



In Vitro Studies on the Relative Sensitivity to Ivermectin of *Necator americanus* and *Ancylostoma ceylanicum*

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Abstract—Richards J. C., Behnke J. M. & Duce I. R. 1995. *In vitro* studies on the relative sensitivity to ivermectin of *Necator americanus* and *Ancylostoma ceylanicum*. *International Journal for Parasitology* 25: 1185–1191. Experiments were carried out to compare the sensitivity of *Ancylostoma ceylanicum* and *Necator americanus* to ivermectin (IVM) and pyrantel *in vitro*. Loss of motility and inhibition of ingestion by IVM were compared and *A. ceylanicum* was found to be approximately 40–50 times more sensitive to IVM than *N. americanus*. Both species showed a similar sensitivity to pyrantel. Uptake of [³H]IVM across the cuticle was compared and shown to be unlikely to account for the differences in sensitivity to IVM between the two species.

Key words: *Ancylostoma ceylanicum*; *Necator americanus*; hookworm; anthelmintic; ivermectin; pyrantel.

INTRODUCTION

There is much interest in the potential of ivermectin (IVM) as a microfilaricide for the treatment of onchocerciasis (Bennett, Williams & Dave, 1988), and mass trials against onchocerciasis are being implemented in 12 African and Central American countries. IVM has proven to be a very effective antiparasitic drug and many of the common gastrointestinal nematodes are highly susceptible to IVM at concentrations much lower than those at which other anthelmintics are effective. However, some parasites, including *Heligmosomoides polygyrus*, *Trichuris trichiura*, and hookworms (Wahid, Behnke & Conway, 1989; Whitworth, Morgan, Maude, McNicholas & Taylor, 1991) have been found to be tolerant to IVM.

In vivo studies on hookworm infections in hamsters revealed that *Ancylostoma ceylanicum* was 300 times more sensitive to IVM than *Necator americanus* (Behnke, Rose & Garside, 1993). Previous work (Rajasekeriah, Deb, Dhage & Rose, 1989) also

indicated that in a hamster model, *Necator* was relatively insensitive to IVM.

The difference in IVM sensitivity is of interest in two respects. First, the drug may be used clinically in situations where *N. americanus* and *Ancylostoma duodenale* are both present in the population and its use may influence epidemiology. Second, if the differential sensitivity of these 2 species reflects a difference in the drug target site, these species of hookworm may provide a good model system to probe further the molecular action of the drug.

In this paper we report *in vitro* experiments which show that the difference in sensitivity of *N. americanus* and *A. ceylanicum* to IVM can be demonstrated *in vitro* and is therefore not attributable to the host–parasite relationship. We further show that the differential responsiveness cannot be explained by differences in the uptake of the drug.

MATERIALS AND METHODS

Parasites and hosts. Infective larvae of *N. americanus* were obtained from Dr Rajasekeriah of CIBA-GEIGY

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Hindustan Ltd, Bombay, India in 1983 and maintained by regular passage through hamsters as described by Sen (1972) and Behnke, Wells & Brown (1986). *A. ceylanicum* was also obtained from Dr Rajasekariah and passed through adult hamsters as described by Garside & Behnke (1989). Worms of both species were recovered from the host (*N. americanus* 5 weeks post-infection, *A. ceylanicum* 14–21 days post-infection), following terminal anaesthesia in chloroform, by cutting open the small intestine and picking out the worms into dishes of warm Hanks's saline (pH 7.0). After several washes with Hanks's saline, approximately 10 µl/ml of penicillin–streptomycin (10,000 IU/ml, 10,000 µg/ml, respectively) were added and the worms were maintained in Hanks's saline at 37°C. For incubations of 24 h, worms were incubated in sterile conditions in Roswell Park Memorial Institute (RPMI 1640 Medium) (GIBCO), containing 10 µl/ml penicillin–streptomycin, 10 µl/ml L-glutamine, 1 µl/ml sodium pyruvate and 0.2 µl/ml monothio glycerol (pH 7.0).

