BENZIMIDAZOLE (BZ) RESISTANCE IN *HAEMONCHUS CONTORTUS*: SPECIFIC INTERACTIONS OF BZs WITH TUBULIN

BY

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Suggested short title

TUBULIN AND BENZIMIDAZOLE RESISTANCE IN *HAEMONCHUS CONTORTUS*

TO MARGARET KANSIIME

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PREFACE

The work described herein was carried out in Prof. Prichard's lab at the Institute of Parasitology, McGill University, Montreal, between September 1986 and September 1990.

The mechanism of BZ-resistance in Haemonchus contortus was investigated, assuming that benzimidazole (BZ) anthelmintics work by binding tubulin. The specific binding of various BZ drugs in supernatants prepared from eggs, larvae or adult worms of BZ-susceptible and -resistant strains of H. contortus was examined and compared. The tubulin content and tubulin isoforms of the susceptible and resistant strains were compared. The binding affinities of several BZs were compared. The binding data was analyzed using computer programs based on the fundamental principles of receptor-ligand interactions. The data presented in this thesis are consistent with tubulin binding being the primary mechanism of action of BZs.

The accounts of the studies have been arranged in 8 Chapters. Chapter I gives a general introduction and literature review of the subject. Because of the fast growth of research knowledge in the fields of tubulin and BZs, only the information directly related to my studies is covered. Chapter II deals with Materials and general Methods used throughout the studies, and Chapters III to X describe the various studies undertaken. Chapter XI gives a general discussion and conclusions of the studies.

Special organizational criteria need to be mentioned. Oxibendazole (OBZ) and mebendazole (MBZ) gave similar results. Therefore, in interest

of clarity, these drugs are discussed together in Chapter III but in subsequent Chapters results of only one of them are presented. Unless otherwise stated it should be assumed that OBZ and MBZ gave similar results. Compared with other tritiated drugs, less work was done using albendazole (ABZ) and for convenience sake the results for ABZ are presented together with those of oxfendazole (OFZ) in Chapter IV.

The selection of the R1, R2, R3 and R4 H. contortus strains used in Chapter VIII was a collaborative effort of Dr. M.E. Scott and her graduate student N. Maingi who subjected the original R strain to selection pressure to give rise to these more resistant strains.

The references are cited in text by first author where there are three or more authors or by both authors where there are only two, and are arranged in alphabetical order in the reference list. The abbreviations used are explained when first introduced in each major section and are given in full in alphabetical order in Appendix I. Appendix II is a list of the publications that have resulted from work described in this thesis.

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The help of my friends and fellow students at the Institute is sincerely acknowledged. Many invaluable discussions with Dr. Siva Ranjan,

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throughout my youth and education. I extend my gratitude to Prof. G.S.Z. SSenyonga, my superior at Makerere University, for his support and encouragement of my further education.

I was supported by a Canadian Commonwealth Scholarship of the association of Universities and Colleges of Canada. This research was supported by a grant (No. A2777) to Dr. R.K. Prichard, from the Natural Science and Engineering Council (NSERC) of Canada. Research at the Institute of Parasitology is supported by the Fonds Fcar pour l'aide à la recherche and NSERC.

ABSTRACT

The mechanism of benzimidazole (BZ) anthelmintic resistance in Haemonchus contortus was investigated. The total binding (TB), low-affinity binding (LAB) and high-affinity (specific) binding (HAB) of [^{3}H]BZs (mebendazole (MBZ), oxibendazole (OBZ), albendazole (ABZ) and oxfendazole (OFZ)) in supernatants derived from BZ-susceptible (S) and BZ-resistant (R) strains were examined and compared. The TB of all [^{3}H]BZs was reduced for the R strain. The TB of OBZ, MBZ and ABZ was separated into LAB and HAB. However, OFZ bound with low-affinity. The binding affinity, K_a, and maximum binding, B_{max}, for the HAB of OBZ and MBZ were calculated using computer programs. Compared with the S strain, the B_{max} of the R strain was reduced but the K_a was not affected. LAB to parasite preparations devoid of tubulin was observed but HAB occurred to preparations containing tubulin only. The HAB per mg protein decreased from egg through larva to adult stage. It was shown by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot and enzyme-linked immunosorbent assay (ELISA) analysis that the tubulin content per mg protein decreased from egg, through larva to adult worm. The ability of various BZs- OBZ, MBZ, ABZ, OFZ, fenbendazole (FBZ), albendazole sulphoxide (ABZSO), albendazole sulphone ($ABZSO_2$), and thiabendazole (TBZ) -to bind tubulin was compared by displacement analysis and their IC_{50} ([BZ] required to inhibit 50% of the [^{3}H]BZ binding) and K_a values were determined. The IC_{50} and K_a values approximately correlated with the known anthelmintic potency (recommended therapeutic doses) of the BZs except for OFZ and ABZSO. Tubulin bound BZs at 4°C with lower K_a than at 37°C. Western blot of tubulin separated by 2-dimensional electrophoresis showed that the ß-tubulin isoform pattern of the S and R strains were dissimilar while the α-tubulin isoform patterns were similar. BZ-resistance was associated with a decrease in high affinity BZ binding and an alteration in ß-tubulin isoform pattern.

Résumé

Le mécanisme de résistance des anthelmintiques du groupe benzimidazole (BZ) a été étudié chez *Haemonchus contortus*. La fixation totale, la fixation à faible affinité (FFA) et la fixation (spécifique) à haute affinité (FHA) des [^{3}H]BZs [mébendazole (MBZ), oxibendazole (OBZ), albendazole (ABZ) et oxfendazole (OFZ)] aux surnageants provenant de parasites de souche sensible (S) et résistante (R) aux BZs ont été examinées et comparées. La fixation totale de tous les [^{3}H]BZs a été réduite chez la souche R. La fixation totale de l'OBZ, du MBZ et de l'ABZ a été séparée en FFA et en FHA, à l'exception de l'OFZ qui ne se fixe qu'avec une faible affinité aux BZs. La constante de fixation K_a et la fixation maximale B_{max} pour la FHA de l'OBZ et du MBZ ont été calculées à l'aide d'un ordinateur. Comparé à la souche S, la B_{max} de la souche résistante a été réduite mais sa K_a n'a pas changé. Il a été observé que les BZs se fixaient avec une faible affinité aux échantillons de parasite dépourvus de tubuline. Par contre, une FHA s'est produite avec les échantillons contenant de la tubuline. La FHA par mg de protéine décroit de l'oeuf à la larve et de la larve au ver adulte. Il a été démontré par électrophorèse de gel de polyacrylamide en présence de sulfate de sodium dodécyl, par immunoblot et par ELISA, que le contenu en tubuline par mg de protéine baisse de l'oeuf à la larve et de la larve au ver adulte. La facilité de divers BZs [OBZ, MBZ, ABZ, OFZ, fenbendazole (FBZ), albendazole sulfoxyde (ABZSO), albendazole sulfone ($ABZSO_2$) et thiabendazole (TBZ)] de se lier à la tubuline a été comparée par analyse de déplacement, et leur IC_{50} (concentration de BZ requise pour inhiber 50% de la fixation de [^{3}H]BZ) et leur K_a ont été évaluées. Les valeurs IC_{50} et K_a correspondaient à l'efficacité connue des anthelmintiques BZs (dosage thérapeutique recommandé) à l'exception de l'OFZ et de l'ABZSO. La tubuline se fixe aux BZs avec une K_a inférieure à 4°C qu'à 37°C. Un immunoblot de la tubuline séparée par une électrophorèse bidimensionnelle a démontré que les points isoélectriques des isoformes de la ß-tubuline des souches S et R sont différents alors que ceux des isoformes de la α-tubuline sont similaires. La résistance aux BZs est associée à la réduction de la fixation à haute affinité et à la modification des isoformes de la ß-tubuline.

CLAIMS OF ORIGINALITY

Much of the work described in this thesis is an original contribution to science:

(1) The specific interaction of BZs with tubulin of the BZ-susceptible and resistant strains of H. contortus is described for the first time.

(2) For the first time, the binding data concerning BZ-resistance was analyzed using computer programs based on the fundamental principals of receptor-ligand interaction.

(3) A specific BZ-binding assay is used for the first time to correlate the binding affinity and the known anthelmintic potency of BZs. A correlation is observed for all the BZs except OFZ and ABZSO. It is suggested that the anthelmintic activity of OFZ and ABZSO might depend on metabolic conversion to FBZ and ABZ respectively.

(4) It is demonstrated for the first time that the BZ-susceptible and resistant strains contain similar total amounts of tubulin per mg protein.

(5) The decline in tubulin content per mg protein from egg through larval to adult stage had not been reported before.

(6) It is also shown for the first time that specific (high-affinity) binding per mg protein decreases from egg through larval to adult stage. This coincides with the parallel decrease in tubulin content per mg protein.

(7) That resistance is associated with a decrease in high-affinity binding but not low-affinity binding is an original finding.

(8) An alteration of β-tubulin isoform pattern that correlates with an

altered BZ-binding had never been reported for any parasitic nematode. This together with the above findings strongly suggest that tubulin may be the pharmacological specific target of BZs. A theory can be advanced that alteration in β-tubulin reduces BZ binding to tubulin and thus causes BZ-resistance in the BZ-resistant strain of H. contortus investigated.

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CHAPTER I

LITERATURE REVIEW

1.1 INTRODUCTION

The most widely used method to control parasitic worms is treatment with anthelmintics (Martin, 1985). Benzimidazoles (BZs) are widely used anthelmintics for man (Cook, 1990) and animals (Campbell, 1990) and are effective against nematodes, adult cestodes and some trematodes. BZs are also used as antifungal, antitumour or anti-mitotic agents (Dustin, 1984). The frequent use of anthelmintic has caused the selection of worms resistant to BZs and other anthelmintic drugs in animals (Donald, 1983). Benzimidazole (BZ) resistance is a major problem for the control of Haemonchus contortus of sheep and other nematodes of sheep and horses (Waller and Prichard, 1986). Current evidence suggests that BZs might exert all of their effects by binding tubulin (Lacey, 1988). Tubulin is the main constituent protein of microtubules and the latter are essential for many functions in eukaryotic cells (Dustin, 1984; Prescott, 1988).

