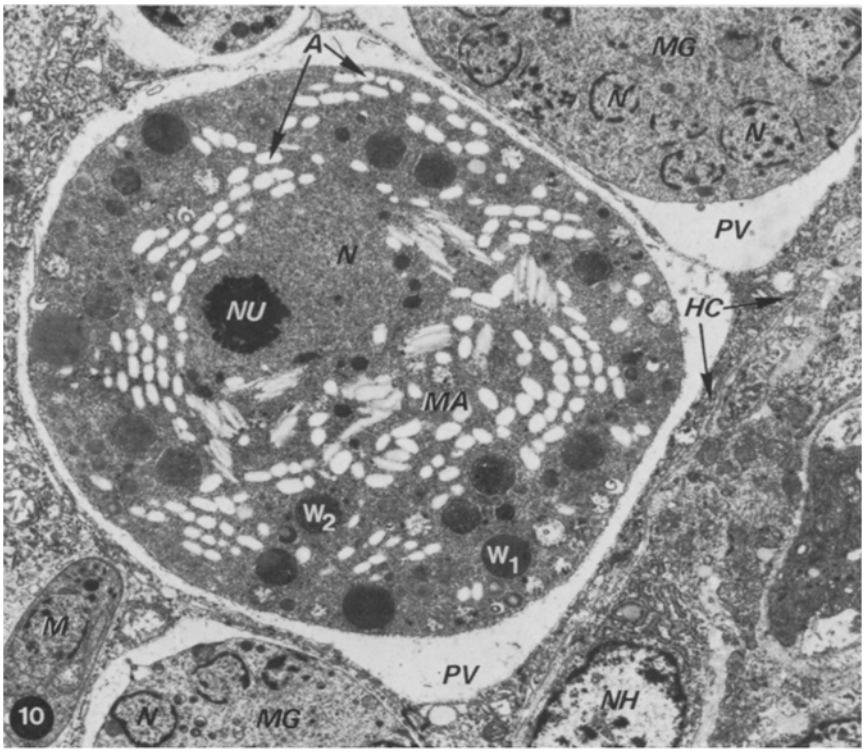


Fig. 8. Control. Section through a mature schizont with differentiated merozoites *M* within the parasitophorous vacuole *PV* on D+4. Note the degenerated host cell (DH). × 5,600

Fig. 9. Section through a mature schizont treated with salinomycin (70 µg/ml). This stage is situated within a parasitophorous vacuole *PV* of a degenerated host cell *DH* and shows alterations such as heavily swollen vacuoles *EV* inside the differentiated merozoites *M* and the residual body (similar damage was seen in schizonts treated with monensin and lasalocid). × 11,250