Chemicals. Pyrantel (Pfizer) was taken from a stock solution of 10 mg/ml pyrantel pamoate in DMSO. Ivermectin (IVM), ivermectin phosphate (IVM-PO₄) and [³H]ivermectin ([³H]IVM) were obtained from Merck and Co. (Rahway, U.S.A.). IVM and IVM-PO₄ were taken from stock solutions of 1 and 2.5 mg/ml, respectively, in DMSO and the [³H]IVM was from a stock with a specific activity of 2.09 MBq/µg; [³H]inulin (specific activity 3.33 GBq/g) was purchased from ICN Biochemicals. All drugs were administered in a final DMSO concentration of 1% v/v.

Toxicity of IVM and pyrantel. Groups of 10 worms (mixed sexes) were incubated at 37–38°C in 1 ml of 1% DMSO Hanks's saline containing IVM, IVM-PO₄ or pyrantel at a range of concentrations. Preliminary experiments showed that male and female worms of both species were similarly affected by all three anthelmintics under these conditions. Although in the control groups 100% of the worms remained active, some loss of motility was observed after 3 h incubation in Hanks's and the level of motility rapidly declined after periods of 6 h or longer. Sterile RPMI 1640 was therefore used as the medium for incubations > 3 h as the control groups of worms incubated in RPMI retained much higher levels of motility after 6–24 h than in Hanks's saline. The activity of the worms at various times (1–24 h) was compared with controls incubated in 1% DMSO in Hanks's saline or RPMI 1640. Worms were classed as either active or inactive, and inactive worms were defined as those which showed no motility and did not respond to a mechanical stimulus (gently prodding and lifting the worms with fine watchmaker's forceps).

Uptake of [³H]IVM. Fine wire ligatures (resin-coated copper wire, approximately 100 µm diameter) were tied as near to the head and tail of each worm as possible. Damage to the cuticle by the ligatures was assessed microscopically, and visibly damaged worms were discarded. The worms were incubated in groups of 10 (mixed sexes) for 3 h at 37–38°C in 1 ml of incubation medium comprising Hanks's saline/1% DMSO; 11.44 µM IVM; 0.033 MBq [³H]IVM. Each worm was washed 3 times in Hanks's saline and then placed on filter paper to remove excess moisture. As the [³H]IVM was found to be adsorbed by the wire ligatures, the

ends (0.5 mm) of each worm (both ligatured and unligatured) were removed. After being left to dry overnight on pieces of foil, individual worms were placed in scintillation vials. The worms were solubilized in 100 µl of 2.5 M NaOH for 1–2 h. HCl, 100 µl 2.5 M, was then added, followed by 4 ml of scintillation fluid (Packard "Emulsifier Safe" liquid scintillation cocktail for aqueous samples). Radioactivity was measured on a Packard Liquid Scintillation Spectrometer. Results were corrected for quenching and expressed as d.p.m./worm. A number of individual worms were incubated without the radioisotope and then analyzed as above to provide background levels; the d.p.m./worm values given are corrected for background.

Uptake of [³H]inulin. As IVM is a highly lipophilic molecule, it readily crosses cellular barriers. Inulin, on the other hand, does not pass across cell membranes, and is not readily taken up by cells. [³H]inulin uptake was therefore used to assess the amount of material taken up from the surrounding medium through ingestion. Ligatured and unligatured worms (mixed sexes) were incubated in 1 ml of Hanks's saline containing 1% DMSO; 0.0333 MBq [³H]inulin for 3 h then analyzed as above.

Inhibition of [³H]inulin uptake. Groups of 10 worms (mixed sexes) were incubated for 24 h in sterile conditions in either 1 ml of RPMI 1640 or HLac (Hanks's saline containing 5 µg/ml lactalbumin, 0.2 M HEPES, 10 µl/ml penicillin–streptomycin, 0.3 mg/ml kanamycin, pH 7.0) and various concentrations of IVM-PO₄ or pyrantel, then analysed as above.

