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INHIBITION OF ACETYLCHOLINESTERASE SECRETION FROM *NIPPOSTRONGYLUS BRASILIENSIS* BY BENZIMIDAZOLE ANTHELMINTICS

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Abstract—Treatment of rats infected with *Nippostrongylus brasiliensis* with a single, oral therapeutic dose of the anthelmintic benzimidazole carbamates oxfendazole or mebendazole resulted, 24 hr later, in a marked reduction (60–90%) in the secretion of a low molecular weight acetylcholinesterase from the parasites when they were incubated *in vitro*. This effect coincided with the expulsion of parasites from the host as a result of the therapy. When parasites were incubated *in vitro* with 0.1 mM oxfendazole, mebendazole, flubendazole, parbendazole, cambendazole or thiabendazole a similar effect was observed; with oxfendazole and mebendazole the effect was apparent within 1 hr and lasted for at least 4 hr after removal to fresh, drug-free medium. Whether treated in the host or *in vitro* the reduction in secretion was balanced by an equivalent rise in acetylcholinesterase activity within the parasites. It is suggested that the inhibition of protein secretion may be a specific manifestation of a general effect of these compounds on microtubule function.

Several species of nematode parasite secrete acetylcholinesterase (AChE) during incubation *in vitro* [1, 2] and compelling immunological evidence indicates that the enzyme is also secreted by the parasites within their hosts [1]. In *Nippostrongylus brasiliensis*, a parasite of rats, the secretion of AChE *in vitro* may account, within an hour, for 4–10% of the total activity normally present in homogenates of worms [1, 3] and it is suspected that this process may constitute a 'biochemical holdfast' within the host gut by the prevention of localised peristalsis [4]. It has been suggested that the benzimidazole anthelmintics oxfendazole and mebendazole, which cause a large, sustained increase in AChE levels in *N. brasiliensis* after oral administration to the host, might facilitate the expulsion of parasites from the rats' intestines by inhibiting the normal secretion of AChE [5].

Considerable detail is now available concerning the different molecular forms of AChE which occur within *N. brasiliensis* but little is known of the secreted enzyme. One form of the enzyme present in homogenates of *N. brasiliensis* has a low molecular weight (61,000), is soluble in aqueous media of low ionic strength and is relatively unaffected by prolonged storage at −20°, by dilution, or by changes in ionic strength [1, 6]. However, the other form is associated with the membrane component of homogenates, has a much higher molecular weight (980,000), is only efficiently extracted by high ionic strength media containing detergent and seems highly labile [6]. The present work sets out to determine which form of the enzyme was affected by drug treatment similar to that previously described by us [5] and whether the increase in activity in worm homogenates could be explained by an inhibition of AChE secretion.

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MATERIALS AND METHODS

Parasites and drugs. Details of the infection of rats with *N. brasiliensis*, the chemotherapy with oxfendazole and mebendazole, the recovery of parasites for experimental purposes and the assay of AChE activity, have been described previously [5, 6]. Oxfendazole and mebendazole were administered orally to infected rats as suspensions in water at doses of 25 mg/kg and 100 mg/kg body wt, respectively. All drugs used *in vitro* were prepared as concentrated solutions (10 mM) in dimethyl sulfoxide (DMSO) which were diluted to final concentrations of 0.1 mM with incubation medium. The final concentration of DMSO was, therefore, 1%. Incubation medium containing 1% DMSO alone was used in control incubations.

Gel filtration of AChE. Gel filtration of AChE in parasite extracts and media was performed on a Sephacryl S-200 Superfine column (Pharmacia K26/70) with a bed height of 67 cm at a flow rate of 21 ml/hr. Fractions of 4.4 ml were collected and 100 μl samples used for the assay of AChE activity with acetylthiocholine iodide as substrate [6]. Parasites were homogenised (20%, w/v) in 10 mM sodium phosphate, pH 7.2, containing 0.5% Triton X-100, 1.0 M NaCl, 0.02% NaN_3 and 1 mM benzethonium chloride to ensure complete extraction of AChE into the soluble phase [6]. Homogenates were centrifuged at 27,000 g_{av} for 30 min at 4° and a sample of the supernatant run on to the column. Incubation medium was applied directly to the column after removal of parasites. Elution of AChE was performed in all cases with 10 mM sodium phosphate,

pH 7.2, containing 0.05% Triton X-100, 100 mM NaCl and 0.02% NaN_3.