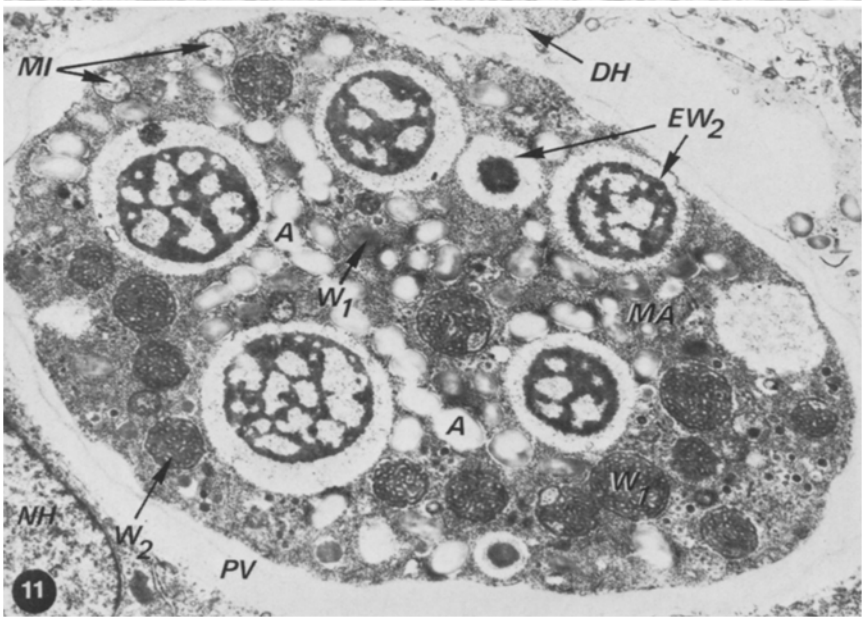
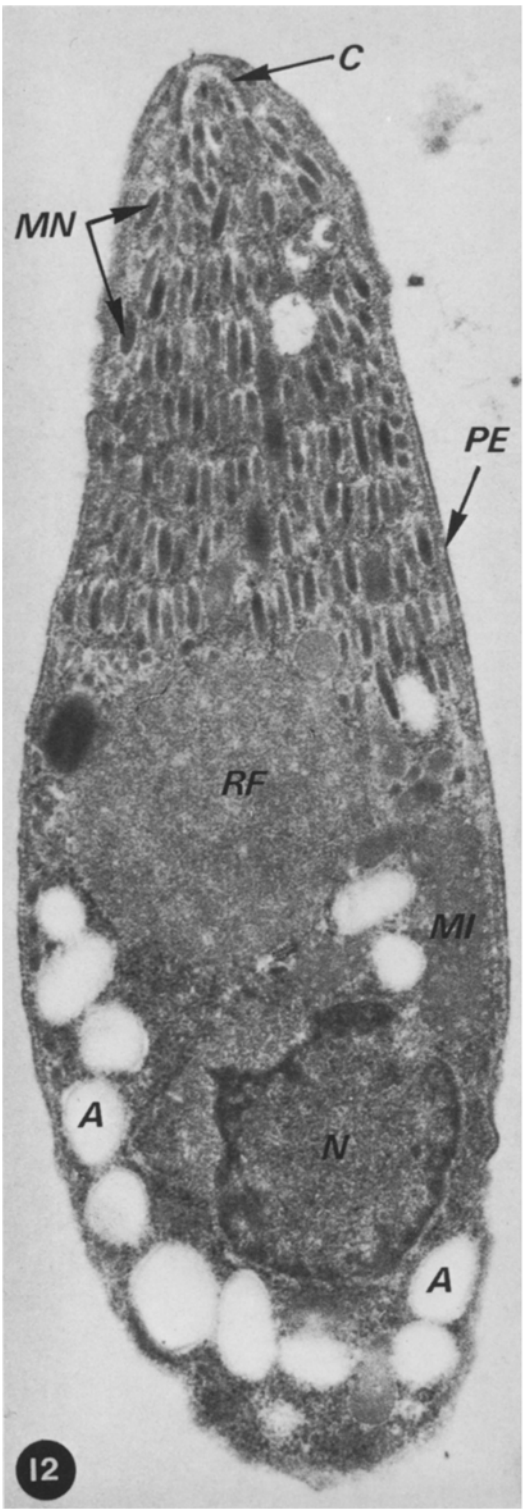


Fig. 10. Control. Section through a macrogamete *M* and two microgamonts *MG* on D+5. Note the nearly completely degenerated host cells *HC*. ×6,000

Fig. 11. Section through the surface of a macrogamete treated with monensin. Note the partly swollen wall-forming bodies II EW_2, probably due to the degeneration of the host cell *DH* (the damage caused by lasalocid and salinomycin was similar). ×1,450