Statistical analysis of results. Non-parametric statistical procedures were used to analyse the data throughout because a normal distribution of data could not be assumed. The results shown in Figs 1a, b and Fig. 2 are presented as mean % of active worms ± standard error (S.E.M.). For each different concentration of drug, the mean number of active worms given is derived from the results of 3–6 experiments in which groups of 10 worms were incubated for 3 h and the number of active worms assessed after 1, 2 and 3 h. The results of the radioisotope uptake experiments are arranged into four groups: (i) uptake of [³H]IVM, (ii) effect of ligatures on uptake of [³H]inulin, (iii) and (iv) effects of IVM and pyrantel on uptake of [³H]inulin. Mean d.p.m. values given for each experimental group are derived from the pooled results from the analysis of individual worms. In order to avoid Type I errors arising from multiple comparisons within experiments, a maximum of two a priori hypotheses were examined within each of the different experimental groups. A non-parametric form of the ANOVA test (analysis of variance by ranks) was used throughout the analysis. A two-way ANOVA (Meddis, 1984) was employed to assess the results of the uptake of [³H]IVM experiments (Experiment 1) specifying species (*A. ceylanicum* vs. *N. americanus*) and treatment (ligatured vs. non-ligatured) as the two factors. The following hypotheses were predicted: non-ligatured > ligatured and *A. ceylanicum* > *N. americanus*. A one-way ANOVA was used to test the hypothesis: ligatured < non-ligatured (both species) for the results of the ligature/uptake of [³H]inulin experiments (Experiment 2). Similarly, in order to test the hypothesis that IVM and pyrantel inhibited the

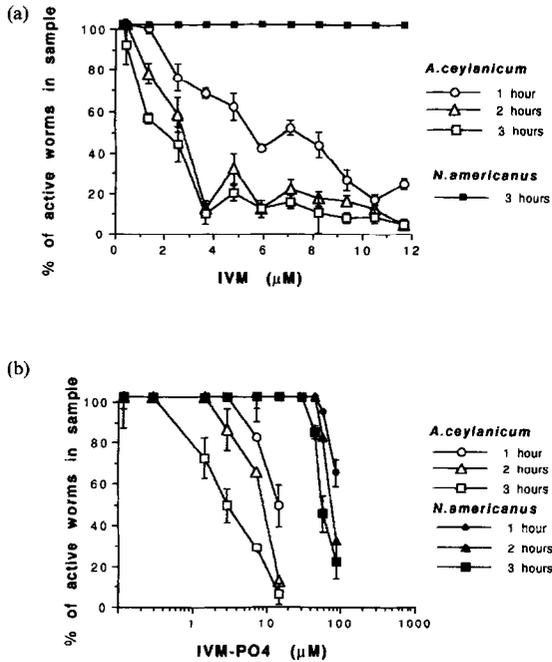


Fig. 1. Comparison of the effects of IVM (Fig. 1a) and IVM-PO₄ (Fig. 1b) on % active *A. ceylanicum* and *N. americanus*. Worms were classed as either active or inactive (see Methods) and the data are presented as the mean percentage (\pm S.E.M.) of worms showing activity after 1, 2 and 3 h incubation in IVM or IVM-PO₄ ($n = 30-60$ worms). IVM-PO₄ was shown to immobilize *N. americanus* dose-dependently (Spearman rank $r_s = -0.898$, $n = 18$, $P < 0.0001$, $t = 3$ h). Similarly IVM and IVM-PO₄ both caused inactivity in *A. ceylanicum* dose-dependently (Spearman rank $r_s = -0.80$, $n = 55$, $P < 0.0001$, $t = 3$ h; $r_s = -0.986$, $n = 13$, $P < 0.0001$, $t = 3$ h, respectively).

uptake of [³H]inulin by *A. ceylanicum* and *N. americanus* (Experiments 3 and 4), one-way ANOVA was used to test the prediction that control > IVM/pyrantel treated for each species. For all a priori hypotheses examined, the test statistic z is given as appropriate. Probabilities (P) of 0.05 or less were considered significant.