Measurement of AChE secretion from whole worms and AChE activity of worm homogenates. Routinely, incubations *in vitro* were performed with nematodes recovered from Sprague–Dawley rats 7 days after infection. They were incubated at 37° in 154 mM NaCl (100 mg wet wt of worms in 5 ml saline) in a vigorously shaking 25 ml Erlenmeyer flask which was loosely stoppered to minimise loss of medium by evaporation. After incubation 10 μl samples of medium were used for AChE assay and the parasites were homogenised at 4° in 10 mM sodium phosphate, pH 7.2, containing 100 mM NaCl, 0.02% NaN_3, 0.5% Triton X-100 and 1 mM benzethonium chloride. The equivalents of 1 μl of homogenate were used for AChE assays [6]. In the series of experiments in which male and female *N. brasiliensis* were exposed separately to oxfendazole and mebendazole for 1 hr the procedure differed slightly from that described above: parasites were recovered from COBS Wistar rats 7 days after infection and incubated in De Jalon's balanced salt solution (154 mM NaCl, 11 mM KCl, 5.55 mM glucose, 12 mM $NaHCO_3$, 0.54 mM $CaCl_2$); homogenates were prepared in 100 mM sodium phosphate, pH 7.2, and were centrifuged at 6000 g_{av} for 15 min prior to AChE assay. The *in vitro* effects of the various benzimidazoles on AChE activity, as opposed to AChE secretion, were assessed by measuring the effect of 0.1 mM drug on the AChE activity in homogenates of the relevant control parasites.

RESULTS

Gel filtration of AChE

A typical profile of AChE elution from the Sephacryl S-200 Superfine column is depicted in Fig. 1 (sample 1) and shows two clear peaks of enzyme activity with markedly different molecular weights. Previous work with identical extracts has shown these apparent molecular weights to be 980,000 and 61,000 [6]. When a similar sample containing considerably less protein (sample 2) was run under identical conditions the high molecular weight (HMW) peak of activity was very much smaller compared to the low molecular weight (LMW) activity. This was in accord with the earlier findings [6] that the HMW AChE activity was labile and sensitive to such factors as dilution, freezing and high ionic strength. Similarly, a sample containing even less protein from a preparation of *N. brasiliensis* recovered from Sprague–Dawley rats treated 16 hr previously with oxfendazole (sample 3) exhibited low HMW AChE but, interestingly, the LMW AChE activity was much higher. The elution of the LMW activity corresponded exactly with the elution profile of the LMW AChE from the other extracts of worm homogenates and of the AChE activity found in the medium in which untreated worms had been incubated *in vitro* (sample 4).

Measurement of AChE secretion and activity in homogenates

Parasites incubated *in vitro* secreted considerable AChE activity into the medium and this secretion