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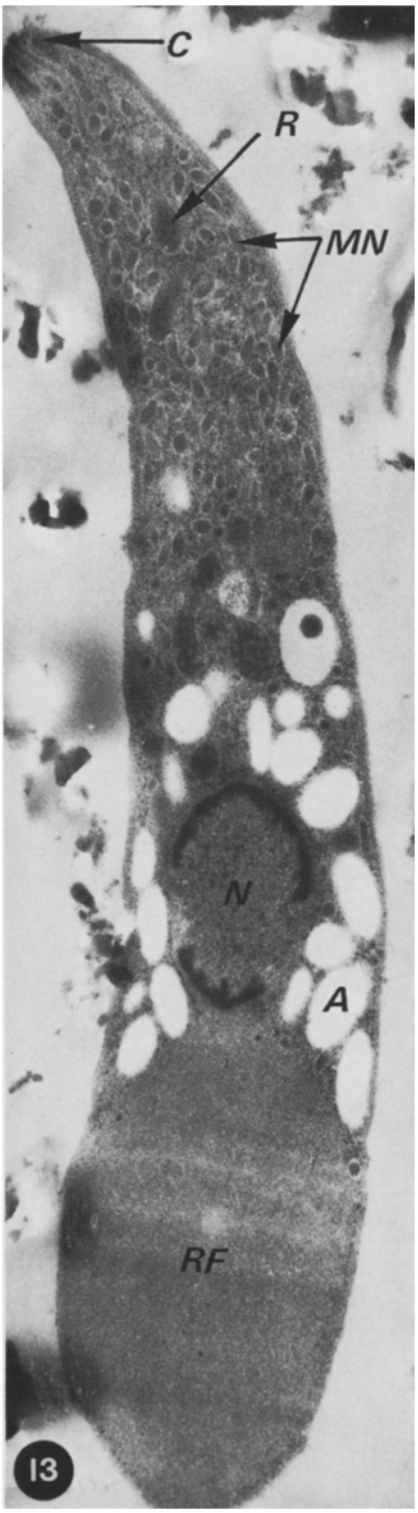

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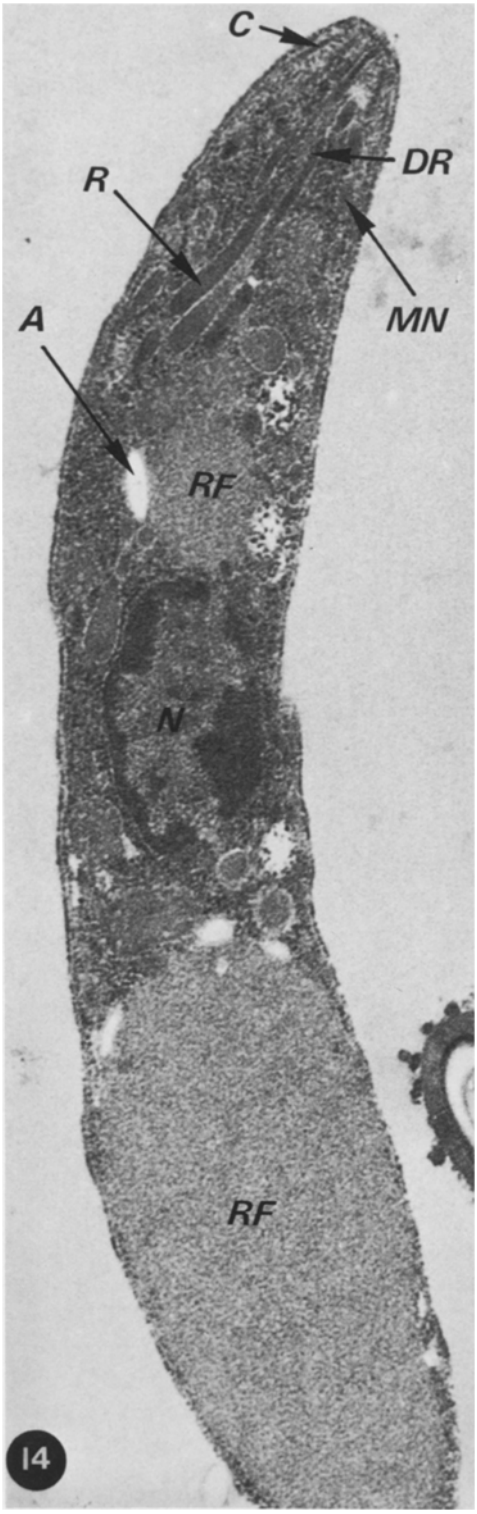


