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SPECIFIC INTERACTION OF BENZIMIDAZOLE ANTHELMINTICS WITH TUBULIN FROM DEVELOPING STAGES OF THIABENDAZOLE-SUSCEPTIBLE AND -RESISTANT *HAEMONCHUS CONTORTUS*

GEORGE W. LUBEGA* and ROGER K. PRICHARD
Institute of Parasitology of McGill University, Macdonald College, Ste-Anne de Bellevue, QC, Canada H9X 1C0

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Abstract—The low- and high-affinity binding of tritiated benzimidazole anthelmintics (mebendazole and oxibendazole) to tubulin-containing supernatants derived from unembryonated eggs, third stage larvae or adult worms of thiabendazole-susceptible and -resistant strains of *Haemonchus contortus* were examined and compared. The displacement of these radioligands by unlabelled benzimidazoles (mebendazole, fenbendazole, thiabendazole and oxibendazole) also was examined. The binding affinity, K_a, and maximum binding, B_{max}, for the high-affinity binding were calculated by non-linear least-square iterative curve fitting using a computer programme (LIGAND) based on the exact mathematical model of ligand–receptor interactions. The K_a was of the same order of magnitude ($\times 10^7\ M^{-1}$) for the susceptible and resistant eggs, larvae and worms. Resistance was associated with a loss of high-affinity binding. There was a 2- to 5-fold loss of B_{max} by the resistant strain. The eggs showed greater high-affinity binding per milligram of protein than the larvae which, in turn, showed greater high-affinity binding than the adult worms. It was shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis that the tubulin content per milligram of protein decreased from egg, through larva to adult worm. Cross-displacement studies indicated that different benzimidazole drugs interacted with the same receptor (tubulin) and that a rank order of affinity of the benzimidazole drugs could be inferred.

Chemotherapy with anthelmintics such as benzimidazoles is the most important means of controlling infections caused by parasitic helminths in humans and animals [1, 2]. Anthelmintics have been used with great success but their frequent use has resulted in resistance to benzimidazoles and other anthelmintic drugs [3]. Benzimidazole resistance is a major problem in the control of *Haemonchus contortus* and other nematodes in sheep and small strongyles in horses [4]. Benzimidazoles exert their effects by binding tubulin [5]. Tubulin polymerizes reversibly to form microtubules which are involved in cell shape, motility, mitosis and transport [6]. Benzimidazoles are effective against adult nematodes and inhibit egg development [7] including that *in utero* [8]. However, they fail to inhibit development of embryonated eggs [9] and are often less effective against immature worms *in vivo* [10]. It has also been reported that development of gastrointestinal parasite eggs *in vitro* is inhibited at doses which are subefficacious against adults *in vivo* [8]. It is, therefore, possible that the interaction of benzimidazoles with tubulin may change with each developmental stage of nematodes.

Diminished total binding by benzimidazole-resistant *H. contortus* compared with the benzimidazole-susceptible strain has been reported for the adult stage [11] and larval stage [12], but no study of benzimidazole resistance has been reported using egg material despite the fact that the egg hatch assay is the most widely used technique for diagnosing benzimidazole resistance [13]. The binding of benzimidazoles to tubulin has been suggested as an alternative technique for diagnosing benzimidazole resistance [12]. *H. contortus* has a high reproductive rate passing 5000 eggs/day/female worm [14]. The eggs are, therefore, easy to obtain making it cheaper and faster to use egg material in the binding assays than adult worms whose recovery involves destruction of sheep. Tritiated benzimidazole binding to supernatants prepared from adult worms of *H. contortus* has been shown to consist of high- and low-affinity binding [15]. Resistance was associated with a loss of the high-affinity component. Tubulin was responsible for the high-affinity binding.

In this study, we compared the tubulin content and high-affinity benzimidazole binding to unembryonated eggs with that to larvae and adult worms for thiabendazole-susceptible (S†) and -resistant (R) strains of *H. contortus*. We have shown that high-affinity binding was lost from the resistant strain at all three stages of development, and that the egg supernatants contained a greater concentration of tubulin per milligram of protein and bound more

* To whom correspondence should be addressed.

† Abbreviations: S, thiabendazole-susceptible; R, thiabendazole-resistant; MBZ, mebendazole; FBZ, fenbendazole; OBZ, oxibendazole; TBZ, thiabendazole; DMSO, dimethyl sulfoxide; MES = 2[*N*-morpholino]ethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; GTP, guanosine-5′-triphosphate; BSA, bovine serum albumin; and SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

drug per milligram of protein than the larvae or adult worms.

MATERIALS AND METHODS

Chemicals and solutions

Pure samples of non-labelled benzimidazoles [oxibendazole (OBZ), fenbendazole (FBZ), thiabendazole (TBZ) and mebendazole (MBZ)] were gifts from Smith Kline Animal Health (Philadelphia, PA, U.S.A.), Hoechst AG (Frankfurt am Main, F.R.G.), Merck Frosst (Kirkland, QC, Canada) and Janssen Pharmaceutica (Mississauga, ON, Canada) respectively. Tritiated mebendazole ([^{3}H]MBZ) and oxibendazole ([^{3}H]OBZ) were donated by Dr. E. Lacey (CSIRO, Sydney, Australia). The other chemicals were reagent grade. MES buffer (pH 6.5) consisting of 0.025 mM MES, 1 mM EGTA, 0.5 mM $MgSO_4$ and 2 mM GTP was routinely used in all binding studies. Stock concentrations of [^{3}H]benzimidazoles were prepared in 20% (v/v) dimethyl sulfoxide (DMSO) in MES buffer, and their specific activities (dpm/pmol) were determined on a beta counter (Rack beta, LKB). Mean values for the specific activities of [^{3}H]MBZ and [^{3}H]OBZ were 3125 and 1193 dpm/pmol respectively. Stock concentrations of unlabelled benzimidazoles were prepared in 100% or 50% (v/v) DMSO in MES buffer.