RESULTS

Comparison of the effects of IVM and pyrantel on the activity of *A. ceylanicum* and *N. americanus*

Over a period of 3 h, *A. ceylanicum* was found to be equally sensitive to IVM and IVM-PO₄ and was immobilized by IVM or IVM-PO₄ dose-dependently with an approximate EC₅₀ at 3 h of 1.14 µM, 2.0 µM (IVM, IVM-PO₄, respectively, EC₅₀s derived from Figs 1a, b).

N. americanus was not visibly affected by the standard therapeutic drug IVM at the range of concentrations used (0.11–11.44 µM) over a period of 3 h. IVM did not remain in solution above this concentration range or within this concentration

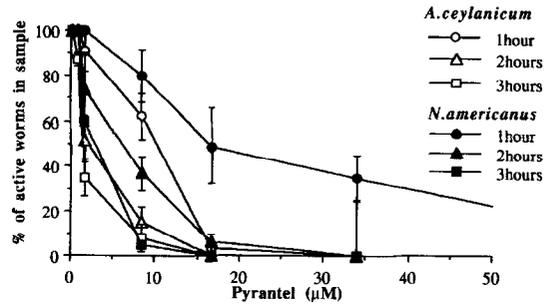


Fig. 2. Comparison of the effects of pyrantel on % active *A. ceylanicum* and *N. americanus*. Worms were classed as either active or inactive (see Methods) and the data are presented as the mean % (\pm S.E.M.) of worms showing activity after 1, 2 and 3 h incubation in pyrantel ($n = 30-60$ worms). *A. ceylanicum* and *N. americanus* were equally sensitive to pyrantel, which immobilized both species dose-dependently (Spearman rank $r_s = -0.78$, $n = 46$, $P < 0.0001$, $t = 3$ h; $r_s = -0.75$, $n = 31$, $P < 0.0001$, $t = 3$ h, respectively).

range for > 8 h. However, experiments with the more soluble phosphate form of IVM (IVM-PO₄) were carried out and IVM-PO₄ was found to paralyse *N. americanus* dose-dependently, with an approximate EC₅₀ of 55 µM after 3 h (Figs 1a, b).

Even after incubation periods of 24–48 h, *N. americanus* was not visibly affected by IVM-PO₄ at concentrations of 11.44 µM compared with controls incubated in normal media. However, this concentration caused > 90% inactivity in *A. ceylanicum* after 3 h.

Pyrantel was found to be similarly effective at paralyzing *A. ceylanicum* and *N. americanus* dose-dependently, with approximate EC₅₀s of 1.34 and 3.5 µM (*A. ceylanicum* and *N. americanus*, respectively) ($t = 3$ h) (Fig. 2).

Uptake of [³H]IVM by *A. ceylanicum* and *N. americanus*

There was no significant effect of ligaturing the worms on the uptake of IVM; however, there was a significant difference between the species with *A. ceylanicum* taking up more [³H]IVM than *N. americanus* (Table 1a). There was no difference in the uptake by male and female worms.

Uptake of [³H]inulin by *A. ceylanicum* and *N. americanus*

The ligatures had a significant effect on the uptake of [³H]inulin by both *A. ceylanicum* and *N. americanus* (ratio non-ligatured vs. ligatured 7:1, 9:1, respectively, Table 1b), proving the effectiveness of the ligatures in preventing the entry of media via anatomical openings (Ho, Geary, Barshun, Sims & Thompson, 1992). *N. americanus* was more active in

Table 1a—The uptake of [³H]IVM by *A. ceylanicum*, *N. americanus* with and without ligatures

Species	Ligated Mean d.p.m. ±S.E.M. (n)	Non-ligated Mean d.p.m. ±S.E.M. (n)
<i>A. ceylanicum</i>	126 ± 11.7 (33)	111 ± 11.0 (53)
<i>N. americanus</i>	78 ± 9.14 (39)	73 ± 17.2 (28)

Statistical analysis for predictions			
<i>A. ceylanicum</i> > <i>N. americanus</i>		Ligated < non ligated (both species)	
z	P	z	P
3.9	<0.0008	-0.88	>0.05

For statistical analysis see text. d.p.m. = disintegrations per min.