If the primary mode of action of BZs is tubulin binding, then the mechanism of BZ-resistance could also be tubulin related. It has been reported for some organisms that homogenates of BZ-resistant strains bind less BZ drug than those of BZ-susceptible strains (Davidse and Flach, 1977; Sangster et al., 1985; Lacey and Prichard, 1986). However in these studies only total binding was considered. The data were not corrected for non-specific binding. The specificity of the reduced BZ binding was not substantiated, although tubulin was theoretically implicated. The cause of the purported reduced tubulin binding in resistant strains of H. contortus

is unknown, nor is it known whether BZ-resistance in all species and strains is similarly caused. Indeed some BZs, such as oxibendazole, are still effective against horse-nematodes resistant to other BZs (Webster et al., 1981; Wescott, 1986; Slocombe et al., 1989). Whilst BZs appear to be equally potent against susceptible strains (when administered at the recommended dose rates), there are several claims of superior performance for some BZs against worms resistant to other BZs (Donald, 1983). Oxfendazole is effective against adult worms in vivo but fails to inhibit egg development in vitro (Lacey et al., 1987a). Studies in vitro suggest that eggs or immature worms may be more susceptible to BZs than are adult worms (Kirsch and Schleich, 1982; Rapson et al., 1985; Rew et al., 1986). Despite extensive evidence of impairment of adult worms, overt toxicity (death) against these stages during time periods of exposure to BZs in vitro has not been documented (see Lacey, 1990, for review). However, in vivo, arrested larvae are less susceptible than adult stages (Prichard, 1978). It is not known whether all of these discrepancies can be explained at the level of BZ-tubulin interaction. In this study, I have (1) investigated the specific binding of various BZs to tubulin from various developmental stages of thiabendazole-susceptible (S) and thiabendazole resistant (R) strains of H. contortus, (2) compared the tubulin content and (3) the tubulin isoforms from these sources in an attempt to substantiate some of the above observations. Therefore, this study addressed the mechanism of BZ-resistance at the biochemical level, compared the tubulin binding affinities of various BZs and compared developing stages of H. contortus with respect to tubulin and BZ-tubulin interaction. This study can lead to further investigations required to unravel the nature of BZ-resistance at the protein and nucleic acid level,

and locate the BZ-binding site on tubulin. Full characterization of the binding site could aid in the development of chemical structures aimed at overcoming BZ-resistance.

1.2 HAEMONCHUS CONTORTUS

1.2.1 General description

Haemonchus is an important genus found in the true stomach (abomasum) of sheep, goats, cattle and other ruminants (Soulsby, 1982). H. contortus is commonly called the stomach worm, the wire worm or the barber's pole worm, all of which are descriptive names of this nematode. The nematodes are 10-30 mm long (0.4 mm diameter) and belong to the Trichostronglid group. The females are larger, more numerous than the males and have white ovaries wound spirally around the intestine which is red from the host's blood, giving a barber's pole appearance. Males have an even red appearance and a large bursa with barbed spicules. The eggs are 90 x 45 μm in size. The larvae are described in the Manual of Veterinary Parasitological Laboratory Techniques (Ministry of Agriculture, Fisheries and Food, London, 1986) and the diagnosis of haemonchosis in mixed infections can be based on the morphology of the third stage larvae.

1.2.2 Life cycle

H. contortus has a typical direct nematode life cycle (Soulsby, 1982). Embryonation starts immediately if there is O_2, moisture and optimal temperature. At 26° C, the first stage larva (L_1) is formed in 20-24 h and development through the second stage (L_2) to the third stage (L_3) takes 4-6 days. There is no development below 9° C. Development of larvae from one

stage to the next is accompanied by moulting and exsheathement. However, the cuticle of the L_2 is retained by the L_3 which does not feed. The infective stage is the ensheathed L_3 and may live on pasture up to 6 months. Infection is by ingestion of the L_3 which will exsheath in the rumen of sheep and migrate down the gut to penetrate the abomasal mucosa and start feeding as the L_4. The L_4 may become hypobiotic before emerging into the lumen of the abomasum to moult to the adult stage and start laying eggs after mating. The time from infection to the first appearance of eggs in faeces of the host (the prepatent period) is 15-20 days. H. contortus has a high biotic potential whereby 5000 eggs/day/female worm may be passed in faeces (Levine, 1978).

1.2.3 Pathology

The geographical distribution of H. contortus is world wide, but it is especially prevalent in warm humid climates and is the most important worm infection wherever sheep are raised for food or wool (Soulsby, 1982). H. contortus is a common parasite that may rapidly kill young sheep. Less severe cases produce weakness in animals, making sheep raising uneconomical.

Anaemia is the basic feature of the infection because the worms feed on blood (Soulsby, 1982). When large numbers of larvae infect sheep, deaths can occur suddenly while the sheep still appear to be in good health. This is termed "acute prepatent disease" because at this time eggs are not seen upon faecal examination. Chronic infections involving smaller numbers of worms may produce oedema (bottle jaw), iron-deficiency anaemia, progressive weakness, wool breaks and sheep death. Diarrhoea or constipation may also occur. Both fourth-stage larvae and adult worms

puncture blood vessels in the stomach wall and feed on the blood that is released. Large quantities of blood spill over during the feeding process. Nutrients are used by the host to replace lost blood elements rather than for growth and wool production Sheep infected with H. contortus may exhibit self-cure, greatly reducing adult parasite loads. Damage by parasites in such cases is minimal after immunity develops.

1.2.4 Management

H. contortus is one of an economically important group of gastrointestinal nematodes of sheep which are closely related taxonomically, epidemiologically and pathologically (Soulsby, 1982). This group includes Haemonchus, Ostertagia, Trichostrongylus, Nematodirus, Oesophagostomum and Charbertia of which there are several species for each of these genera. In general animals are parasitized by a mixture of species so that management is the same for all of them. Collectively, several or all of these nematode species cause a disease entity known as parasitic gastroenteritis (PGE) (a term which otherwise means inflammation of stomach and intestines by parasites). In spite of the intensification of grazing livestock industries in the recent past, one of the major limitations to increasing productivity has been the great range and severity of animal diseases, of which PGE has been prominent (Waller and Prichard, 1986). The greatest challenge to this nematodiasis has been the production of broad spectrum anthelmintics, such as BZs (Arundel, 1985). The traditional approach to suppress the effects of PGE on animal production has been the use of frequent treatment, the timing of which has been largely guided by convenience and economic considerations, rather than epidemiological principles (Brunsdon, 1980). This approach led to

considerable inefficiency. Resistant nematodes have, therefore, been selected (Waller, 1985). This probably explains why drug resistance is mostly confined to the economically important strongylid nematodes (Kelly and Hall, 1979). Few reports have been made in plant nematodes (Foot, 1980) and none in man. However, there are reports of resistance in small strongyles of horses and, even then, its frequency is directly related to the intensity of drug use (Webster et al., 1981). Generally, the clinical picture caused by gastrointestinal nematodes is similar, but the primary pathology is varied (Soulsby, 1982). Infection with Ostertagia spp., for example, is associated with destruction of the gastric glands in the abomasum. Haemonchus is a blood sucker and its principle effects are due to loss of blood. When present in low numbers, the infestation is inapparent but, nevertheless, causes economic losses, such as reduced meat and wool production (Soulsby, 1982).

The economic losses due to inapparent infestation (helminthiasis) appear to be more significant than losses caused by overt disease (helminthosis) or parasite-induced host mortality (Soulsby, 1982). This awareness has been one of the factors behind the overuse of anthelmintic. Indeed, frequent treatment of apparently healthy animals can have significant economic advantages (Johnstone et al., 1979). Anthelmintic treatment is likely to continue as the first and most important line of defense against worm parasites (Martin, 1985).

1.3 BENZIMIDAZOLES (BZs)

1.3.1 Classification and uses of BZs

Anthelmintics are used therapeutically or prophylactically (Martin,

1985). Therapy requires that the drug removes sufficient worms to eliminate signs of disease while prophylaxis requires removal of enough worms to prevent significant contamination of pastures (Arundel, 1985). It is both convenient and logical to classify drugs according to their chemical structure, mode of action and spectrum of activity. Accordingly, Prichard et al. (1980) have grouped the most commonly used anthelmintics of sheep (Table 1.1). These same groups are also the most important in cattle (Prichard, 1983), horses (Barragry, 1984) and to a large extent man (James and Gilles, 1985).

BZs have broad spectrum activity (Campbell, 1990; Cook, 1990). They are active against nematodes, adult cestodes and some trematodes and show antifungal and antitumour activity (Dustin, 1984; Atasi and Tagnon, 1975; Lacey, 1985). The commercially available BZs are safe (Goldsmith, 1984). The structures of benzimidazoles and probenzimidazoles developed for experimental or commercial use are organised in groups and shown in Table 1.2. With the exception of triclabendazole which has been developed as a flukicide specific for Fasciola hepatica (Boray et al., 1983), all BZs have broad spectrum activity. Probenzimidazoles undergo cyclisation in vivo to form BZ-carbamates (Prichard, 1978). For example, thiophanate undergoes cyclisation in vivo to form a BZ-ethyl-carbamate. Similarly febantel is metabolised to oxfendazole while netobimin is cyclised to albendazole. BZ-methyl-carbamates as a group are newer and more potent than the BZ-thiazolyls.

1.3.2 The anthelmintic action of BZs

The study of the pharmacological mode of action of a drug comprises a linkage of its biochemistry and pharmacokinetics with in vivo and in

Table 1.1 Groups of commonly used anthelmintics.