Parasites

S and R strains of *H. contortus* were obtained from Smith Kline Animal Health Research Farm (Applebrooke, PA, U.S.A.). Cross-bred sheep were orally infected with 10,000–15,000 third stage larvae of either strain. Benzimidazole resistance was verified previously [15] using the egg hatch assay.

Unembryonated eggs were recovered 28 days post-infection from faeces by a modification of the flotation, sedimentation and centrifugation procedure of Weston *et al.* [16]. Canvas bags were tied over the anus of infected sheep and faeces obtained from these bags within 6 hr and transferred to a refrigerator. About 10 kg of faeces was blended with 10 vol. of water, at 4°, and the resultant fecal slurry sieved sequentially through metal meshes of apertures 2 mm, 1 mm, 0.4 mm, 0.15 mm and 0.09 mm, discarding the residue at each step. The 0.09 mm filtrate was left to stand for 2–3 hr to sediment the eggs. The supernatant was then decanted off and the sediment concentrated by pouring it onto a 38 μm metal sieve. The 38 μm metal sieve residue was dispensed in cold sucrose solution (sp. g. 1.2) and the flotation, sedimentation and centrifugation procedure [16] followed to obtain unembryonated eggs free of fecal debris. All operations were conducted at 4°. The eggs were concentrated by centrifugation and stored in liquid N_2 or briefly on ice before use.

Infective third stage larvae (L_3) were recovered by modification of the ordinary cultural procedure [17]. Fecal pellets obtained as described above were placed, together with several petri dishes half-filled with water, into closed plastic containers (24 × 12 × 12 cm) and kept at 27° for 1 week. The larvae migrated into and onto the petri dishes from where they were recovered free of fecal matter using a fine spray of water. To further clean and free the L_3 of free living nematodes, the recovered larval suspension was poured onto a large filter paper (Whatman No. 1) and blotted completely dry [17]. Free-living nematodes died of dehydration within 12 hr, and the L_3 were recovered after placing (for 1 hr) the dry filter paper face down onto a Baermann funnel [17]. Dead larvae and fecal debris remained adherent onto the filter paper while the clean and motile *H. contortus* L_3 migrated to the base of the funnel from where they were recovered 1 hr later. The mobile and infective L_3 could be stored in tap water at 10° for up to 6 months.

The recovery of adult worms was described previously [15].

Preparation of crude tubulin extracts

The eggs or L_3 were washed several times with cold distilled water and then cold MES buffer, and homogenised on ice with a Polytron cell disrupter (PT 10, OD Kinematica GMBH Luzern-Schweiz) in MES buffer (1 mL/200 mg of parasites) until seen by microscopic examination to be disrupted completely. Eggs and larvae required 2 × 5 min and 2 × 10 min homogenisation time respectively. The homogenate was centrifuged at 100,000 *g* at 4° for 1 hr to produce a clear supernatant that was used in binding and electrophoretic studies. The preparation of adult worm-derived supernatants was described elsewhere [15]. The supernatants were stored in liquid N_2 or briefly on ice before use. Protein concentrations of the supernatants were measured by the method of Bradford [18]. The egg-derived supernatants and larva-derived supernatants were used for drug binding or electrophoretic analysis before or after enrichment for tubulin using poly-L-lysine affinity chromatography as described previously for the adult worm-derived supernatants [15].

Binding studies

Binding conditions. The optimal protein and drug concentrations used in the binding assays were determined as described previously [15].

Saturation studies. These studies were designed to resolve the total binding (TB) by egg and larval supernatants into low-affinity binding (LAB) and high-affinity binding and to determine the K_a and B_{max} values of the high-affinity binding. High-affinity binding is the difference between total and low-affinity binding. The assay was carried out as described previously [15]. Five microliters of 50% DMSO (TB) or 5 μL of 200 μM unlabelled MBZ or OBZ in 50% DMSO (LAB) was preincubated in triplicate with 90 μL of either buffer (blank) or S or R supernatants for 30 min at 37° as described previously [15]. Then 10 μL of [^{3}H]MBZ or [^{3}H]OBZ (0.156 to 20 μM) was added to each tube and incubated for a further 30 min. The assay was terminated by addition of a charcoal suspension and the bound drug (dpm) quantified as described previously [15]. Counts (dpm) were calculated for TB and LAB before computer analysis as follows:

$$TB = (dpm_{1s} - dpm_{1b}) \times 1.3$$

and

$$LAB = (dpm_{us} - dpm_{ub}) \times 1.3$$

where dpm_{ls} and dpm_{lb} = dpm of sample and blank, respectively, incubated with the labelled ligand only; dpm_{us} and dpm_{ub} = dpm of sample and blank, respectively, incubated with the unlabelled and labelled ligand; and 1.3 is the correction factor for the fraction of the assay counted (0.45 mL out of 0.6 mL final assay volume was counted).

Mathematical analysis of data from saturation studies. The data were analysed using the recent versions [19] of the computer programs EBDA and LIGAND as described before [15]. Using EBDA [20], Scatchard and Hill analyses were performed to obtain initial estimates of K_a and B_{max} and predict the model (one site vs two sites) required by LIGAND [21]. The Scatchard and Hill transformed data were presented graphically for visual inspection and selection of the site model. The initial estimates of K_a and B_{max} were supplied to LIGAND to calculate final estimates by non-linear least-square iterative fitting of the curve of total ligand concentration versus bound, based on the exact mathematical model of ligand–receptor interactions. After fitting, the data were presented in a Scatchard plot for visualisation.