Table 1b—the uptake of [³H]inulin by *A. ceylanicum*, *N. americanus* with and without ligatures

Species	Ligated Mean d.p.m. ±S.E.M. (n)	Non-ligated Mean d.p.m. ±S.E.M. (n)
<i>A. ceylanicum</i>	1.05 ± 2.86 (22)	7.17 ± 2.96 (21)
<i>N. americanus</i>	5.52 ± 3.16 (23)	51.2 ± 7.41 (51)

Statistical analysis for predictions			
Ligated < Non-ligated			
z	P	z	P
		4.74	<0.01
		1.86	<0.05

For statistical analysis see text. d.p.m. = disintegrations per min.

terms of uptake, and ingested approximately 6 times more [³H]inulin than *A. ceylanicum*. In the medium used, the basal level of ingestion of [³H]inulin was lower in *A. ceylanicum* than *N. americanus*, suggesting that *N. americanus* is more active in terms of oral uptake under these conditions.

Comparison of the effect of IVM-PO₄ on the uptake of [³H]inulin by *A. ceylanicum* and *N. americanus*

Although *N. americanus* fed more actively than *A. ceylanicum* over a period of 24 h (RPMI & HLac medium, Tables 2a, b) a significant inhibition of oral uptake of [³H]inulin by IVM-PO₄ was demonstrated for both species (Tables 2a, b).

There was a highly significant difference between the two species with inulin uptake by *A. ceylanicum* being inhibited by much lower concentrations of IVM-PO₄ than *N. americanus* (approximate EC₅₀s 4 and 0.1 μM, *N. americanus* and *A. ceylanicum*, respectively, Tables 2a, b, Fig. 3).

Pyrantel was found to inhibit the uptake of [³H]inulin by *N. americanus*, *A. ceylanicum* dose-

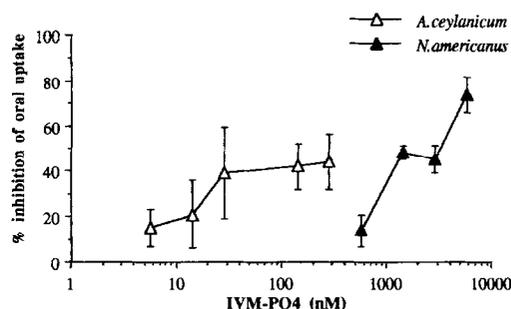
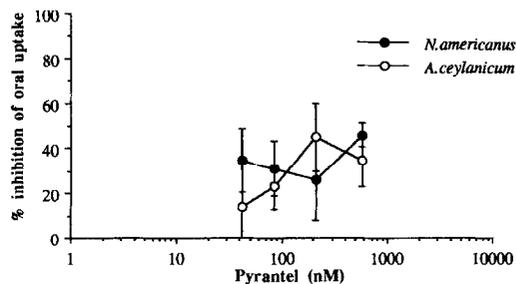


Fig. 3. Comparison of the effects of pyrantel (Fig. 3a) and IVM-PO₄ (Fig. 3b) on % inhibition of oral uptake of [³H]inulin by *A. ceylanicum* and *N. americanus* (*t* = 24 h).

independently (Tables 2c, d, Fig. 3), with both species showing similar sensitivity (EC₅₀s approximately 0.6 and 0.4 μM, *N. americanus* and *A. ceylanicum* respectively).

DISCUSSION

The results reported in this paper provide the first direct *in vitro* comparisons of the sensitivities of two species of hookworm from the important genera *Ancylostoma* and *Necator* to IVM. Previous studies (Behnke *et al.*, 1993) showed that in the hamster host, *A. ceylanicum* was on average 300 times more sensitive to IVM than *N. americanus*. Studies by Rajasekeriah *et al.* (1989) also reported the relative insensitivity of *N. americanus* to IVM in the hamster model. The results of the above *in vitro* experiments in which the effects of IVM and pyrantel on motility were assessed support the results of these previous *in vivo* experiments (Behnke *et al.*, 1993; Rajasekeriah *et al.*, 1989) as *A. ceylanicum* and *N. americanus* were shown to have similar sensitivities to pyrantel, whereas *A. ceylanicum* was found to be approximately 50 times more sensitive to IVM (*t* = 3 h) than *N. americanus*. The difference in responsiveness to IVM does not appear to be related to response time as concentrations of IVM-PO₄ that immobilized *A.*