BROAD SPECTRUM ANTHELMINTICS

Group 1. **Benzimidazoles and probenzimidazoles**

Proposed mode of action: Binding tubulin

Examples: see Table 1.2

Group 2. **Levamisole and morantel**

Proposed mode of action: Cholinergic agonists

Examples: levamisole, morantel

Group 3. **Avermectins**

Proposed mode of action: GABA agonists

Examples: ivermectin

NARROW SPECTRUM ANTHELMINTICS

Group 4. **Salicylanilides and substituted nitrophenols**

Proposed mode of action: Uncoupling oxidative phosphorylation

Examples: rafoxanide, niclosamide, nitroxynil, clioxanide.

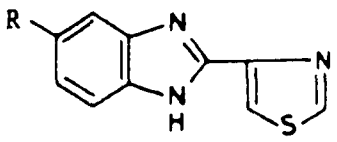
Group 5. **Organophosphates**

Proposed mode of action: Inhibiting acetylcholinesterase

Examples: naphthalophos, dichlorvos, metrifonate.

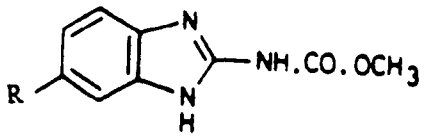
Table 1.2 Groups and structures of benzimidazoles and pro-benzimidazoles developed for experimental or commercial use.

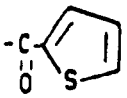
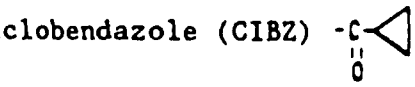
BENZIMIDAZOLE-THIAZOLYLS



		-R
Thiabendazole	(TBZ)	H
Cambendazole	(CBZ)	$-NHCO_2CH(CH_3)_2$

BENZIMIDAZOLE-2-METHYLCARBAMATES

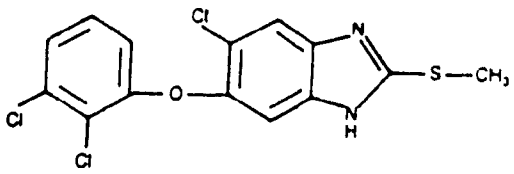


		-R			-R
Carbenazim	(MBC)	H	Mebendazole	(MBZ)	$-C(=O)-C_6H_5$
Parbendazole	(PBZ)	$-CH_2CH_2CH_2CH_3$	Nocodazole	(NDZ)	
Oxibendazole	(OBZ)	$-SCH_2CH_2CH_3$	Flubendazole	(FLBZ)	$-C(=O)-C_6H_4-4-F$
Albendazole	(ABZ)	$-OCH_2CH_2CH_3$	Fenbendazole	(FBZ)	$-S-C_6H_5$
Rycobendazole	(RBZ)	$-S(=O)CH_2CH_2CH_3$	Oxfendazole	(OFZ)	$-S(=O)-C_6H_5$
Ciclobendazole	(CIBZ)		Luxabendazole	(LBZ)	$-OSO_2-C_6H_4-4-F$

continued.....

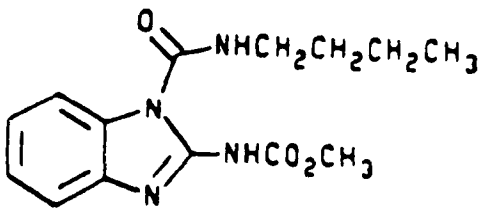
Table 1.2 .....continued.

HALOGONATED BENZIMIDAZOLE THIOLS

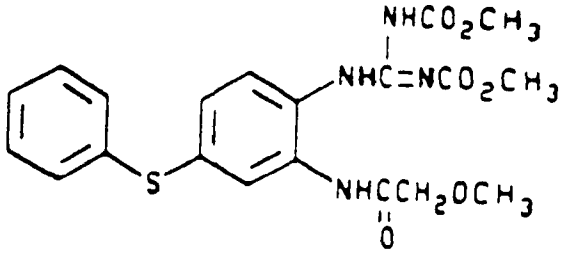


Triclabendazole

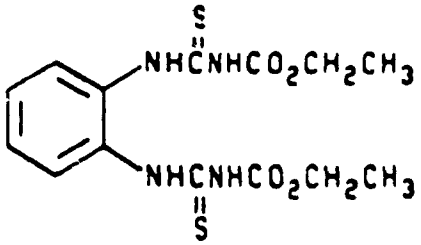
PROBENZIMIDAZOLES



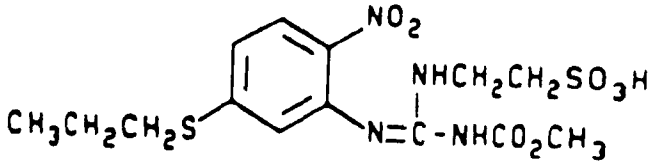
Benomyl



Febantel



Thiophanate



Netobimin

vitro efficacy studies. The biochemistry of BZs is reviewed later after their putative receptor, tubulin, is discussed. In this section I will review the pharmacokinetics and the in vivo and in vitro efficacy studies of those BZs with which I will be mostly concerned.

1.3.2.1 Pharmacokinetics of BZs

Pharmacokinetics is concerned with drug absorption, distribution, metabolism and excretion and the relationship of these processes in vivo to the intensity and time course of pharmacological effects (such as efficacy and toxicity) (Wagner, 1968). The study of drug-receptor interactions, pharmacodynamics, is the major thrust of this study. However, since the pharmacokinetic behaviour of a drug can be important in modulating its anthelmintic efficacy, I will review some pharmacokinetic aspects of the BZs I will be concerned with in this thesis.

Since the introduction of thiabendazole (Brown et al., 1961), benzimidazoles and probenzimidazoles with improved efficacy and extended spectra of activity have been developed. Parbendazole (Actor et al., 1967), cambendazole (Hoff et al., 1970), mebendazole (Brugman et al., 1971) and oxibendazole (Theodorides et al., 1973) were products of an empirical search for more potent drugs. Each new compound came with a claim of superiority in performance, compared to pre-existing drugs. The improvement in performance could be attributed to new structures substituted into the BZ-nucleus with resultant greater affinity for the specific receptor (Lacey and Watson, 1985). On the other hand, the improved efficacy could simply be due to improved pharmacokinetic behaviour of the compound leading to increased drug bioavailability. For example, compared to earlier BZs, the poorly soluble sulphide and

sulphoxide BZs, fenbendazole (Baeder et al., 1974), oxfendazole (Averkin et al., 1975) and albendazole (Theodorides et al., 1976), have improved efficacy against lungworms and inhibited larvae. Febantel (Delatour and Euzeby, 1983) and netobimin (Nafissi-Varchei, 1983) are prodrugs that are metabolised to fenbendazole/oxfendazole and albendazole/albendazole sulphoxide, respectively, and may have improved efficacy owing to easier formulation/solubility. Drugs such as thiabendazole and cambendazole which are highly soluble in the aqueous rumen fluid tend to be rapidly absorbed and to have shorter elimination half-lives than the less soluble BZs (Prichard et al., 1978a). The less soluble BZs remain in the plasma for longer periods, and since it is assumed that an equilibrium exists between plasma and the gastrointestinal tract, the time during which parasites are exposed to effective concentrations of the drug is extended However, at the extreme limits of insolubility, the drugs again become less effective as larger proportions fail to be absorbed and are excreted unchanged in the faeces.

Solubility is not the only criterion for bioavailability; the nature and rate of metabolism and rate of excretion can play a role too. Parbendazole has low solubility but is extensively metabolised in the rumen fluid and achieves very low plasma concentrations (reviewed by McKellar and Scott, 1990). Anatomical features such as the ruminant rumen or equine caecum, which slow the passage of digesta in these species, may enhance the activity of BZs by extending their transit time (Marriner and Bogan, 1981a). Oesophageal groove closure and thus ruminal by-pass of drugs may influence drug availability (Duncan et al., 1977; Ngomuo et al., 1984). The pharmacokinetics of BZs differ between species of animals (Weir and Bogan, 1985; Marriner and Bogan, 1980, 1981a, b; Ngomuo et al., 1984;

McKeller and Scott, 1990). There are some ill-explained modulations of pharmacokinetic profile of drugs. For example the metabolism and excretion of oxfendazole, when co-administered with parbendazole, were found to be reduced which resulted in increased efficacy of these drugs against BZ-resistant nematodes in sheep (Hennessy et al, 1985). Gastrointestinal parasitism itself has been shown to reduce the bioavailability of BZs in ruminants (Marriner and Bogan, 1984). Drug preparation or formulation and route/mode of administration may influence drug bioavailability (see McKeller and Scott, 1990). In this study the affinity of various BZs for their specific receptor(s) was compared in order to determine how much of the anthelmintic performance of these drugs can be explained by the strength (affinity) of receptor binding or by pharmacokinetic behaviour.

1.3.2.2 Efficacy of BZs in vivo

From previous reviews of in vivo efficacy of BZs (Van den Bossche et al., 1985; Campbell and Rew, 1986; Campbell, 1990) a number of general principles can be established: (1) The potency of BZs (mg per kg weight) based on in vivo efficacy studies (see Marriner and Armour, 1986) demonstrate a rank order efficacy of,

ABZ = ABZSO = FBZ = OFZ > MBZ > OBZ > PBZ > CBZ > TBZ.