The binding data were also manually transformed into picomoles bound per milligram of protein and graphically analysed for total, low- and high-affinity binding.

Displacement studies. These studies were designed to determine whether a series of unlabelled benzimidazoles bound to the same classes of receptor sites in S and R supernatants and to compare the affinities of these drugs for tubulin derived from S and R strains. The procedure was a modification of that described previously [15]. Two microliters of unlabelled benzimidazoles (0–1 mM for MBZ, FBZ and OBZ and 0–10 mM for TBZ) in 100% DMSO was added in triplicate to 90 μL of either buffer (blank) or S or R samples. Then 10 μL of 1 μM [^{3}H]benzimidazole was added to each tube and incubated for 30 min at 37° [11]. The assay was continued as described previously [15] to determine the bound dpm in each tube. Counts (dpm) were calculated for B_m and B_i as follows:

$$B_m = (dpm_{ls} - dpm_{lb}) \times 1.3$$

$$B_i = (dpm_{us} - dpm_{ub}) \times 1.3$$

where B_m = maximum dpm bound in presence of 0.1 μM labelled benzimidazole only and B_i = dpm bound in presence of a given concentration, i, of the unlabelled benzimidazole and 0.1 μM labelled benzimidazole. The values of B_m and B_i were fed into EBDA/LIGAND to determine the low-affinity binding, the specific displacement, the K_a of the unlabelled ligand and the high-affinity receptor concentration (B_{max}).

Mathematical analysis of displacement data. The displacement data were analysed as described elsewhere [15] to obtain the K_a, B_{max} and low-affinity binding. However, the K_a of the labelled ligands was fixed at 10^7 M^{-1} in order to directly compare the affinities of the unlabelled ligands for the S and R receptors.

Statistical analysis of binding data. The binding data were analysed statistically utilising Student's *t*-test for unpaired observations, ANOVA and Duncan's multiple range test.

SDS–PAGE and immunoblotting

SDS–PAGE and immunoblotting were performed in order to obtain an indication of the relative concentrations of tubulin in the supernatants derived from unembryonated eggs, larvae and adult worms of S and R strains. The supernatants were analysed by SDS–PAGE utilising the discontinuous system in slabs (Mini-protean II, Biorad) as described previously [22, 23]. Four percent and 10% polyacrylamide gels were used as stacking and separating gels respectively.

Electroblot transfer from SDS-gels to nitrocellulose paper (Biorad) was performed utilising the Mini-transblot apparatus (Biorad) following the Biorad protocol.

Monoclonal anti-chicken α- or β-tubulin mouse IgG (Amersham) were used in Western blot assays as described previously [22] for the visualisation of tubulin bands on SDS-transblots. The nitrocellulose blots were washed with phosphate-buffered saline (PBS) (pH 7.4) containing 0.4% (v/v) Tween-20 for 3 × 15 min following 1 hr of incubation with the primary or the peroxidase-conjugated anti-mouse IgG secondary antibody (BioCan). The diluted primary (1 : 10,000) and secondary (1 : 1,000) antibody contained 1% (w/v) BSA and 0.1% (v/v) Tween-20 in PBS (pH 7.4). The transblots were incubated in 10% (w/v) BSA in PBS for 2 hr at room temperature to block nonspecific sites. The substrate was diaminobenzidine (Sigma). In other experiments the secondary antibody was biotinylated anti-mouse IgG (Amersham) [see Ref. 15]. Mammalian (sheep brain) and parasite (*H. contortus* eggs) tubulin purified by polymerisation/depolymerisation [24] and Taxol [23], respectively, were used as standards to identify and compare tubulin bands in parasite supernatants. The purity of the tubulin was estimated by densitometry [22, 23] to be 85 and 70% for the mammalian and parasite tubulin respectively. Parasite tubulin (0.07 or 0.14 μg), mammalian tubulin (0.17 μg) and protein (5 μg) of S and R egg-, larval- and adult worm-derived supernatants in 10 μL of reducing buffer were loaded onto a 1.5 mm thick 12% SDS–polyacrylamide separating gel.

SDS–PAGE and immunoblotting were also performed on fractions derived from poly-L-lysine chromatography to identify those fractions which contained tubulin.

RESULTS

Standardisation of the binding assays

The results for adult worms are described elsewhere [15]. However, the K_a and B_{max} estimates for adult worms are shown in Table 1, and some other features are mentioned for comparison with eggs and larvae. The normalised binding (pmol/mg protein) was uniform for protein concentrations above 10, 30 and 100 μg protein/assay for eggs,

Table 1. LIGAND-generated parameters for [^{3}H]MBZ and [^{3}H]OBZ high-affinity (specific) binding to preparations of eggs (E), larvae (L) and adult worms (A) of TBZ-susceptible (S) or resistant (R) *Haemonchus contortus*

Drug	Stage	Strain	K_a^* ($\times 10^6 M^{-1}$)	B_{max}^* (pmol/mg protein)	SF†
MBZ	E	S	4.2 ± 3.0[a]	108 ± 3[c,d]	1.6[e]
		R	3.2 ± 4.0[a]	67 ± 5[a]	
	L	S	3.8 ± 9.0[a]	53 ± 5[c]	2.5[e]
		R	3.4 ± 4.0[a]	21 ± 4[b]	
	A‡	S	12.0 ± 1.0[b]	30 ± 1[c]	1.8[e]
		R	10.0 ± 2.0[b]	17 ± 2[b]	
OBZ	E	S	6.9 ± 2.0	147 ± 8[c,d]	3.5[f]
		R	6.6 ± 0.6	42 ± 3[a]	
	L	S	5.0 ± 1.2	59 ± 4[c]	7.4[f]
		R	4.8 ± 1.3	8 ± 2[b]	
	A‡	S	7.0 ± 1.0	44 ± 4[c]	4.9[f]
		R	5.3 ± 1.0	9 ± 2[b]	