Figs. 12–14. Sporozoites in vitro.
12 Untreated control. × 36,000.
13 30 min incubation in 100 µg/ml lasalocid. × 30,000.
14 30 min incubation in 100 µg/ml salinomycin. × 30,000. Note the normal fine structure of the treated stages (as control)

2. Experiment II

During this experiment the animals were constantly treated with one of the three drugs (see Materials and Methods). After this procedure it was noted that the number of schizonts and particularly the number of gamonts decreased considerably, so that it was hard to find them on histological sections. The fine structure of young schizonts and young gamonts within parasitophorous vacuoles of their relatively intact host cells was similar to that of the controls. Some destruction was seen in mature schizonts (with differentiated merozoites) and in gamonts (micro- and macrogamonts), which were all situated in degenerating host cells (Figs. 9, 11). In the differentiated merozoites residual bodies of schizonts and macrogamonts showed a considerable vacuolization, enlarged perinuclear spaces and degenerating mitochondria. In macrogamonts the wall-forming bodies II were also partly destroyed (Fig. 11, W2). On the other hand, the oocysts appeared intact, as in the controls or in experiment I. The cytological results of experiments I and II led to the suggestion that intact host cells are a barrier for the polyether antibiotics and that their effect is directed against free stages such as sporozoites or merozoites, as studied in experiments III and IV.

3. Experiment III

Free sporozoites were incubated for 30 min at 39° C in media containing 1, 10, 100, 1,000 µg/ml salinomycin, monensin and lasalocid, respectively. It was found that salinomycin and lasalocid in a concentration of 1,000 µg/ml immediately destroyed most of the sporozoites, which remained intact in controls and after incubation in 1,000 µg/ml monensin. In the lower concentrations (1, 10, 100 µg/ml) no effect was seen cytologically on the incubated sporozoites (Figs. 12–15). As in the controls these stages remain intact and were intensively motile before fixation and embedding.

4. Experiment IV

The merozoites that had been incubated in media containing 1, 10 or 100 µg/ml salinomycin, monensin and lasalocid, respectively, were significantly affected (Figs. 16–19). The effect started even after 20 min in media containing 1 µg/ml by the loss of motility and a clear vacuolation (fissuration) in the cytoplasm (Fig. 17). Merozoites incubated in media containing 10 µg/ml had a heavily swollen endoplasmic reticulum and a considerably enlarged perinuclear space as well as swollen mitochondria (Fig. 18). Merozoites incubated for 20 min in media containing 100 µg/ml or for 1 h in the lower concentrations were completely disintegrated by constantly degenerative processes such as destruction of micronemes, rhoptries, golgi apparatus and finally by the bursting of the limiting cell border (i.e. the typical pellicle, Fig. 19). The severity of effect was clearly dependent on the drug concentrations. However, there was no obvious difference between the polyethers investigated with respect to the intensitivity of damage in the merozoites.