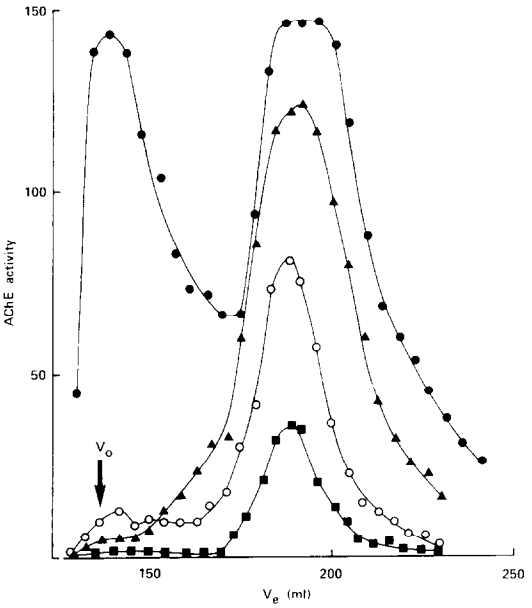


Fig. 1. Gel filtration of AChE from *N. brasiliensis*. The elution profile of AChE activity from a Sephacryl S-200 Superfine column is depicted (nmoles acetylthiocholine hydrolysed/100 μl sample/15 min) for the following samples. (1) ●——●, sample of the 27,000 g_{av} supernatant from a homogenate of control worms (40 mg protein); (2) ○——○, as for (1) above but only 9.7 mg protein; (3) ▲——▲, as for (2) but the worms had been treated 16 hr previously in their host with 25 mg/kg oxfendazole (8.6 mg protein); (4) ■——■, *N. brasiliensis* was incubated in 154 mM NaCl for 5 hr at 37° (100 mg wet wt/ml medium) and after removal of parasites 3.5 ml of incubation medium were run onto the column (the sample had been stored at −20° overnight with no loss of enzyme activity). See Materials and Methods for further details.

was reduced by 62% in worms removed from rats 24 hr after treatment with oxfendazole (Table 1). Experiments with parasites from mebendazole-treated rats revealed a similar inhibition of AChE secretion (Fig. 2). When male and female parasites were incubated separately under comparable conditions there was no difference from the results obtained above with joint incubations; if incubated *in vitro* 48 hr after treatment of the host there was a considerable restoration of the secretory process

Table 1. AChE activity* of *N. brasiliensis* and of incubation media following exposure to oxfendazole in the host

Sample	Control worms	Treated worms
Fresh homogenate	320 ± 67	1150 ± 230
Homogenate after incubation of worms	400 ± 120	1190 ± 130
Incubation medium	170 ± 40	64 ± 35

* μmoles acetylthiocholine hydrolysed/g wet wt or per 50 ml medium/hr. The results are the means ± S.D. of 3 separate experiments. Parasites were homogenised immediately after recovery from the host or after incubation *in vitro* for 5 hr. The treated worms were recovered from rats to which an oral dose (25 mg/kg) of oxfendazole had been administered 24 hr previously.

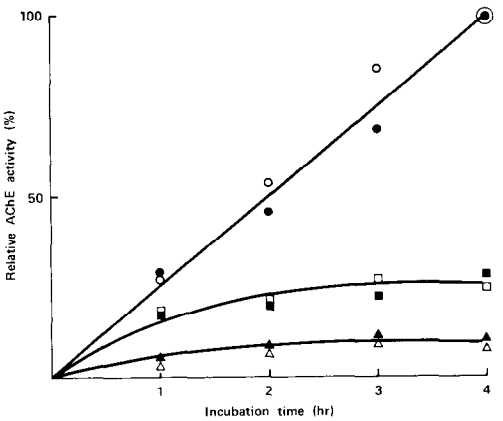


Fig. 2. The secretion of AChE by *N. brasiliensis in vitro*. Parasites were incubated *in vitro* in 154 mM NaCl as described in Materials and Methods. The secreted AChE activity relative to the activity secreted by control worms after 4 hr is plotted against elapsed time of incubation. The control 4 hr values were 915 and 655 μmoles acetylthiocholine hydrolysed/g wet wt/hr: ●, ○ = control worms; ■, □ = control worms exposed during incubation to 0.1 mM mebendazole and oxfendazole, respectively; ▲, △ = worms removed from hosts treated 24 hr previously with 100 mg/kg mebendazole and 25 mg/kg oxfendazole, respectively.

in both sexes although it was still somewhat reduced (−21%) in male parasites exposed to oxfendazole. In support of earlier observations [5] and as reported above (Fig. 1), the AChE activity of parasites from rats treated with oxfendazole was much higher than the activity in worms from untreated rats (Table 1).