* Mean ± SE of 4–8 experiments.
† The ratio of B_{max} of S to that of R at each stage of development.
‡ Data from Ref. 15.
[a,b] Within the column and drug, values sharing the same letter were not significantly different (K_a, $P > 0.05$; B_{max}, $P > 0.01$).
[c] B_{max} of S significantly higher than R ($P < 0.01$).
[d] B_{max} of S significantly different among developmental stages ($P < 0.01$).
[e,f] Within the column, values sharing the same letter were not significantly different ($P > 0.05$): values with different letters were significantly different ($P < 0.01$).

larvae and adult worms respectively. Below these limits, binding was irregular. Subsequent experiments were done using protein concentrations above these limits. The ranges of drug concentrations used were governed by the low aqueous solubility of benzimidazoles and the effect of the co-solvent, DMSO, on binding [see Ref. 15].

Saturation studies

The K_a and B_{max} estimates of high-affinity binding to egg-, larva- and adult worm-derived supernatants are shown in Table 1. The relationships between total, low- and high-affinity binding are presented in Fig. 1 and those of the larvae (Fig. 1, A and B) were similar to those of adult worms [15] but different from those of eggs (Fig. 1, C and D) for both MBZ and OBZ. Like that for adult worms, total binding for larvae was not saturable, within the range of drug concentrations used, due to the presence of a considerable amount of low-affinity binding. However, total binding for the eggs was saturable because they contained relatively little low-affinity binding. The low-affinity binding of adults and larvae was nearly linear for both drugs but that of the eggs increased curvilinearly with the drug concentration. The low-affinity binding of the S and R strains was not significantly different for both drugs and all developmental stages. The high-affinity binding of the R strain was significantly lower ($P < 0.01$) than that of the S strain for both drugs and all stages (Fig. 1 and Table 1). Like those of adults [15], the Scatchard plots of total binding (not shown) were curvilinear, indicating that total binding of adult worm and larval supernatants cannot be described by a single site model. However, the Scatchard plots of total binding of the eggs were linear and similar in form and slope (but a little different in x-intercept) to those of high-affinity binding, indicating that low-affinity binding in the eggs was little and that the total binding can be described by a single site model. The Scatchard plots of high-affinity binding of the S and R strains were linear and had similar slopes but differed in their x-intercepts, indicating that their K_a values were similar but their B_{max} values were different (Fig. 1E) for all stages and both drugs.

Susceptibility factor (SF)

The susceptibility factor (SF) [11, 12] is the ratio of B_{max} of the S strain to that of the R strain. The SF was calculated and chosen in preference [11] to its reciprocal (the resistance factor) as a measure of the degree of resistance. The SF values (Table 1) collaborated the egg hatch assay data [15] using TBZ which showed that the ratio of EC_{50} (effective concentration of TBZ causing 50% egg hatch inhibition) of the R strain compared to that of the S strain was 4.4.

Statistical analysis

B_{max} *values.* The B_{max} values of the S strain were significantly higher ($P < 0.01$) than those of the R strain for all developmental stages and both drugs (Table 1). For the S strain and both drugs, B_{max} values were significantly different ($P < 0.01$) among developmental stages (Table 1). For the R strain and both drugs, B_{max} values of eggs were significantly higher ($P < 0.01$) than those of larvae and adults but