Table 2a—The effect of IVM-PO₄ on the uptake of [³H]inulin by *N. americanus* (*t* = 24 h)

IVM (μM)	Mean d.p.m. ± S.E.M.	No. worms (<i>n</i>)
0	279 ± 17	48
0.57	242 ± 21	15
1.43	146 ± 19	14
2.86	153 ± 16	16
5.72	73 ± 21	6
Statistical analysis for prediction		
Control > 0.57 μM > 1.43 μM > 2.86 μM > 5.72 μM		
	<i>z</i>	<i>P</i>
	5.72	< 0.0003

For statistical analysis see text. d.p.m. = disintegrations per min.

Table 2b—The effect of IVM-PO₄ on the uptake of [³H]inulin by *A. ceylanicum* (*t* = 24 h)

IVM (nM)	Mean d.p.m. ± S.E.M.	No. worms (<i>n</i>)
0	92 ± 9	26
28.6	60 ± 11	7
143	53 ± 8	14
286	60 ± 7	23
Statistical analysis for prediction		
Control > 28.6 nM > 143 nM > 286 nM		
	<i>z</i>	<i>P</i>
	2.59	< 0.0045

For statistical analysis see text. d.p.m. = disintegrations per min.

ceylanicum in 3 h (1.14 μM, 50% worms immobilized) had no detectable effect on *N. americanus* over 24 h.

The experiments reported here used concentrations ≥ 1 μM, which have been previously criticized as being unrepresentatively high compared to effective *in vivo* concentrations (Geary, Klein, Vanover, Bowman & Thompson, 1992; Geary, Sims, Thomas, Vanover, Davis, Winterrowd, Klein, Ho & Thompson, 1993). The difference in sensitivity between the two species *in vitro* is also relatively lower than the difference in sensitivity shown *in vivo* (*A. ceylanicum* 40–50 times more sensitive than *N. americanus in vitro*, compared to a 200–300-fold difference *in vivo*).

When comparing these *in vitro* experiments with experiments carried out *in vivo*, it is important to take into account a number of factors. For example, the *in vitro* experiments required a dose of IVM that had an acute physiological effect, resulting in a single end-point (e.g. loss of motility) being demonstrated within the limited timescale of a few hours. In comparison, *in vivo* it is likely that a given drug affects worm viability by inhibiting or interfering with a range of processes in the parasite, e.g. inhibition of egg production, feeding, or movement.

Table 2c—the effect of pyrantel on the uptake of [³H]inulin by *N. americanus* (*t* = 24 h)

Pyrantel (nM)	Mean d.p.m. ± S.E.M.	No. worms (<i>n</i>)
0	243 ± 51	9
42	160 ± 36	9
84	169 ± 29	10
210	180 ± 44	7
420	131 ± 14	9
Statistical analysis for prediction		
Control > 42 nM > 84 nM > 210 nM > 420 nM		
	<i>z</i>	<i>P</i>
	1.71	< 0.0401

For statistical analysis see text. d.p.m. = disintegrations per min.

Table 2d—The effect of pyrantel on the uptake of [³H]inulin by *A. ceylanicum* (*t* = 24 h)

Pyrantel (nM)	Mean d.p.m. ± S.E.M.	No. worms (<i>n</i>)
0	140 ± 17	5
42	121 ± 32	5
84	109 ± 14	7
210	78 ± 22	6
420	93 ± 17	8
Statistical analysis for prediction		
Control > 42 nM > 84 nM > 210 nM > 420 nM		
	<i>z</i>	<i>P</i>
	1.99	< 0.0202

For statistical analysis see text. d.p.m. = disintegrations per min.