The speed of onset and duration of this inhibition of secretion were examined by incubating parasites *in vitro* either after prior exposure to oxfendazole or mebendazole in the host or during exposure to these benzimidazoles *in vitro* (Fig. 2). These experiments were repeated several times with very similar results although the absolute levels of AChE in the incubation media varied somewhat from one experiment to another. In the particular experiments depicted, the AChE activities of media in which untreated parasites had been incubated were 915 and 655 μmoles acetylthiocholine hydrolysed/g wet wt/hr (1 g worm wet wt was equivalent to 50 ml incubation medium when parasites were incubated at 100 mg/5 ml medium). Untreated parasites continued to secrete AChE into the medium at a constant rate for up to 4 hr after the commencement of incubation. Parasites exposed to 0.1 mM oxfendazole or mebendazole for the duration of the incubation showed a sharp decline in secretion within 1 hr of exposure which continued to decrease during subsequent exposure. Parasites removed from hosts treated 24 hr previously with either drug exhibited a slow initial release of AChE which stopped completely after 2 hr. The efficacy of these two compounds in inhibiting AChE secretion and boosting the AChE activity within the parasites was emphasised by a series of experiments in which male and female *N. brasiliensis* were exposed in separate groups to 0.1 mM drug for 1 hr *in vitro* and then transferred to fresh, drug-free medium for a further 4 hr incubation (Table 2). Secretion of AChE was reduced by 40–50% and the AChE activity in worm homogenates was significantly increased. The different strain of host used in this particular series of experiments and the modified incubation conditions did not significantly affect the results: since 1 g wet wt of parasites contained approximately 16,000 males and 16,000 females on days 7–8 after infection of rats (E. B. Rapson, unpublished results) the values for control worms in Table 2 can be converted to 394 and 242 μmoles acetylthiocholine hydrolysed/g wet wt/hr for homogenate and incubation medium AChE activities, respectively, and are comparable with the values reported in Tables 1 and 3.

Treatment of *N. brasiliensis in vitro* with other benzimidazole anthelmintics at a concentration of 0.1 mM resulted in effects closely similar to those produced by oxfendazole and mebendazole (Table 3). Examination of AChE activity in worm homogenates after incubation revealed that whereas there had been a considerable reduction in the value for untreated worms during a 5 hr incubation, the activity in worms exposed to the benzimidazoles was comparable with that of unincubated, fresh, untreated worms. The total AChE activity recovered from homogenates and corresponding media was similar in all cases; only the *distribution* of AChE between parasites and media was different for

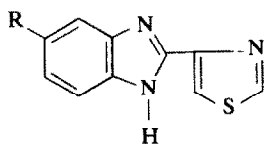
Table 2. AChE activity of male and female *N. brasiliensis* after exposure to oxfendazole and mebendazole *in vitro* (0.1 mM) for 1 hr*

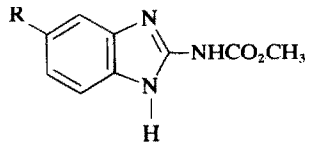
	AChE activity†			
	Male		Female	
Treatment	Homogenate	Incubation medium	Homogenate	Incubation medium
None (DMSO alone)	8.1 ± 0.7	3.54 ± 0.16	16.5 ± 0.4	11.59 ± 0.28
Oxfendazole	13.9 ± 1.3	2.04 ± 0.10	22.0 ± 1.6	6.38 ± 0.15
Mebendazole	12.4 ± 0.9	2.05 ± 0.23	19.9 ±1.2	5.14 ± 0.48

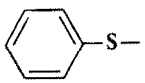
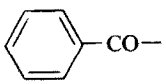
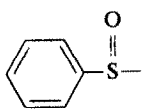
* Parasites were grown in COBS Wistar rats. 200 male or female worms were preincubated for 1 hr in De Jalon's BSS containing 0.1 mM drug before transfer to 0.5 ml fresh drug-free medium and incubation for a further 4 hr.

† nmoles acetylthiocholine hydrolysed/nematode/hr. Values listed are means ± S.E. (n = 10).

Table 3. AChE activity of *N. brasiliensis* and incubation media following exposure to various benzimidazoles *in vitro**

(1) 

(2) 

Treatment	Homogenate activity†	% Change	Incubation medium activity†	% Change	Total activity recovered†
None (DMSO only)	537 ± 78 (7)		497 ± 151 (7)		1034
(1a) Thiabendazole, R = —H	746 ± 94 (3)	+39	124 ± 8 (3)	−75	870
(1b) Cambendazole, R = —$NHCO_2CH(CH_3)_2$	809 ± 136 (3)	+51	148 ± 31 (3)	−70	957
(2a) Fenbendazole, R = 	686 ± 112 (3)	+28	175 ± 19 (3)	−65	861
(2b) Mebendazole, R = 	742 ± 252 (3)	+38	136 ± 63 (3)	−73	878
(2c) Oxfendazole, R = 	773 ± 300 (3)	+44	181 ± 91 (3)	−64	954
(2d) Parbendazole, R = $CH_3(CH_2)_3$—	754 ± 87 (3)	+40	180 ± 35 (3)	−64	934

* Parasites were incubated for 5 hr in 0.1 mM drug as described in the text. The AChE activity of parasites which were homogenised directly after removal from the host was 833 ± 206 (7) μmoles/g wet wt.