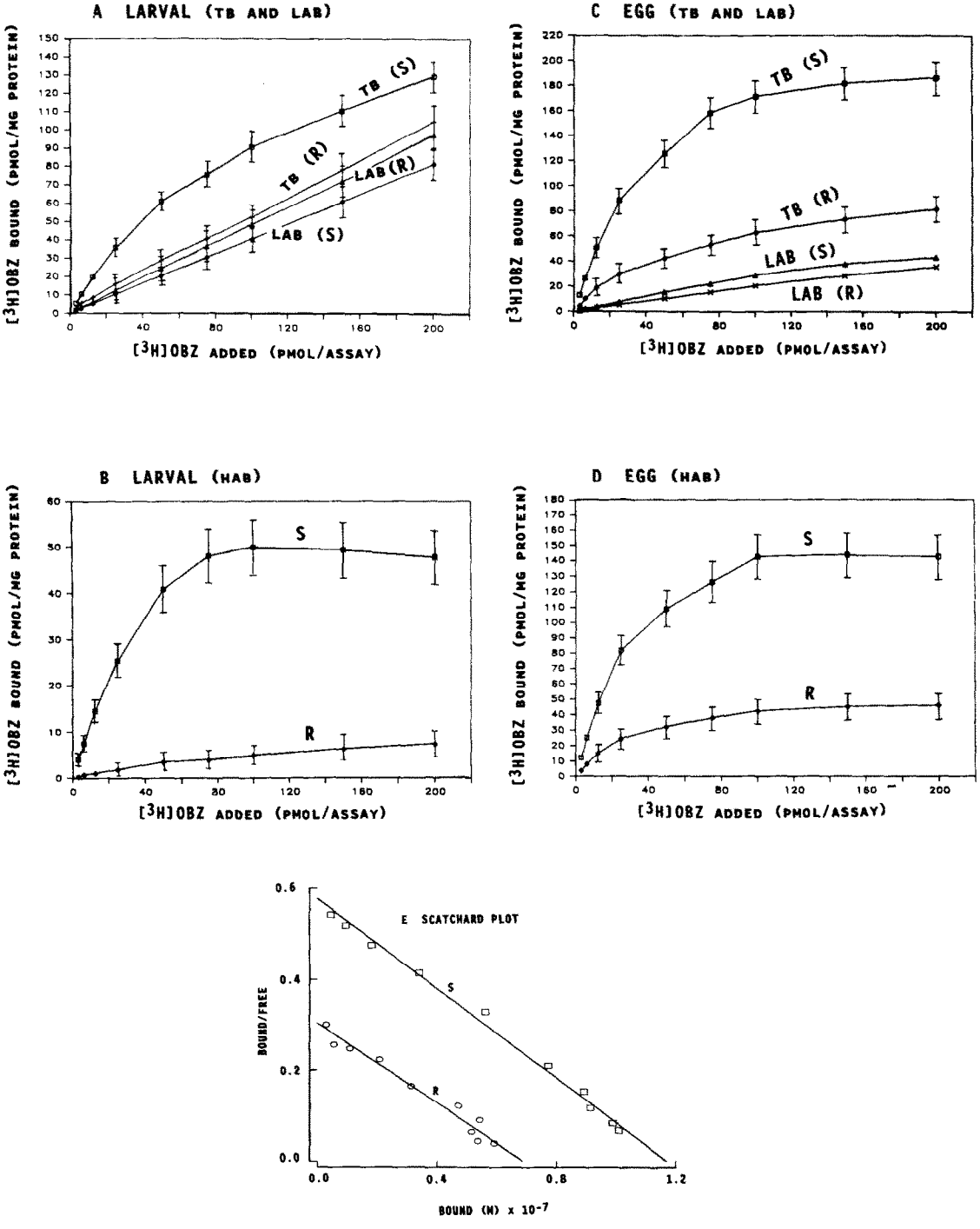

Fig. 1. Total binding (TB), low-affinity binding (LAB) and high-affinity binding (HAB) by [^{3}H]OBZ to S- or R-derived supernatants of *H. contortus* third stage larvae (A and B) and eggs (C and D). Five microliters of 50% (v/v) DMSO in MES buffer (TB) or 5 μL of 200 μM unlabelled OBZ in 50% (v/v) DMSO in MES buffer (LAB) was preincubated in duplicate with 90 μL of either MES buffer (blank) or S or R supernatants for 30 min as described in Materials and Methods. Then 10 μL of 0.156 to 20 μM [^{3}H]OBZ was added to each tube and incubated for a further 30 min. Disintegrations per minute for TB and LAB were determined by scintillation counting. The data were transformed into picomoles per milligram of protein. HAB was determined by subtracting LAB from TB. Each point is the mean ± SE of 4–5 separate experiments. Similar graphs were obtained using [^{3}H]MBZ. The results of [^{3}H]MBZ high-affinity binding to S or R egg supernatants are presented in Scatchard plots (E) generated by LIGAND after fitting the respective curves of total ligand concentration versus ligand bound. The Scatchard plots for [^{3}H]OBZ or other developing stages and either drug were similar to the ones shown here.

Table 2. Displacement of tritiated benzimidazoles by unlabelled benzimidazoles from S or R supernatants prepared from unembryonated eggs

Radioligand	Unlabelled ligand	IC_{50}^{*} (nM)	K_a^{*} ($\times 10^6 M^{-1}$)	B_{max}^{*} (pmol/mg protein)
Susceptible				
[^{3}H]MBZ	FBZ	125 ± 5	34 ± 7	107 ± 7
	MBZ	161 ± 8	24 ± 4	105 ± 6
	OBZ	229 ± 10	8 ± 2	104 ± 9
	TBZ	3344 ± 451	1 ± 0.4	97 ± 5
[^{3}H]OBZ	FBZ	98 ± 7	38 ± 7	102 ± 12
	MBZ	127 ± 11	32 ± 7	109 ± 14
	OBZ	165 ± 13	19 ± 4	95 ± 11
	TBZ	3080 ± 560	0.9 ± 0.5	89 ± 17
Resistant				
[^{3}H]MBZ	FBZ	129 ± 7	27 ± 8	41 ± 4
	MBZ	105 ± 10	29 ± 5	39 ± 3
	OBZ	325 ± 14	7 ± 3	42 ± 7
	TBZ	2617 ± 198	1 ± 0.3	35 ± 6
[^{3}H]OBZ	FBZ	42 ± 3	50 ± 11	22 ± 2
	MBZ	87 ± 8	32 ± 9	22 ± 1
	OBZ	106 ± 11	28 ± 7	22 ± 2
	TBZ	2600 ± 576	1.2 ± 0.2	21 ± 3

* Values are means ± SE of 3–4 experiments.

there was no significant difference between larvae and adults.

K_a *values.* There were no significant differences in K_a between the S and R strains for any developmental stage and between developmental stages except for the adult worms which showed a significantly higher ($P < 0.05$) K_a for MBZ (Table 1) than for the other stages.

SF values. The SF values were significantly higher ($P < 0.01$) for OBZ than for MBZ but were not significantly different between stages (Table 1).