(2) The doses required to achieve efficacy against nematodes are lower than those used for cestode and trematode control. For the latter parasites, control is normally obtained at higher doses or multiple treatment compared with nematodes (Van den Bossche et al, 1982). (3) Extra-intestinal parasites, particulary interstitial dwelling parasites, are less sensitive than gastrointestinal nematodes (Van den Bossche et al., 1982). (4) Activity against developing stages is superior to that

against arrested or adult stages in comparable habitats (Kirsch and Schleich, 1982; Forsyth et al., 1984). (5) There are host-related principles affecting in vivo efficacy: (i) Host toxicity is nil at the efficacious anthelmintic doses (Seiler, 1975). (ii) Efficacious BZ doses are host-dependent probably due to host differences in handling of the drug in vivo (pharmacokinetics) (Van den Bossche, 1985). (iii) BZs are extensively metabolised in host species to more potent, less potent or non-potent forms. (iv) BZs are hydrophobic and water insoluble and therefore bioavailability and pharmacodynamics and thus efficacy can be altered by formulation and presentation (Hennessy, 1985).

1.3.2.3 Efficacy of BZs in vitro

The most popular test of anthelmintic efficacy in vitro is the egg hatch assay (Boersema, 1984). Such a procedure assumes that the host plays no role in modifying drug potency and that drug uptake by the egg is similar to that by the parasitic stages. BZs, in vitro, inhibit development, motility and survival of various transitions between L_2 to L_3 or L_3 to L_4 (Rew et al., 1986) and L_4 to young adults (Jenkins, 1982; Rapson et al., 1985). But inhibition of egg development is better documented (see Waller and Lacey, 1985). Inhibition of development and population growth of free-living species has also been reported (Platzer et al., 1977). Techniques which involve the isolation of obligatory parasitic stages for evaluating efficacy of BZs are less successful (reviewed by Lacey, 1988). However, Folz et al., (1987) have recently developed an in vitro test for assessing the motility of adult and non-developing stages which could be used in future to test efficacy of drugs against these stages. Use of cultured cells or tissue sections has also

been reported (Rubino et al., 1983; Howells and Delves, 1985).

Lacey et al., (1987a) reported that inhibition of hatching of H. contortus eggs and inhibition of mammalian brain-tubulin polymerization by BZs were correlated, suggesting that the mode of action of BZs is by inhibiting tubulin polymerization. Nevertheless, at the recomended dose rates, BZS are selectively toxic to parasites leaving the host unharmed. Therefore, demonstration of the selectivity of tubulin inhibition by BZs between helminth and mammalian tubulins would provide the fundamental argument for tubulin as the site of BZ action. It has not been shown that BZs will inhibit parasite tubulin more strongly than it does mammalian tubulin. Nevertheless, tubulin binding and subsequent inhibition of its polymerization to microtubules is the putative mode of action of BZs (Lacey, 1988, 1990).

1.4 TUBULIN AND MICROTUBULES

1.4.1 Composition and Structure

Although the existence of microtubules in eukaryotic cells has been sporadically recorded over the last century, their intensive study in cell biology is relatively recent. The term "microtubules" was only coined in 1961 (Dustin, 1984). Since then several reviews have been published as monographs (Dustin, 1978; 1984; Roberts and Hyams, 1979) or conference proceedings (De Brabander and De May, 1980; Soifer, 1975, 1986). The following information has been deduced from these reviews but more specific articles are also cited.

Dustin (1984) defined microtubules on basis of their structure and function. Microtubules are hollow proteinaceous organelles present in all

eukaryotic cells. They are made up of tubulin molecules assembled into long tubular structures with an average diameter of 24 nm (Shelanski and Taylor 1968) and their basic structure is similar in all animals (Thomas and Henley, 1971).

Tubulin is an acidic hetero-dimeric protein molecule composed of α and β types. The dimer has a molecular weight of 110 Kd (55 Kd for the monomer) and a sedimentation constant of 4.8 s at 20° C. Alpha- and β-tubulins are closely related proteins with a sequence length of 450 and 445 aminoacids, respectively, for pig brain tubulin. Structural homology between the proteins is normally > 40%; both α and β are highly acidic (pI 4-6) with 40% of the final 30 COOH-terminus residues being acidic (Ponstingl et al., 1981). Although tubulins isolated from most species fall within these broad parameters, considerable size, amino-acid sequence and acidity heterogeneities exist between tissues, isolates or species. Both α- and β-tubulins are heterogeneous and can be resolved into several isoforms each (Little and Luduena, 1985; Tang and Prichard, 1988, 1989). The precise nature and roles of isotubulins have not yet been fully elucidated, although several groups have demonstrated that many in vivo functions of tubulin are, to varying degrees, isoform-specific (Gundersen et al., 1984). The work of Lewis et al., (1987) and Lopata and Cleveland (1987) suggests that while isotubulin specific functions may exist, communal association in microtubule matrix occurs.

In a freely reversible manner, GTP or GDP, microtubule-associated proteins (MAPs) and other stabilizing factors and α- and β-tubulin co-polymerize in a concentration dependent process to form microtubules (Fig. 1.1). Because of the concentration dependence, anything that will remove or reduce free-tubulin in solution will promote depolymerisation.

Colchicine, BZs and other specific tubulin binding molecules promote depolymerization by removing free tubulin. Microtubules are sensitive to cold and high hydrostatic pressure in addition to these specific molecules. On the other hand high free-tubulin concentration, high temperature (25-37° C), glycerol and the tubulin specific molecule, taxol (Vallee, 1986) will promote polymerization (Fig. 1.1).

Tubulin and MAPs build into complex assemblies such as the mitotic spindle, centrioles, cilia, flagella, axonemes and neurotubules. However, the MAPs differ in different microtubules and the stability of different microtubules therefore varies (Behuke and Zelender, 1967). The mitotic spindle is of universal importance in eukaryotic cells.

1.4.2 Function

A number of discrete functions are ascribed to microtubules at the cellular level (Fig. 1.2): (i) formation of the mitotic spindle during cell division (ii) maintenance of cell shape (iii) cell motility (iv) cellular secretion (v) nutrient absorption and intracellular transport. Tubulin or microtubules may perform these functions by virtue of their association with most cellular organelles: mitochondria, Golgi apparatus, ribosomes, lysosomes, cell membranes and the nucleus. It is via these interactions and probable direct association of tubulin and microtubules with physiological mechanisms of hormone, neurotransmitter, nutrient, enzyme and receptor action that the cellular responses of microtubule disruption are manifested (Karkhoff-Schweizer and Knull, 1987; Durrieu et al., 1987; Mizel and Wilson, 1972; Azhar et al., 1983; McKay et al., 1985; Faulkner et al., 1984). The disruption of the tubulin-microtubule equilibrium can be seen as leading to a cascade of direct and indirect

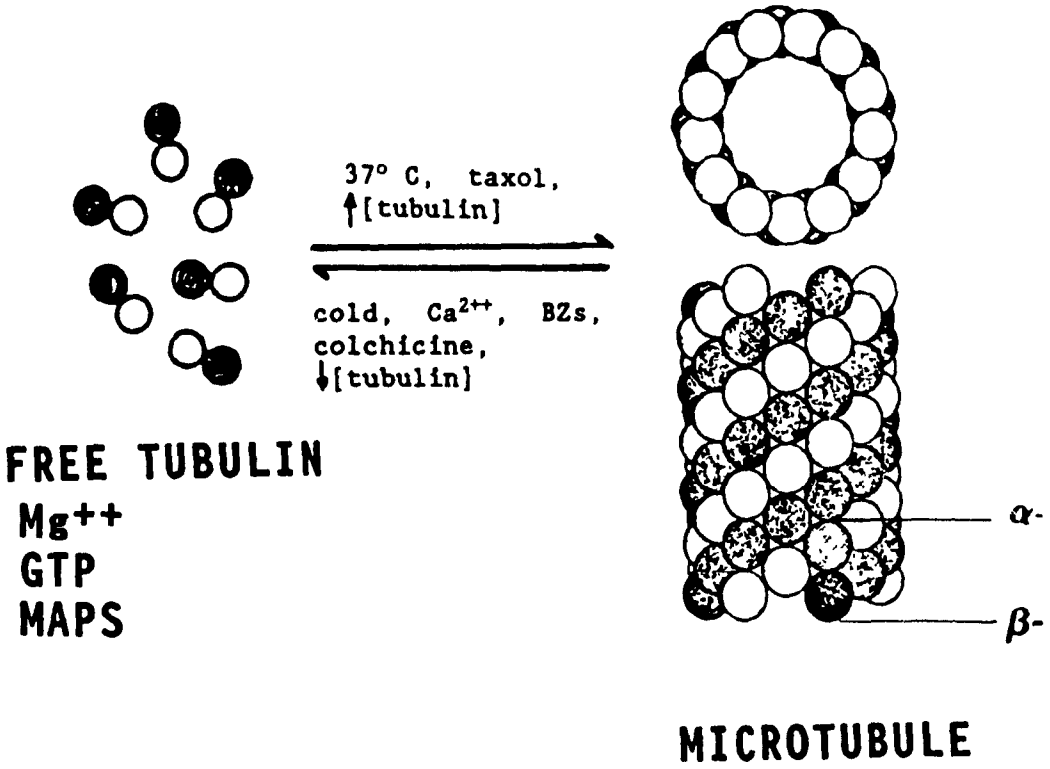


Fig. 1.1. Tubulin polymerization. Free tubulin, Mg^{2++}, GTP and MAPS polymerize to microtubles. High temperature (25-37°C), taxol or an increase in [tubulin] promote polymerization while cold (4°C), Ca^{2++}, BZs, colchicine and other microtubule active substances or a reduction in [tubulin] promote depolymerization.