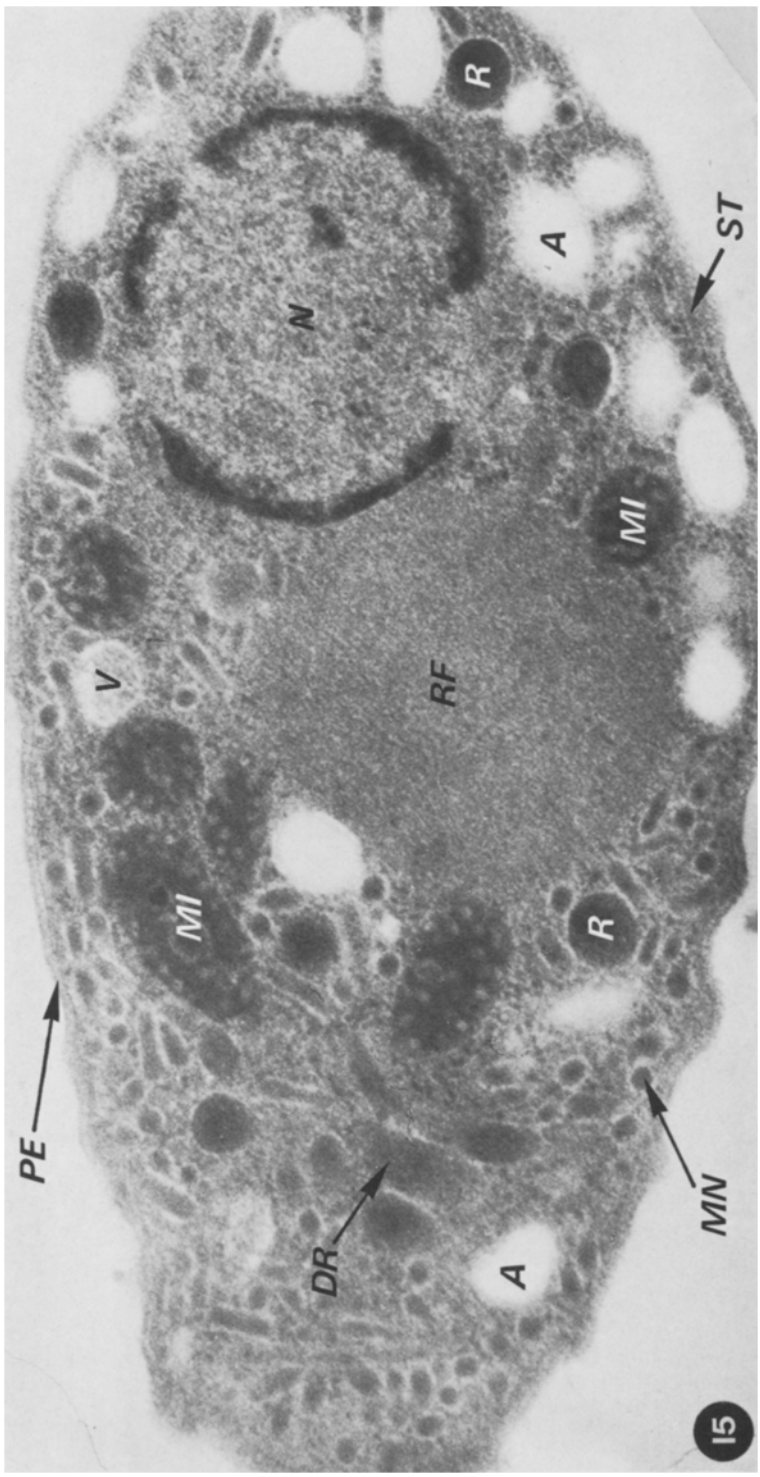