† AChE activity is listed as μmoles/g wet wt/hr for homogenates after incubation or as μmoles/50 ml incubation medium/hr which is an equivalent value in terms of the parasites involved. Values listed are the means ± S.D. and the number of experiments is in parentheses.

treated and untreated preparations. The benzimidazoles had no inhibitory effect on the AChE activity of control worm homogenates and, therefore, the observed results could be attributed to effects on protein secretion *per se*.

DISCUSSION

The secretion of AChE by *N. brasiliensis* in the present study occurred at rates comparable to (Table 2) or somewhat higher than (Table 3) the rates reported previously and, in accord with the earlier observations [1], the amount of enzyme secreted also varied in direct proportion with the levels of AChE activity found in the relevant worm homogenates. When expressed as a percentage of the homogenate values, the hourly secretion rates were in good agreement with those of earlier work [1, 3]. Although no complete explanation can be offered for the variation in homogenate AChE activity, it is known that this enzyme is very sensitive to changes in the environment of the worm; including the immunological status of the host [7] and the effects of treatment with certain benzimidazole anthelmintics [5]. It would seem that the HMW enzyme recently described in *N. brasiliensis* and associated with the membrane fraction of worm homogenates [6] does not constitute an important part of the secreted enzyme, *in vitro*. On the other hand, the LMW (61,000) form which was found in the incubation media clearly was affected by treatment with oxfendazole as the levels of activity in homogenates of treated worms were elevated considerably. The molecular weight of the AChE secreted by *Stephanurus dentatus*, a parasite which lives in pigs' kidneys, was recently reported to be around 100,000 [2].

The pronounced inhibition of AChE secretion caused by exposure of *N. brasiliensis* to oxfendazole and mebendazole either in the host or for a brief period *in vitro* supports the suggestion [5] that this phenomenon might explain the elevated levels of AChE activity in treated parasites. It is possible to demonstrate, assuming a constant hourly rate of secretion within the rat, that the difference between the hourly secretion rate *in vitro* for treated and untreated parasites (which for the latter is constant, at least, for 4 hr *in vitro*—see Fig. 2) when multiplied by the number of hours after treatment and added to the AChE activity of a control worm homogenate approximates to the AChE activity of treated worm homogenates (Table 1). Indeed, even after only relatively brief exposure the same effect was apparent *in vitro* (Tables 2 and 3).

Although this is the first time that an effect of benzimidazoles on secretion in nematodes has actually been measured, in ultrastructural studies mebendazole has been reported to lead to an accumulation of secretory vesicles within the intestinal cells of nematodes such as *Ascaridia galli* [8], *Ascaris suum* and *Syngamus trachea* [9]. These effects were thought to be due to the disruption of microtubule function by the drug, and indeed more recent work [10, 11] has shown that mebendazole and fenbendazole do bind to nematode tubulin. Such an explanation for the ultrastructural effects and for the present observations is highly plausible since the benzimidazoles as a group of chemicals have been shown to affect microtubules in fungi [12–15], plants [15] and mammalian cells [16–19]. Biochemical studies have shown that these compounds actually bind to tubulin in the relevant species [20, 21] and that several inhibit the polymerisation of mammalian brain tubulin into microtubules [22–24]. The observation in the present study that thiabendazole was as effective as the benzimidazole carbamates in inhibiting AChE secretion is of particular interest since although it is known to bind to fungal tubulin [13] no experimental evidence has ever been presented to suggest that it might also interfere with tubulin function in helminths. Indeed, there is no unifying hypothesis which adequately explains the mode of action of the whole range of benzimidazole anthelmintics. The possibility that they might all owe their therapeutic effect to antagonism of tubulin function is attractive. Specific manifestations of an effect on a basic element of cell physiology such as microtubule function might well differ between susceptible species in addition to being dependent upon the pharmacokinetic characteristics of a given drug in a particular host.

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