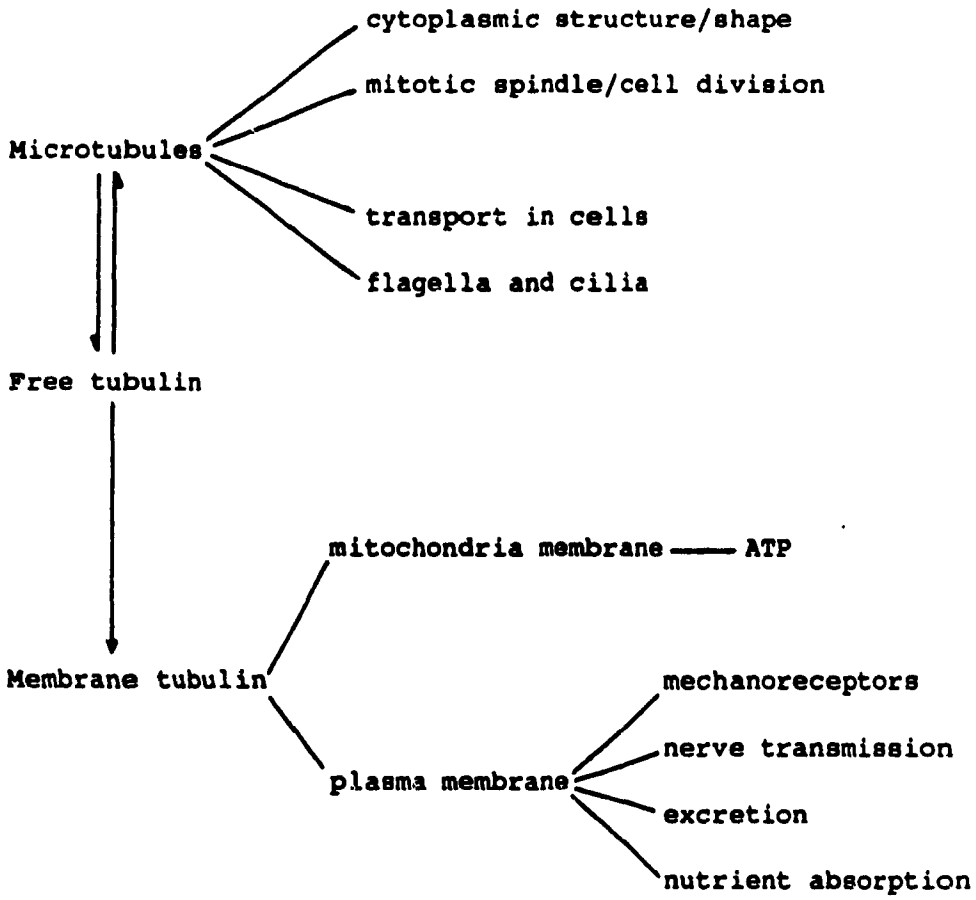


Fig. 1.2. Functions of tubulin.

biochemical/physiological changes resulting in the loss of cellular homeostasis. Loss of homeostasis, if maintained, can be lethal. Lethal effects are most evident in actively dividing or growing cells. While it may be postulated that such cells require greater utilisation of tubulin and are therefore more sensitive to microtubule inhibitors, it is also probable that it is the timing of critical physiological events (such as cell division) which is lethal. One limitation is that the study of the cellular role of tubulin and microtubules is heavily dependent on the use of pharmacological probes such as colchicine, vinblastine and podophyllotoxin. The use of these approaches is based on the assumption that the drugs act only by microtubule disruption and that in vitro microtubule activity reflects the nature and extent of the drug-tubulin interaction affecting particular cellular functions in vivo.

1.4.3 Purification

Tubulin has been isolated from a wide range of organs and species of eukaryotes-from invertebrates through cartilaginous fish to man (Dustin, 1984) and from fungi to higher plants (Lloyd, 1982). Most strategies for the isolation of tubulin rely on three basic characteristics of tubulin: temperature-dependent cycles of polymerization and depolymerisation, interaction with basic ligands and affinity for specific ligands such colchicine and vinblastine. Some of the specific methods which have been used to isolate tubulin may be summarised: (1) DEAE chromatography: pig brain 16,000 x g supernatant was precipitated with 38-49% $(NH_4)2SO_4$ and then followed by DEAE-Sephadex chromatography in the presence of 10 mM NaH_2PO_4 (pH 6.4), 10 mM $MgCl_2$ and 0.1 mM GTP and then eluted with a NaCl gradient (Weisenberg et al., 1968). There are several modifications of

this approach (Luduena, 1979; Lacey and Snowdon, 1990). (2) The temperature dependent cycling of polymerization and depolymerization of tubulin in vitro (Shelanski et al., 1973; Borisy et al., 1975) is the most successful approach for tubulin-rich sources and there are several modifications of it for purification of tubulin from other sources (Lacey, 1988). (3) Affinity sepharose chromatography in which colchicine (Morgan and Seeds, 1975) or antiserum to tubulin (Ikeda and Steiner, 1976) was used as ligand to purify tubulin from mammalian brain or human platelets, respectively. (4) Vinblastine precipitation with formation of tubulin crystals (Bryan and Wilson, 1971). (5) Differential centrifugation to prepare tubulin from the stable microtubules of flagella or cilia (Farrell, 1982). (6) Taxol-induced assembly (Vallee, 1986). (7) Separation of α- and ß-tubulin using hydroxyappatite chromatography or electrophoresis (Luduena, 1979).

These methods are not successful for all organisms (Lacey, 1988). Preparation of pure tubulin from high eukaryotes, especially from mammalian brain, in which the tubulin concentration is high, is much easier compared with purification from lower eukaryotes such as helminths. The main reasons are that (i) the amount of tubulin as percentage of total soluble protein is usually very low (< 1%) in helminths (Friedman et al., 1980; Köhler and Bachmann, 1981) (ii) endogenous inhibitors of tubulin polymerization, such as proteolytic activity and GTP-ase activity may degrade the already limited tubulin pool (Roobol et al., 1980) (iii) Frequently, the starting material itself is limited. Purifications of nematode tubulin, reported previously, have used relatively large nematodes such as Ascaris suum (Barrowman et al., 1984) and Ascaridia galli (Dawson et al., 1983, 1984). However, Tang and Prichard (1988, 1989)

have reported the recovery of tubulin from Nippostrongylus and Brugia species using taxol.

There are limitations which relate to the suitability of the purified tubulin for drug binding studies and other applications. For example, using the temperature dependent cycling, only tubulin above the critical concentration is removed at each cycle while sizeable losses of "cold stable" microtubules occur during depolymerization on ice (Margolis and Rauch, 1981) and other proteins, especially MAPS, co-purify with tubulin (Vallee, 1986). The use of DMSO for further purification or salt to elute tubulin can impair [^{3}H]ligand stoichiometric studies (Croom et al., 1986). In general, the use of purified helminth tubulin in direct [^{3}H]BZ binding assays has not been successful. However, inhibition of purified nematode tubulin polymerization by BZs (Dawson et al., 1984) or inhibition of [^{3}H]colchicine binding to purified nematode tubulin (Ireland et al., 1982) have been reported. It is not clear how inhibition of polymerization or [^{3}H]colchicine binding of pure tubulin is achieved. Tang (1988) observed that taxol-purified tubulin did not bind [^{3}H]MBZ while whole parasite extracts readily bind [^{3}H]BZs (Sangster et al., 1985; Lacey and Prichard, 1986; Tang et al., 1987). However, tubulin isolated by poly-L-lysine affinity chromatography (Lacey and Prichard, 1986) will bind [^{3}H]BZ but tubulin prepared this way contains a sizeable amount of impurities (Tang and Prichard, 1988, 1989). The major limitations of the specific ligand techniques relate to their lack of general applicability to different tubulin sources (Farrell, 1982).

1.4.4 Microtubule inhibitors

Microtubule inhibitors are chemical agents which alter cellular

characteristics of microtubules discussed above. Accordingly, microtubule inhibitors may cause metaphase arrest and inhibit cell growth or polymerization/depolymerization of tubulin in vitro or in vivo or the binding of ligands already known to bind microtubule-protein. Microtubule inhibitors may be subdivided, on the basis of relative-selectivity, into non-selective and selective inhibitors.

1.4.4.1 Non-selective microtubule inhibitors

Non-selective microtubule inhibitors will inhibit many of the cellular functions of microtubules and at the same time, exhibit similar levels of activity against many other proteins at comparable concentrations. Such compounds include alkylating or acylating agents, thiols, heavy metal ions, organometals, aldehydes, ketones, quinones and basic amines (see Lacey, 1988).

1.4.4.2 Selective microtubule inhibitors

Whereas 100% selectivity is unlikely, a selective inhibitor must be specific for a known receptor, at relatively low concentrations, without altering activities of other proteins at the same concentration. Naturally, the reason for this selectivity, in kinetic terms, is that the selective inhibitor has greater affinity for a specific receptor compared with other receptors. Effectively, specificity is a concentration dependent phenomenon, which decreases (i.e other types of receptors with progressively less affinity are bound) as the concentration of the pharmacological agent is increased. Thus overdosage may cause toxicity or side effects. In terms of ligand binding, the selectivity of BZs for parasite tubulin has not been demonstrated. Nevertheless, three

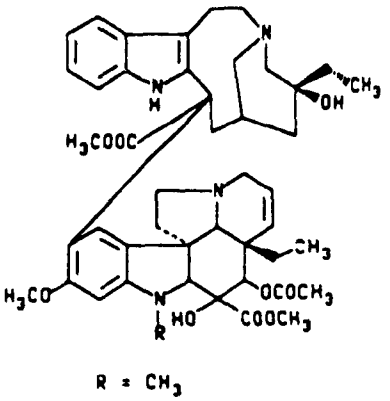
pharmacologically selective sites on mammalian tubulin have been defined using various methods and are denoted according to the protype inhibitor (see Fig. 1.3) as the vinblastine, taxol and colchicine binding sites. BZs have been classified with the colchicine-site binding drugs because they inhibit the binding of [^{3}H]colchicine.