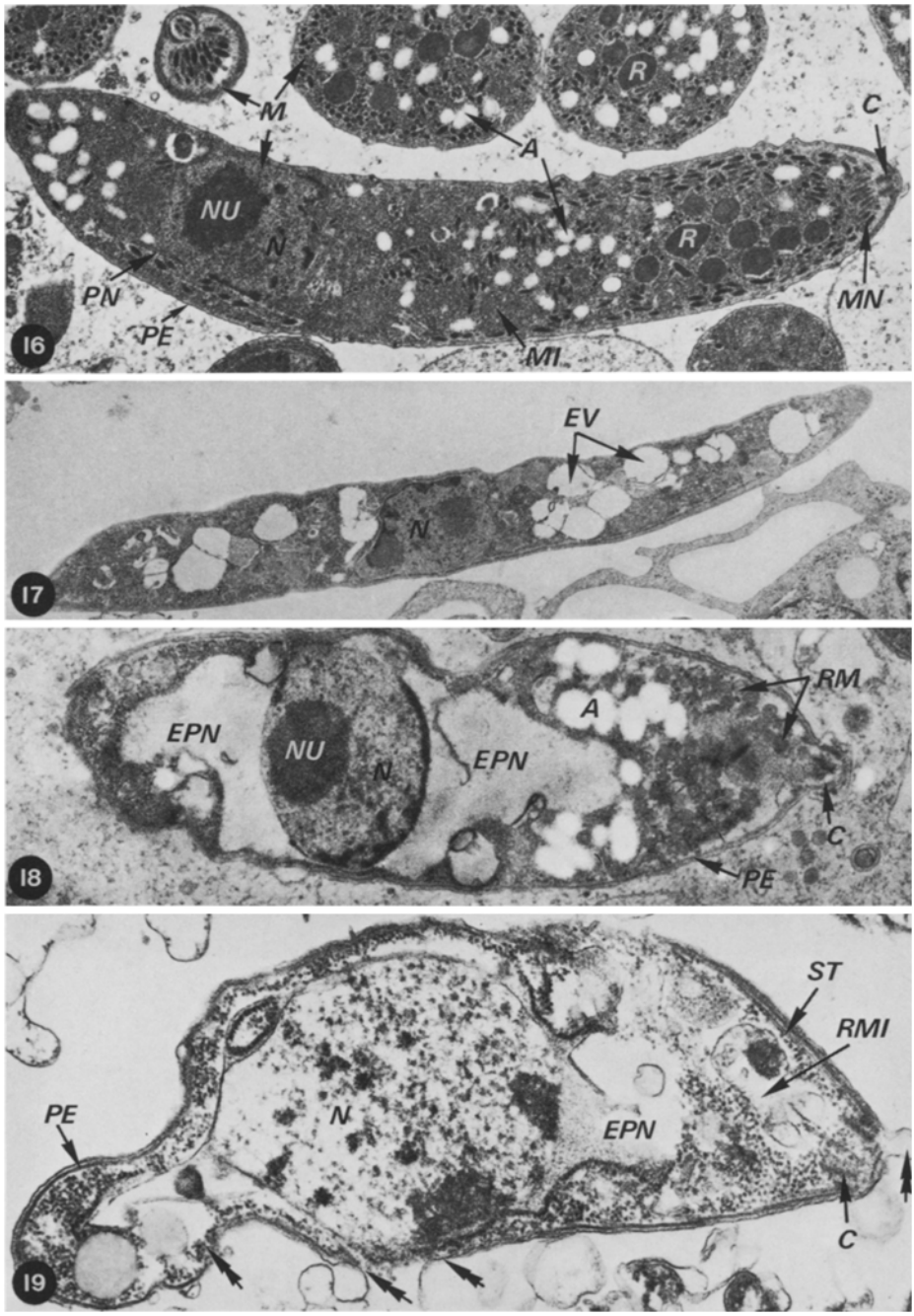