Displacement studies

High-affinity binding by the adult worm and larval preparations of the R strain was so low that mathematical computer analysis of the displacement data for this strain was not possible in most cases [see Ref. 15]. Displacement studies, therefore, were completed using egg-derived supernatants which showed a substantial amount of high-affinity binding for the R strain. Unlike the previous studies [5, 15], in which the unlabelled ligands were preincubated with the samples before adding the labelled ligands, in this study the unlabelled and labelled ligands were added to the samples at the same time to allow direct competition between ligands. The K_a values of the radioligands ([^{3}H]MBZ and [^{3}H]OBZ) were fixed at $10^7 M^{-1}$ and the displacement data iteratively fitted using EBDA/LIGAND to compare the K_a values of the unlabelled benzimidazoles. (From the saturation studies, the K_a values of [^{3}H]MBZ and [^{3}H]OBZ were of the order of $10^7 M^{-1}$.) The results are therefore relative values and refer to high-affinity binding (see Table 2). The B_{max} values calculated at the same time were lower for the R strain than for the S strain. The B_{max} values of the R strain depended on the radioligand displaced, with [^{3}H]OBZ showing lower values than [^{3}H]MBZ (Table 2). The B_{max} values were independent of the unlabelled ligand used, indicating that the latter displaced the labelled ligands to a similar extent. (In the displacement study, B_{max} values represent the concentration of the high-affinity receptors recognised by both the radioligand and the unlabelled ligand.)

Based on the IC_{50} and K_a values, the displacement of [^{3}H]MBZ or [^{3}H]OBZ from S or R eggs gave an approximately similar rank order of affinity (see Fig. 2):

$$FBZ \geq MBZ > OBZ \gg TBZ.$$

The K_a values derived from the displacement of [^{3}H]OBZ were generally higher (i.e. the IC_{50} values were lower) than those derived from [^{3}H]MBZ displacement. Using LIGAND, it was estimated that the amount of low-affinity binding was insignificant at 0.1 μM final ligand concentration and was, therefore, ignored (fixed to 0).

SDS–PAGE and immunoblotting

The Western blots of egg-, larva- and adult worm-derived supernatants of S and R strains using anti-α- or anti-β-tubulin monoclonal antibodies (Amersham) and peroxidase conjugated IgG (BioCan) are shown in Fig. 3. The width and intensity of the S and R strains were visually similar for α- or β-tubulin at each stage of development. However, the tubulin bands in the egg supernatants were thicker and more intense than those in larval supernatants which were, in turn, thicker and more intense than those in adult worm supernatants. When biotinylated IgG (Amersham) followed by streptividin-peroxidase (Amersham) was used as second antibody, two other bands plus tubulin were seen in the adult worm [15] and larval supernatants (not shown). It was concluded that the other bands were due to this particular second antibody

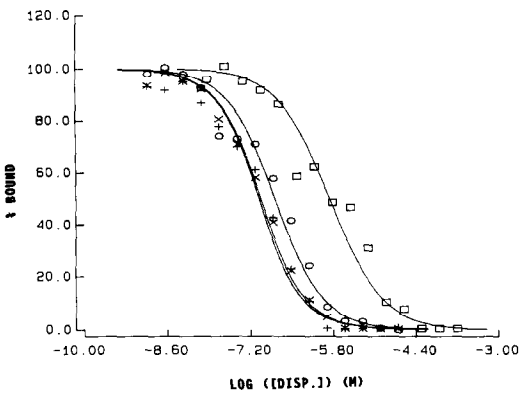

Fig. 2. LIGAND-generated displacement curves for the specific displacement (inhibition) of [^{3}H]MBZ binding by unlabelled benzimidazole drugs [TBZ (□), OBZ (○), MBZ (+) and FBZ (×)] from S unembryonated egg-derived supernatants. Two microliters of unlabelled benzimidazole and 10 μL of 1 μM [^{3}H]MBZ were added to 90 μL supernatants and incubated at 37° for 30 min. See Materials and Methods and Table 2 for details. Curves of total ligand concentration versus bound were fitted using LIGAND before transforming the data into the coordinate system shown here. The data refer to high-affinity binding. Similar data were derived for the displacement of [^{3}H]MBZ from R- or [^{3}H]OBZ from S- or R-derived egg supernatants.

(Amersham) which was not used any further. As reported previously [15, 22], poly-L-lysine chromatography fractions devoid of specific (high affinity) binding contained no tubulin.

DISCUSSION

Our results show that differences in high-affinity binding between S and R strains of *H. contortus* can be demonstrated using eggs and infective larvae (L_3), as well as adult worms [15]. This suggests that the receptors for benzimidazole drugs may be similar at each stage of development. The concentration of the receptor(s) per milligram of protein (B_{max}) was greater in the eggs than in the larvae which, in turn, was greater than that in the adults for the S and R strains respectively (Table 1). Compared with the eggs (Fig. 1), more of the binding in the larvae (Fig. 1) and adult worms [15] was low-affinity binding. On the other hand, compared with the larvae and adult worms, more of the binding in the eggs was high-affinity binding. High-affinity binding occurred in poly-L-lysine chromatography fractions containing tubulin. There was no high-affinity binding in fractions devoid of tubulin. Resistance was associated with a reduction in amount (pmol/mg protein) of high-affinity binding (Table 1). Benzimidazole resistance was not associated with low-affinity binding. Most of the low-affinity binding of the larvae and adult worms was probably non-specific binding since it was nearly linear when plotted versus ligand concentration and most of it occurred in fractions devoid of tubulin. However, low-affinity binding was detectable in both tubulin-enriched and tubulin-free supernatants. There was little low-affinity binding of the egg supernatants, and the plot of this low-affinity binding versus ligand concentration was curvilinear (Fig. 1C) and could be found, together with high-affinity binding, in poly-L-lysine chromatography fractions containing tubulin. Some of the low-affinity binding may represent specific low-affinity binding to tubulin. The source of the greater amount of low-affinity binding in the larval and adult worm supernatants than in those of eggs is obscure. It may involve tubulin or other proteins or lipids which are not present in the eggs.