(i) The vinblastine-binding site inhibitors

Vinblastine is a clinically used anticancer agent originally isolated from the periwinkle (Vinca rosa). It is the prototype of the class of indoles known as vinca alkaloids incorporating both natural and semi-synthetic derivatives (Zavala et al., 1978). Examples of vinblastine-binding site ligands include vincristine, maytansine, phomopsin A and rhizoxin (Dustin, 1984; Lacey, 1988). Vinblastine and other vinca alkaloids inhibit tubulin polymerisation in vitro. Vinblastine binding to tubulin has been established (see Luduena et al., 1986) but the association constants recorded are highly variable ranging from 0.16 to 45 μM^{-1}. Despite the evident importance of this site in tubulin activity, evidence of its existence outside mammals or echinoderms (sea-urchin eggs) is limited (Luduena et al., 1986). There are no published reports of ligands which are active at the vinblastine-site on helminth tubulin.

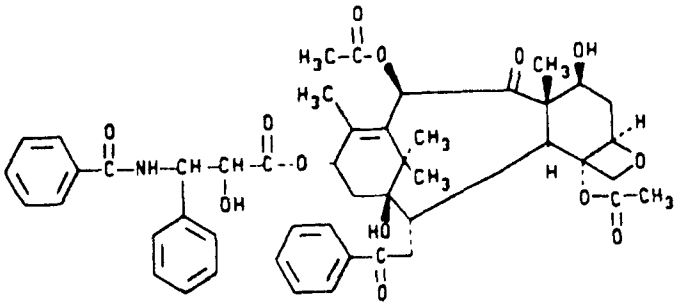
(ii) The taxol binding-site inhibitors

Taxol is a natural product isolated from yew tree, Taxus brevifolia (see Horwitz et al., 1982). Taxol's action as a microtubule inhibitor is unique in that it is achieved by inducing tubulin to polymerize, increasing both the rate and yield of microtubules in the polymerization-competent and incompetent tubulin and effectively reducing the critical

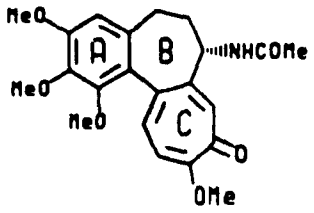


Vinblastine

R = CH_3



Taxol



Colchicine

Fig. 1.3. Structure of the prototype microtubule inhibitors. There are three pharmacologically distinct classes of microtubule inhibitors, each with a selective site on mammalian tubulin denoted according to the prototype inhibitor as the vinblastine, taxol and colchicine binding sites. Because they can inhibit [^{3}H]colchicine binding, BZs have been classified with colchicine binding-site drugs.

concentration of tubulin for assembly by greater than 20-fold. This action renders possible the isolation of microtubules from sources that were previously impossible (Vallee, 1986; Tang and Prichard, 1988, 1989). [^{3}H]taxol seems to bind exclusively to microtubules at a site distinct from those of vinblastine, colchicine, Ca^{2+} and GTP (Parness and Horwitz, 1981). Compounds, other than taxol itself, which interact with this site have not been reported.

(iii) The colchicine-binding site inhibitors

Competitive inhibition studies suggest that three main groups of compounds interact with this site: colchicine, podophyllotoxin and BZs.

a) COLCHICINE (CLC)

Colchicine (CLC) is a tricyclic acetylated alkaloid originally isolated from the meadow saffron (Colchicum automnale). CLC (Fig. 1.3) slowly binds to mammalian brain tubulin with an equilibrium time > 4 h, in a highly temperature dependent (optimum 37° C) manner with virtually no binding at 4° C. The CLC-tubulin association is not readily dissociable and is referred to as 'pseudo-irreversible'. However, the complex dissociates readily upon tubulin denaturation by solvent extraction, heat or detergent and is therefore non-covalent. The CLC-binding site on tubulin is unstable and decays with first order kinetics. The mechanism of this decay has not been elucidated. Fluorescence studies (see Dustin, 1984; Lacey, 1988) suggest that conformational changes occur both to CLC and tubulin upon interaction. The CLC-tubulin complex formation is associated with changes in the circular dichroism spectrum, accessibility of altered protease sites, reactivity of cysteinyl residues, GTPase activity, antibody

recognition and electron paramagnetic resonance spectrum which, together with fluorescence studies, support the occurrence of conformational changes both to CLC and tubulin upon interaction. The kinetics of complex formation may involve a minimum of two-steps:

$$\text{Tubulin} + \text{CLC} \underset{\text{fast}}{\rightleftharpoons} (\text{Tubulin-CLC}) \xrightarrow[\text{slow}]{} (\text{Tubulin-CLC})^*$$

The first step is rapid leading to the formation of a reversible complex which undergoes a slow CLC-induced conformational change in tubulin to give a final complex. This final step is thought to be responsible for the observed pseudo-irreversibility of CLC binding (Garland, 1978).

b) PODOPHYLLOTOXIN (PDT)

Podophyllotoxin (PDT), a naturally occurring lignan isolated from the May Apple (Podophyllum peltatum), inhibits tubulin polymerisation and [^{3}H]colchicine binding. However, the [^{3}H]PDT-tubulin interaction is quite different in that it is freely reversible, equilibrates rapidly (< 30 min) and can occur at 4° C (Cortese et al., 1977). There are a number of PDT analogues that induce similar changes as PDT itself (see Dustin, 1984; Lacey, 1988).

c) BENZIMIDAZOLES (BZS)

The involvement of BZs in microtubule inhibition was first indicated by studies of the mode of action of the prodrug benomyl and its active principle, carbenazin (methyl-benzimidazole carbamate, MBC), in fungi (Clemons and Sisler, 1971). This was supported by cytological observations

and analogies to other well known microtubule inhibitors (Hammerschlag and Sisler, 1973; Davidse, 1973). Similar cytological studies on mammalian cell lines and in rats (Styles and Garner, 1974) and the nematode Ascaris suum (Borgers and De Nollin, 1975) confirmed the anti-mitotic and microtubule inhibitory actions. However, the first in vitro potent inhibition of tubulin polymerization by BZs was reported for nocadozole (NDZ) using purified rat brain tubulin (Hoebeke et al., 1976). In vitro inhibition of purified bovine-tubulin polymerization by thiabendazole and cambendazole were later reported (Friedman and Platzer, 1978; Ireland et al., 1979; Lacey and Watson, 1985). Oxibendazole and fenbendazole were found to be competitive inhibitors of [^{3}H]CLC binding (Friedman and Platzer, 1978) while mebendazole (Laclette et al., 1980) was also found to inhibit bovine-tubulin polymerization.

Studies with NDZ-tubulin interaction, by both equilibrium and non-equilibrium methods, indicate that the binding is a two-step reaction, analogous to that of CLC, with an initial fast step followed by a slow phase (Head et al., 1985):

$$\text{NDZ} + \text{Tubulin} \underset{\text{fast}}{\rightleftharpoons} (\text{NDZ.tubulin}) \underset{\text{slow}}{\rightleftharpoons} (\text{NDZ.tubulin})^*$$

Unlike CLC, however, this reaction was freely reversible.

(d) OTHER CLC-BINDING SITE INHIBITORS

Other possible groups of CLC-site inhibitors, such as griseofulvin, are discussed elsewhere (Lacey, 1988).

1.5 BENZIMIDAZOLE ANTHELMINTIC RESISTANCE

1.5.1 Introduction

BZs have served humans well in the continuing battle to control nematode parasites in livestock (Martin, 1985). However, the use of these drugs has been seriously undermined in various countries, particulary in the southern hemisphere, by the development of resistance in the target parasite species (Waller, 1987). BZ-resistance is just one aspect of a larger problem of anthelmintic resistance. Anthelmintic resistance encompasses other broad spectrum anthelmintics such as the levamisole/morantel group and the recently released ivermectin (Waller, 1987, 1990).

Anthelmintic resistance was defined (Prichard et al., 1980) as the presence of a heritable greater frequency of worms within a population able to tolerate recommended doses of a drug than in a normal population of the same species. Therefore, resistance is not an absolute phenomenon but the LD_{50} or LD_{90} value (dose required to kill 50% or 90%, respectively, of the worms) is higher in the resistant population than in the susceptible population. Probably, resistance reflects an increase in the proportion of resistant individuals in a population, rather than a reduced susceptibility by every member in the population.

It is well known that strains resistant to one BZ drug may automatically become resistant to other BZs (Berger, 1976; Colglazier et al., 1975; Waller and Prichard, 1986; Lacey, 1988). Prichard et al. (1980) described this situation, whereby a resistant strain selected by one drug becomes resistant to other drugs of the same mode of action, as side resistance. If drugs of different modes of action become involved the phenomenon is described as cross resistance. The term multiple resistance

embraces both side resistance and cross resistance. Reversion is the loss of resistance following removal of the selecting drug. These situations, when they arise, may have important implications regarding the development or mechanism of drug resistance.