Fig. 15. Sporozoite in vitro; 30 min incubation in 100 μg/ml monensin. ×45,000



Figs. 16–19. Merozoites in vitro. **16** Fine structure of control. ×11,250. **17** 20 min incubation in 1 µg/ml salinomycin. ×7,500. **18** 20 min incubation in 10 µg/ml salinomycin. ×13,500. **19** 20 min incubation in 100 µg/ml salinomycin. ×15,000

Discussion

The results obtained indicate that the anticoccidial effect of the polyether ionophorous antibiotics studied is due to the immediate killing of free merozoites, after exposure to low drug concentrations even within a short time. No effect, however, was found in intracellular stages situated within an intact host cell such as in the developmental stages of young schizonts and gamonts. This effect may be due to the slow penetration rate of these polyether compounds into intact cells, as described by various authors (Lit. cf. McDougald 1982). The alterations observed in those intracellular developmental stages (such as differentiated schizonts, old gamonts), which were situated in the degenerating host cells, suggest this mode of action.

The findings of Long and Jeffers (1982) and Smith et al. (1981) that polyether antibiotics also destroy sporozoites were not supported by our results. However, in the present study shorter exposure periods to drugs (30 min instead of 2 h or 4 h) were chosen, since it is known from tissue culture experiments that sporozoites penetrate the cells relatively soon after excystation and thus decrease the influence of polyether drugs. Even after the relatively long incubation periods demonstrated by Long and Jeffers (1982) some of the sporozoites avoided the action of the drug because haemorrhage, as a result of infections, occurred in some of the embryos inoculated with sporozoites incubated in this way. Our observation that sporozoites were able to survive the very high concentrations of 1,000 µg/ml monensin, even if only for short periods, underlines the fact that these stages accumulate and retain polyether antibiotics (Smith and Strout 1979) as do host cells to a lower degree, whereas many sporozoites died immediately after exposure to 1,000 µg/ml salinomycin or lasalocid. However, sporozoites are apparently much less susceptible than merozoites as shown in our experiments. The latter are destroyed after short exposure periods and even in lower concentrations. From this it is understandable that polyether antibiotics have to be fed continuously to prevent an outbreak of coccidiosis since the infectious merozoites are set free periodically and thus may be destroyed by the drug only during this period. Therefore the polyether drugs are only used prophylactically, whereas sulfonamides (e.g. sulfaquinoxaline) are used exclusively as therapeutic drugs. On the other hand, various compounds are known to act on intracellular stages, e.g. robenidine (Lee and Millard 1972), aprinocid (Wang et al. 1981; Long and Jeffers 1982), symmetrical triazinone (Aichinger et al. 1978; Mehlhorn et al. 1983) and halofuginone (Greuel and Raether 1979), and therefore they might be used also as chemotherapeutic drugs.

It is known that the potency of the polyether antibiotics varies. The feed concentrations recommended for control of coccidiosis in broiler chicks are different. Efficacy of the most commonly used level of monensin is 100 ppm, whereas lasalocid was recently approved for use in the range 75–125 ppm; the optimal activity of narasin appears to be 70 ppm[1] (Ruff et al. 1979), whereas salinomycin is used at 50–70 ppm[2]. To avoid a delay

1 See prospect of Monteban (Elanco Ltd.)
2 See prospect of Sacox (Hoechst AG)

of immunity salinomycin and narasin would be preferred according to the results of Reid et al. (1977) and Long et al. (1979) who recommended the use of the lowest possible drug concentrations that still give sufficient anticoccidial protection.

The damage caused by salinomycin, monensin and lasalocid on the fine structure of *Eimeria tenella* is basically the same in all developmental stages attacked by the polyethers. On the other hand, controls showed the typical fine structural aspects of Eimerians as described in previous papers by numerous authors (Ryley 1969; Scholtyseck et al. 1971, 1972; Hoppe 1973; Vetterling et al. 1973; Chobotar and Scholtyseck 1982; Mehlhorn and Piekarski 1981; Mehlhorn et al. 1983). However, treated parasites showed typical disintegration. These reactions were mainly due to the ionophorous nature of the drugs causing an osmotic shock which led to vacuolisation of intracellular organelles and finally to the bursting of the whole parasitic cell. A suppression of nuclear divisions, as observed after treatment with symmetrical triazinone by Aichinger et al. (1978) and Mehlhorn et al. (1983), was not seen in our studies, which may be explained by the barrier function of the host cell.

Abbreviations

A – Amylopectin; C – Conoid; DH – Degenerating host cell; DP – Developmental stages; DR – Ductules of rhoptries; DS – Developmental schizonts; EPN – Enlarged perinuclear space; EV – Enlarged vacuoles; EW_2 – Enlarged wall-forming bodies II; HC – Host cell; LU – Interior of crypts; M – Merozoites; MA – Macrogametes; MG – Microgamonts; MI – Mitochondria; MN – Micronemes; N – Nucleus; NH – Nucleus of host cell; NU – Nucleolus; OC – Oocyst; PE – Pellicle; PN – Perinuclear space; PV – Parasitophorous vacuole; R – Rhoptries; RB – Residual body; RF – Refractile body; RM – Remnants of micronemes; RMI – Remnants of mitochondria; S – Mature schizont; ST – Subpellicular microtubules; V – Vacuole; W_1 – Wall-forming body I; W_2 – Wall-forming body II

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