OBZ was more affected by the reduction in high-affinity binding than MBZ. The susceptibility factor (SF) values (Table 1) of eggs, larvae and adult worms were not significantly different, although the SF values of OBZ were significantly higher ($P < 0.01$) than those of MBZ. The results suggest that benzimidazole resistance can be diagnosed accurately using eggs, larvae or worms. They also suggest that benzimidazole resistance is drug dependent, with OBZ being more affected than MBZ. The SF values reported here (Table 1) are in good agreement with the egg hatch assay data [15], indicating the validity of both methods for diagnosing benzimidazole resistance.

The B_{max} values derived from the saturation study were similar to those derived from the displacement study and were broadly independent of the drug used (Tables 1 and 2). This indicates that all the benzimidazole drugs studied bind to the same class of sites, albeit with different affinity. It also indicates that the K_a of 10^7 M^{-1} chosen to fit the displacement data is a good estimate of the actual K_a of these benzimidazole drugs for *H. contortus* tubulin.

Benzimidazole resistance did not affect the K_a values. Also the K_a values for embryonic, larval and adult worm supernatants were of the same order of magnitude, although the K_a values for [^{3}H]MBZ were apparently higher for adult worm supernatants than for the other supernatants (Table 1). This unique behaviour in affinity of [^{3}H]MBZ for adult worm supernatants cannot be explained easily. The main differences between stages and strains were associated with the amount of drug bound (B_{max}). Egg supernatants bound more drug, perhaps because egg cells contain more tubulin than the cells of larvae and adult worms. Tubulin is the principal protein of the mitotic spindle [6] and may be abundant in rapidly dividing cells, such as eggs. Western blot analysis indicated that supernatants prepared from unembryonated eggs contained more tubulin per unit weight of protein than the supernatants derived from larvae or adult worms (Fig. 3). The larvae were intermediate between eggs and adult worms in tubulin content and high-affinity binding. We have observed a similar trend by the Enzyme-Linked-Immunosorbent-Assay (ELISA) (manuscript in preparation). Therefore, high-affinity binding may depend on tubulin content which may, in turn, depend on the mitotic requirement of a developmental stage. However, since a difference in tubulin content between the S and R strains was not apparent, high-affinity binding may also depend on the type(s) of tubulin present. We have observed (manuscript in

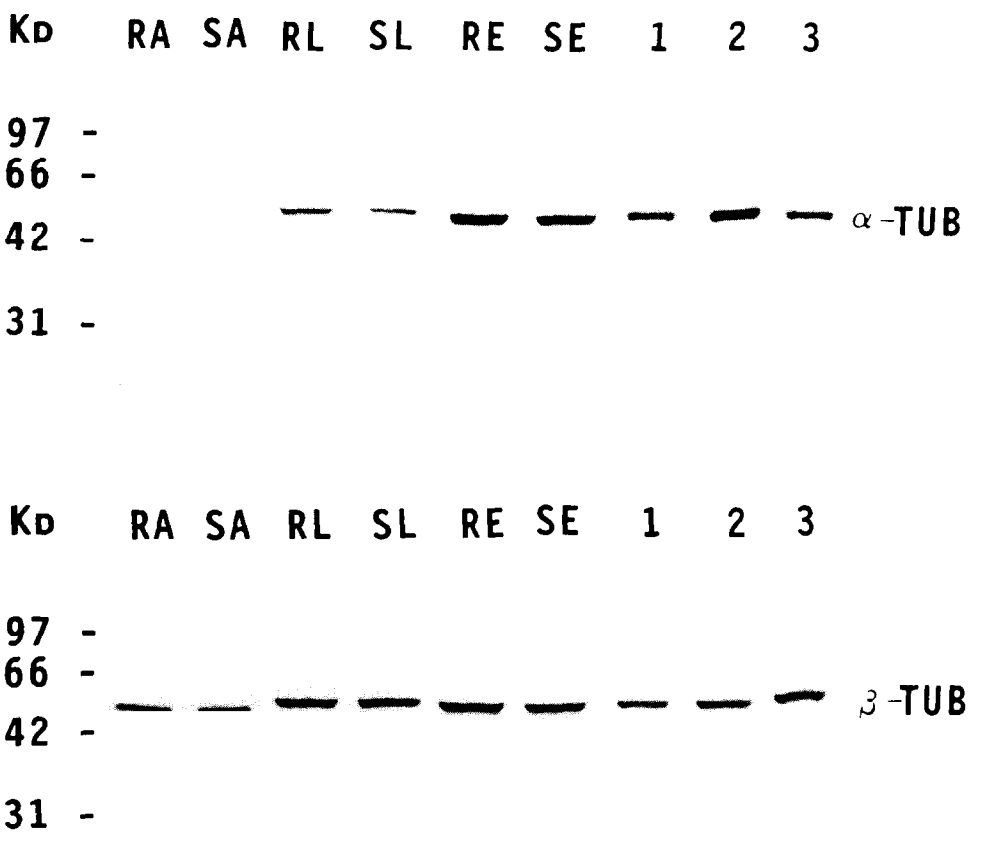

Fig. 3. Western blots of α- and β-tubulin (TUB) in supernatants prepared from unembryonated eggs, infective larvae and adult worms of the susceptible (S) and resistant (R) strains of *Haemonchus contortus*. Mammalian (sheep brain) and parasite (*H. contortus* eggs) tubulin purified by polymerisation/depolymerisation and Taxol, respectively, were used as standards to identify and compare tubulin bands in parasite supernatants. Parasite tubulin, 0.07 (lane 1) or 0.14 μg (lane 2), mammalian tubulin, 0.17 μg (lane 3), and protein, 5 μg, of supernatants derived from unembryonated eggs (E), infective larvae (L) and adult worms (A) were loaded onto a 1.5 mm thick 12% SDS–polyacrylamide separating gel. The transblots on nitrocellulose paper were immunoblotted with mouse anti-chicken α- (top panel) or β-tubulin (bottom panel) monoclonal antibodies (Amersham) and peroxidase-conjugated antimouse IgG (BioCan) as outlined in Materials and Methods. Key: RE, resistant E; SE, susceptible E; RL, resistant L; SL, susceptible L; RA, resistant A; and SA, susceptible A.