1.5.2 Extent of BZ-resistance

The extent of anthelmintic-resistance, including BZ-resistance, has been previously reviewed by Prichard et al., 1980; Coles, 1986; Waller and Prichard, 1986; Waller, 1987, 1990 and Prichard, 1990. Drudge et al. (1957) were the first to report anthelmintic resistance and this was by H. contortus to phenothiazine. H. contortus developed resistance to TBZ (Drudge et al., 1964), the first BZ on the market (Brown et al., 1961), three years after it was introduced. Since then, BZ-resistance has developed slowly but steadily in nematodes of sheep, goats and horses (Prichard et al., 1980). Anthelmintic resistance may not have developed or is still insignificant in nematodes infecting cattle and humans or in cestodes or trematodes (Donald, 1983; Prichard, 1990). Today anthelmintic resistance involves all broad spectrum anthelmintics and has been demonstrated for some narrow spectrum anthelmintics as well (Van Wyk and Gerber, 1980; Van Wyk and Malaan, 1988). Nematocide resistance has been widely reported in Australia, New Zealand, U.S.A., Canada, U.K., Switzerland, France, Brazil and South Africa (Waller and Prichard, 1986). There is evidence that the list of countries is becoming longer and longer (Njanja et al., 1987; Van Wyk et al., 1989; Ngomuo et al., 1990; Waller, 1990). The relative differences in frequencies of reports of drug resistance in nematodes between countries or regions or host and parasite species may be attributed to differences in the relative importance

(impact on health and productivity) of the parasite species, differences in management or anthelmintic use and, most likely, the extent to which drug resistance has been investigated (Waller and Prichard, 1986). BZ-resistance in sheep and goat parasites, H. contortus, Ostertagia circumcincta and Trichostrongylus colubriformis, has been found in many parts of Australasia, Africa, Europe, North and South America and Asia. In virtually all areas where sheep or goats are treated with BZ anthelmintics and adequate investigations have been made, resistance has been found on some, but not all farms (see Prichard, 1990 for review). BZ-resistance has also been found in Nematodirus spathiger in New Zealand (Middleberg and McKenna, 1983), in Cooperia curticei in The Netherlands (Borgsteede, 1986) and in Oesophagostomum spp. in sheep in Australia (Edwards et al., 1986). OFZ-resistance has been reported in Trichostrongylus axei in cattle in Australia (Eagleson and Bowie, 1986); however, the development of BZ-resistance has not been a concern for parasite control in cattle so far. In the horse, anthelmintic resistance is apparently confined to Cyathostoma spp. (Kelly et al., 1981; Webster et al., 1981) but has been reported world wide (see Prichard, 1990).

1.5.3 Development of resistant strains

The process by which anthelmintic resistance develops has been addressed by several workers (see Le Jambre, 1985; Prichard, 1990). The process may be likened to the development of insecticide resistance (Donald, 1983). It is thought that the genes expressing resistance are normally present in a population at a low frequency but their frequency increases since the survivors of treatment (the resistant worms) make a greater contribution to succeeding generations. The intensity of selection

pressure depends on the frequency of treatment, the dose rate and the infection rate (Prichard et al., 1980). Theoretically, susceptibility returns if the selection pressure is removed but in anthelmintic resistance reversion is slow and reselection is rapid (Kelly and Hall, 1979). There has been no agreeable theory regarding the inheritance of BZ-resistance. Le Jambre et al. (1979) reported that thiabendazole resistance in H. contortus was not sex linked but semi-dominant and probably involved more than one gene. Herlich et al. (1981) found that BZ-resistance in H. contortus was not sex linked but recessive and multi-genic. BZ-resistance in T. colubriformis was reported to be controlled by more than one gene and semi-dominant with some maternal influence (Martin et al., 1988).

Unlike insecticide resistance, which usually occurs rapidly to involve entire industries or regions, anthelmintic resistance is strikingly patchy in distribution. The reasons for this difference may relate to the relative immobility of nematodes and the fact that selection in nematodes is restricted only to the parasitic stages by non-persistent and highly efficacious drugs (Waller, 1985). Most reports of BZ-resistance come from those regions of the world where the highly pathogenic, H. contortus, is endemic and where sheep and goats graze all year round on permanent pastures (Waller, 1985). Where there is little diversification of farming enterprise and limited opportunity for pasture spelling, alternate grazing or provision of new leys, there is a tendency by farmers to rely more on frequent anthelmintic treatment. And because the BZs were the first in the new generation of broad-spectrum anthelmintics, they were often used to the exclusion of all other classes of drugs. When flock sizes tend to be larger, the value of individual animals tend to be less and the risk of owners under-dosing their animals increases. Under-dosing

may increase the chances for survival of moderately resistant worms. Theoretically, however, over-dosing might select strongly for resistance. Although frequent treatment, under-dosing and failure to alternate with other drug classes are most commonly associated with selection of BZ-resistance (Donald, 1983; Waller, 1987), there are other factors. For example, frequent dosing during an episode of clinical parasitism, when larval availability on pasture is enormous, may not be as strong a selector for resistance as fewer doses at a time when most parasites are within the host (Waller, 1990). The extent to which the host regulates the survivors of anthelmintic treatment varies with parasite species and with host age, nutritional status and prior exposure to infection (Waller, 1990). Therefore, depending on the host regulation, the survivors of anthelmintic treatment may make a very limited or a very profound contribution to the evolution of resistance. The impact of new technology, such as controlled release devices, on selection of BZ-resistance needs investigation.

1.5.4 Significance of BZ-resistance

BZ-resistance is the most widespread of all anthelmintic resistances (Prichard et al., 1980; Waller, 1987). BZ-resistance is most serious in regions of the world where the most pathogenic nematode, H. contortus, is endemic. Although widespread occurrence of BZ-resistance has been reported in the other important parasites of sheep, Ostertagia and Trichostrongylus spp., BZ-resistance by H. contortus causes most concern (Waller, 1990).

The degree of economic loss from anthelmintic resistance is not known. Moreover, the total economic impact of parasitic disease is impossible to quantify accurately. The losses in animal production by

clinical and subclinical parasitism are only one part in the whole economic context. One important part of the economic cost is the investment in control measures. And anthelmintic treatment is likely to continue as the first and most important line of defence against worm parasites (Martin, 1985). Hundreds of millions of dollars are spent annually in an effort to reduce effects of parasitism (reviewed by McKellar and Scott, 1990). Naturally, anthelmintic resistance will enormously increase the cost of management of parasitic worms.

In addition, there is evidence, although still controversial, that resistant nematodes can be more pathogenic, have increased establishment rates in the host and increased survival rates of free living stages (Kelly et al., 1978). However, other workers have found contradictory evidence (Maingi et al., 1990; MacLean et al., 1987). Experience with insecticide resistance suggests that the problem is not only likely to persist but may escalate unless effective counter-measures are undertaken. Anthelmintic resistance has previously been dubbed as a problem of the grazing industries of Australia, Africa and South America, an exception being the horse industry where BZ-resistance in small strongyles has been reported world-wide (Bauer et al., 1986). Waller (1990) argued that there is no grounds for complacency in regions or countries where it is not considered a problem, because in common with high levels of drug resistance in many other invertebrate pests, most of the important nematode genera of domestic livestock have shown that they posses the genetic capacity to develop resistance. Therefore, it is not a question of working for solutions if resistance occurs but of having the answers when it occurs and ideally, acting on them before hand to prevent development of resistance. The remedies discussed by Donald (1985) include the short

term ones such as introducing new anthelmintics and extending the life of the existing ones and the long term ones such as development of vaccines or sustained helminth control systems. The long term solutions are more sound but very far from being achieved. To be able to develop alternative drugs or adopt sustained practices and substantiate claims of drug efficacy against resistant strains, one needs accurate information on the biochemical aspects, selection and extent of anthelmintic resistance (Donald, 1985). Accurate early detection of anthelmintic resistance is also necessary.

1.5.5 Detection of BZ-resistance

Accurate diagnosis is important because if the resistance is not detected early, continued administration of an anthelmintic from the same group can increase the problem until ultimately there is an irreversible failure of control by the entire group (Waller, 1985). Also, lack of control may be inaccurately attributed to resistance when it is due to other causes (Waller and Prichard, 1986).

The methods which have been used (Waller and Prichard, 1986) to diagnose BZ-resistance include the indirect ones, such as the faecal egg-count depression test and the *in vitro* egg hatch assay and the direct ones, such as the slaughter and worm count method. The *in vitro* egg hatch assay (Coles and Simpkin, 1977) is the fastest and most efficient but it assumes that the resistance mechanism in developing eggs is identical to that in the adults and that the drug metabolism in the two systems is identical.

1.6 MECHANISM OF DRUG RESISTANCE

1.6.1 Basic mechanisms of drug resistance

Studies, particularly with antibiotics and insecticides, have led to the recognition of several basic mechanisms by which organisms can become resistant to drugs:

(i) Decreased rate of uptake or increased rate of excretion. The drug permeability across the limiting membrane becomes altered so that the drug no longer enters the pathogen or the excretion rate is increased so that the drug leaves the pathogen very quickly before exerting any effect against the pathogen. Chloroquine resistance in Plasmodium bergei may be due to a decreased amount, affinity or accessibility of chloroquine on the constituent of the parasite (Fitch, 1969). Trypanosoma brucei resistant to arsenicals lack a carrier-mediated mechanism for the uptake of melarsen oxide (Ojeda and Flynn, 1982). Permeability of resistant strains of Sporobolomyces roseus to carbenazim (MBC) is less than in a susceptible strain (Nachmias and Barash, 1976). Coles (1977) has suggested that decreased drug uptake by eggs of thiabendazole resistant worms may confer resistance, but Rew et al. (1982) and Sangster and Prichard (1984) have suggested that differential uptake is not a mechanism of resistance in nematodes.

(ii) Drug metabolism. The rate of drug inactivation or activation can become altered in the resistant organisms. This mechanism is well recognised in penicillin resistant bacteria or DDT resistant insects. Penicillin-resistant bacteria or DDT-resistant insects secrete great quantities of the enzymes, penicillinase and DDTase, respectively that

quickly detoxify the drug. Elucidation of this mechanism of penicillin-resistance has led to the discovery and marketing of specific penicillinase-inhibitors which have gone a long way towards extending the life of penicillin on the market. Kawalek et al. (1984) reported that a cambendazole-resistant H. contortus had greater activity of glutathione-S-transferase (a drug metabolising enzyme) than a susceptible strain. However, Sangster and Prichard (1986) found no evidence for the involvement of drug metabolism in the mechanism of BZ-resistance.

(iii) Alternative metabolic pathway. A resistant organism may use an alternative metabolic pathway which by-passes the action of the drug. When the fumarate reductase was considered as the primary mode of action of BZs, Bennet (1981) reported that a thiabendazole-resistant strain of H. contortus relied less on glycogen reserves, took up less glucose and produced more CO_2 than a susceptible strain. These results are consistent with increased use of the TCA cycle and thus reduced dependence on fumarate reductase. However, the inhibition of fumarate reductase is now considered as secondary to microtubule inhibition by BZs rather than a primary mechanism of BZ action (Lacey, 1988).