In vivo experiments with IVM also tend to involve much longer periods of exposure to the drug. For example, Campbell & Benz (1984) found that the anthelmintic efficacy of IVM in cattle persisted for 2 weeks after treatment. Similarly, the microfilaricidal activity of IVM was shown to persist in rodents for at least 30 days after a single dose (Zahner, Sanger, Lammler & Muller, 1987).

Using a micromotility meter, Geary *et al.* (1993) demonstrated that the motility of *Haemonchus contortus* was affected by concentrations of IVM ≥ 10⁻⁸ M, much lower than the concentrations of IVM at which loss of motility could be determined by observation (10⁻⁶ M). It is likely that IVM affects motility in *A. ceylanicum* and possibly *N. americanus* at much lower concentrations than those at which the end-point was determined in these experiments. Nevertheless, in these acute *in vitro* experiments both motility and ingestion showed a significantly greater sensitivity to IVM in *A. ceylanicum* than in *N. americanus*.

The cuticle of nematodes can act as an important permeability barrier and it is possible that the differences in sensitivity to IVM between the two

species may reflect differences in the ability of the drug to cross the cuticle. This possibility was investigated by measuring the uptake of [³H]IVM in worms which were ligatured to prevent the entry of material via anatomical openings (Ho *et al.*, 1992). The results of our experiments suggest two things; first, the rate of IVM uptake across the cuticle is higher for *A. ceylanicum* than *N. americanus*, although the difference in uptake (resulting in an approximate internal IVM concentration 1.6 × greater) is unlikely to be large enough to account for the differences in sensitivity (50-fold) between the two species. Second, as ligatured worms of both species took up considerable amounts of the radiolabelled ivermectin, the results also suggest that the cuticle is an important route of IVM uptake in hookworms. This is supported by studies in which the rate of absorption of drugs across the cuticle was postulated to depend on their lipophilicity (Thompson, Ho, Sims & Geary, 1993; Ho, Geary, Raub, Barshun & Thompson, 1990).

Geary *et al.* (1993) showed that at lower IVM concentrations than those at which motility is affected, IVM caused the paralysis of pharyngeal muscles in *H. contortus*, thus reducing the ingestion of erythrocytes. These authors proposed that this action of IVM may be important in the ability of IVM to control *H. contortus*.

We carried out experiments to establish if IVM caused reduction of ingestion of [³H]inulin in *N. americanus* and *A. ceylanicum* and also to determine if there was any difference in the sensitivity to IVM of the ingestion process between the two species. Our results indicate that IVM-PO₄ causes a dose-dependent inhibition of ingestion in both *N. americanus* and *A. ceylanicum* and that the ingestion process in *A. ceylanicum* is approximately 40 times more sensitive to IVM than in *N. americanus* (EC₅₀ approx. 0.1 μM, 4 μM, respectively, *t* = 24 h). In contrast pyrantel inhibited ingestion in both species at the same concentration. The effect of IVM on ingestion has an EC₅₀ approximately 1 order of magnitude less than for loss of motility. This implies some process involved in ingestion; possibly the action of the pharyngeal muscle is differentially affected by IVM.

It has been determined that the primary physiological effect of ivermectin is an increase in permeability of cell membranes to chloride ions (Turner & Schaeffer, 1989). Specific high affinity avermectin binding sites have been identified and characterized in *Caenorhabditis elegans* (Schaeffer & Haines, 1989; Cully & Paresse, 1991; Cully, Vassilatis, Liu, Paresse, Van Der Ploeg, Schaeffer & Arena, 1994) and a clear correlation has been shown between the binding affinity of a series of avermectin analogues for *C.*

elegans membranes and *in vivo* efficacy, suggesting that the binding site is physiologically important (Rohrer, Meinke, Hayes, Mrozik & Schaeffer, 1992). It is therefore possible that the differential sensitivity to ivermectin observed between *A. ceylanicum* and *N. americanus* may reflect differences in chloride channels or chloride channel binding sites between the two species. This intriguing possibility is currently under investigation in our laboratory using electrophysiological techniques.

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