preparation) that the tubulin isoform pattern of the S strain is different from that of the R strain. The R strain may consist of mainly the resistant genotype but with a proportion of the susceptible genotypes. This would explain why the binding affinities (K_a) of the S and R strains are similar while the B_{max} of R is less than that of S.

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REFERENCES

1. Barragry T, Anthelmintics—A review. *N Z Vet J* **32**: 161–164, 1984.
2. Barragry T, Anthelmintics—Review, part II. *N Z Vet J* **32**: 191–199, 1984.
3. Donald AD, The development of anthelmintic resistance in nematodes of grazing animals. In: *Facts and Reflections* (Eds. Borgsteede FHM, Henriksen SvAa and Over HJ), pp. 15–28. Central Veterinary Institute, Lelystad, The Netherlands, 1983.
4. Prichard RK, Hall CA, Kelly JD, Martin ICA and Donald AD, The problem of anthelmintic resistance in nematodes. *Aust Vet J* **56**: 239–251, 1980.
5. Lacey E, The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazole carbamates. *Int J Parasitol* **18**: 885–936, 1989.

6. Dustin D, *Microtubules*. Springer, Berlin, 1984.
7. Samizadeth-Yazd A and Todd AC, Anthelmintic activities of fenbendazole against strains of *Nematodirus helvetianus* in cattle: Effect on egg production, embryogenesis and development of larval stages. *Am J Vet Res* **39**- 1668–1671. 1978.
8. Kirsch R and Schleich H, Morphological changes in trichostrongylid eggs after treatment with fenbendazole. *Vet Parasitol* **11**: 375–380, 1982.
9. Lacey E, Brady RL, Prichard RK and Watson IR, Comparison of inhibition of polymerization of mammalian tubulin and helminthic ovicidal activity by benzimidazole carbamates. *Vet Parasitol* **23**: 105–119, 1986.
10. Prichard RK, Sheep anthelmintics. In: *The Epidemiology and Control of Gastrointestinal Parasites of Sheep in Australia* (Eds. Donald AD, Southcott WH and Dineen JK), pp. 75–107. CSIRO Division of Animal Health, Melbourne, Australia, 1978.
11. Lacey E and Prichard RK, Interaction of benzimidazoles (BZ) with tubulin from BZ-sensitive and BZ-resistant isolates of *Haemonchus contortus*. *Mol Biochem Parasitol* **19**: 171–181, 1986.
12. Lacey E and Snowdon KI, A routine diagnostic assay for the detection of benzimidazole resistance in parasite nematodes using tritiated benzimidazole carbamates. *Int J Parasitol* **27**: 309–324, 1987.
13. Boersema JH, Possibilities and limitations in the detection of anthelmintic resistance. In: *Facts and Reflections* (Eds. Borgsteede FHM, Henriksen SvAa and Over HJ), pp. 207–218. Central Veterinary Institute, Lelystad, The Netherlands, 1983.
14. Levine DN, *Textbook of Veterinary Parasitology*, pp. 183–186. Burgess Publishing, Minneapolis, MN, 1978.
15. Lubega GW and Prichard RK, Specific interaction of benzimidazole anthelmintics with tubulin: High affinity binding and benzimidazole resistance in *Haemonchus contortus*. *Mol Biochem Parasitol* **38**: 221–232, 1990.
16. Weston KM, O'Brien RW and Prichard RK, Respiratory metabolism and thiabendazole susceptibility in developing eggs of *Haemonchus contortus*. *Int J Parasitol* **14**: 159–164, 1984.
17. Ministry of Agriculture, Fisheries and Food, *Manual of Veterinary Parasitological Laboratory Techniques*. Reference Book 418, pp. 20–31. Her Majesty's Stationery Office, London, 1986.
18. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
19. McPherson GA, *KINETIC, EBDA, LIGAND, LOWRY*. A collection of radioligand binding programs. Elsevier Science Publishers, Amsterdam, The Netherlands, 1988.
20. McPherson GA, A practical computer based approach to the analysis of radioligand binding experiments. *Comput Prog Biomed* **17**: 107–114, 1983.
21. Munson PJ and Rodbard D, LIGAND: A versatile computerized approach for the characterization of ligand-binding systems. *Anal Biochim* **107**: 220–239, 1980.
22. Tang L, Prichard RK and Lacey E, Identification of tubulin isoforms from *Brugia malayi*, *Brugia pahangi* and *Nippostrongylus brasiliensis* and comparison with mammalian brain tubulin. In: *Molecular Paradigms for Eradicating Helminth Parasites* (Ed. MacInnis AJ), pp. 311–320. Alan R. Liss, New York, 1987.
23. Tang L and Prichard RK, Characterization of tubulin from *Brugia malayi* and *Brugia pahangi*. *Mol Biochem Parasitol* **32**: 145–152, 1989.
24. Shelanski ML, Gaskin F and Cantor CR, Microtubule assembly in the absence of added nucleotides. *Proc Nat Acad Sci USA* **70**: 765–768, 1973.