(iv) An increased importance of a stage in the life cycle which is less susceptible to the drug. An increase in the incidence of hypobiosis has been observed in Ostertagia circumcincta under selection by levamisole (Le Jambre et al., 1978). There is no evidence of this mechanism in H. contortus.

(v) Altered receptor. The drug-receptor may become altered so that the

affinity of the drug for its specific target is reduced or abolished altogether. Pyrimethamine-resistance in P. berghei is associated with an increased production of a dihydrofolate reductase enzyme with decreased affinity for the drug (Ferone, 1970). A knowledge of this mechanism of resistance has allowed the synergistic combination of a sulphone (an analogue of para-aminobenzoic acid) with pyrimethamine, which is highly effective against field cases of pyrimethamine resistant Plasmodium falciparum. The drug-receptor may become altered by reduction in the number of binding sites. Levamisole-resistance, which has been induced in the free-living nematode, C. elegans, may be associated with fewer acetylcholine binding sites at which levamisole can act (Lewis et al., 1980). Alternatively the target receptor can be altered by increasing its quantity so that the pathogen can survive normal drug doses but may be susceptible to elevated doses. Evidence, reviewed below, has consistently pointed to an altered site of action (tubulin) as the possible mechanism of BZ-resistance. In this thesis, the role of this basic mechanism in BZ-resistance by H. contortus will be explored.

1.6.2 Mode of action of BZs and mechanism of BZ-resistance

Whereas the mode of action of BZs has been hypothesized to be tubulin binding, the basis of selectivity of BZs against parasites and the molecular mechanism of BZ-resistance have not been established.

Studies on the mode of action of BZs as antifungal and anthelmintic agents have demonstrated an inhibitory effect on fumarate reductase (Allen and Gottlieb, 1970; Malkin and Camacho, 1972; Prichard, 1973; Romanowski et al., 1975), glucose uptake (Van den Bossche, 1976, 1985), protein secretion (Watts et al., 1982) and other ill-defined effects (Rew

et al., 1982; Van den Bossche, 1985). Of all these apparently unrelated suggested modes of BZ action, inhibition of fumarate reductase attracted attention first. These early studies demonstrated that mitochondrial electron transport, especially the fumarate system, was inhibited by a number of BZs at concentrations between 10 and 100 μM in fungi and in BZ-susceptible but not resistant nematodes (Allen and Gottlieb, 1970; Malkin and Camacho, 1972). However, it was observed later on that when mebendazole was fed to pigs and turkeys infected with Ascaris suum and Syngamus trachea, respectively, it caused a loss of microtubules in intestinal cells of these nematodes (Borgers et al., 1975). Microtubules disappeared from BZ-susceptible but not from BZ-resistant worms treated with BZs (Sangster et al., 1985). Some, but not all, BZs inhibit colchicine binding or the in vitro polymerization of mammalian brain tubulin (Friedman and Platzer, 1978; Ireland et al., 1979; Laclette et al., 1980; Köhler and Bachmann, 1981). Subsequently, Friedman and Platzer (1980) demonstrated that colchicine binding by A. suum embryo preparations was inhibited by MBZ and FBZ, with apparent inhibition constants 250-400 times lower than those for bovine brain tubulin, and they postulated that tubulin was the primary target of BZs in helminths. The ratio of the apparent inhibition constants between mammalian and helminth tubulin could then easily account for selective toxicity against parasites. In contrast, the ratio of the apparent inhibition constants for a preparation from A. suum intestine and porcine brain tubulin for mebendazole was only 2 (Köhler and Bachmann, 1981). These workers suggested that the selective toxicity be explained by the pharmacokinetic behaviour of the drug in the host. Other studies have shown [^{3}H]mebendazole (Kohler and Bachmann, 1981; Tang and Prichard, 1989) and [^{3}H]parbendazole (Ireland et al., 1982) to

bind tubulin enriched preparations. In addition, BZs inhibit acetycholinesterase secretion by Nippostrongylus brasiliensis (Watts et al., 1982), a process which is thought to be microtubule dependent and important for allowing the worm to maintain itself in the host's intestine (Rapson et al., 1981). Inhibition of acetylcholinesterase secretion has been linked to BZ-resistance in Trichostrongylus colubriformis (Sangster et al., 1985). In the strains of the fungus Aspergillus nidulans, BZ-resistance correlates inversely with the affinity of [^{14}C]methylbenzimidazole carbamate for tubulin (Davidse and Flach, 1977). Homogenates of BZ-resistant worms bind less [^{3}H]BZ than those of BZ-susceptible worms (Sangster et al., 1985; Lacey and Prichard, 1986; Lacey et al., 1987b). Resistance to parbendazole by a fungus, Physarum polycephalum was associated with an alteration of a β-tubulin isoform compared with the wild type (Foster et al., 1987). BZ-resistance in a free-living nematode, C. elegans, has been ascribed to deletion of a β-tubulin gene (Driscoll et al., 1989). Other studies also suggest that differences in β-tubulin genes may be responsible for resistance to BZs (Sheir-Neiss et al., 1978; Thomas et al., 1985; Orbach et al., 1986; Roos et al., 1990).

1.7 RESEARCH OBJECTIVES

The literature cited above suggests that: (a) anthelmintic treatment is the major method of worm control in animals, (b) anthelmintic resistance can be expected to become more wide-spread, (c) BZ-resistance is the biggest problem of anthelmintic-resistance, (d) BZ-resistance is most widely reported in H. contortus in sheep and goats, (e) H. contortus

is the most pathogenic nematode of sheep, (f) there has been extensive extrapolation from studies on mammalian tubulin to explain the behaviour of nematode tubulin, (g) the mode of action of BZs needs further substantiating especially because the basis of selective toxicity is not explained and the molecular basis of differences in anthelmintic efficacy among the BZs is unknown, (h) BZ-resistance has been studied in fungi and the free-living nematode C. elegans more than parasitic nematodes but this information may not be adequate to explain BZ-resistance in helminth parasites whose epidemiology is different and (i) the mechanism of BZ-resistance in helminth parasites has not been explained precisely in terms of the altered properties of tubulin, the hypothesized target of BZs.

We need to know the biochemical mechanism of BZ-resistance in H. contortus because knowledge of the mechanism will help define and monitor resistant strains and allow assessment as to whether tubulin binding is the sole mechanism of drug action by all BZs. It may assist in making decisions on which drugs to use in the field and whether new BZ drugs will be effective against resistant strains. Biochemical characteristics may also be helpful markers for genetic research. Most importantly, if the mechanism of resistance is understood, other drugs or synergists may be designed to improve worm control. Elucidation of the mechanism of resistance to penicillin and to pyrimethamine, for example, led to the discovery and marketing of counteracting drugs. The work presented in this thesis was based on the assumption that BZs act by binding tubulin. My study has, therefore, focused on tubulin. Unlike previous work, in this study BZ binding to parasite homogenates is separated into high-affinity and low-affinity binding as a basis for determining pharmacological specificity.

The objectives of this study were as follows:

(i) To compare the specific binding of the S and R strains

Although reduced total BZ binding to whole parasite homogenates of BZ-resistant H. contortus was previously reported, the binding had not been separated into specific and nonspecific binding to determine whether one or both of these two components were reduced. Ligand binding is never 100% specific. Specificity is based on the relative binding affinity of the ligand to one or other receptor. The ligand should have greatest affinity for the pharmacologically relevant receptor. Specificity is concentration dependent and will reduce as the latter increases. A method was devised which would separate specific binding from non-specific binding over a wide range of ligand concentrations.

(ii) To compare the total tubulin content in S and R preparations

As already discussed in this chapter, an organism can become resistant by reducing its dependency on the molecule which is a target of the drug. Alternatively, the resistant organism can have elevated amounts of the target molecule so that normal doses of the drug cannot inhibit all the molecules. The tubulin content in S and R strains were compared in order to determine whether tubulin content was altered in the resistant strain.

(iii) To compare the tubulin isoforms of the S and R strains

Evidence from the literature that BZ-resistance in fungi or C. elegans may be due to an alteration of the tubulin genes or protein was reviewed above. Alteration of this type should be evident in tubulin

isoform patterns on two-dimensional SDS-PAGE gels.

(iv) To compare developing stages

All the stages in the nematode life cycle are sensitive to BZs (Samizadeth-Yazd and Todd, 1978) and BZ-resistance seems to affect all stages (Donald, 1983). At the same time, the sensitivity to BZs *in vitro* seems to decline with development from the egg to the adult stage (Kirsch and Schleich, 1982). I was interested in determining whether some or all of these observations could be explained in terms of tubulin, the putative target of BZ anthelmintic action. This would provide further evidence for tubulin being a target of BZ anthelmintic action. Tubulin is ubiquitous to all stages and tissues, although different stages or tissues may differ in tubulin isotypes they contain. It is not known whether the sequences responsible for BZ binding are common to all tubulin isotypes.

(v) To compare BZ drugs in terms of binding affinity

The overall anthelmintic efficacy of a drug may be determined primarily by the binding affinity for the target receptor or may be modulated by the pharmacokinetic behaviour of the drug. If all BZs have a common primary mode of action, their anthelmintic efficacy should be proportional to the binding affinity plus or minus any difference in pharmacokinetic behaviour.

CHAPTER II

GENERAL MATERIALS AND METHODS

2.1 PARASITES

Thiabendazole-susceptible (S) and thiabendazole-resistant (R) strains of H. contortus were provided by Dr. R. S. Rew (formerly at Smith Kline Beecham Animal Health, Applebrooke, PA). The S strain used in this thesis was isolated in 1962 at Beltsville Parasitological laboratory, USDA, Maryland, and had not been exposed to any BZ drug before (Colglazier et al., 1970). Since then the S strain has been maintained by passage in sheep (Dr. R. S. Rew, personal communications) and was found to be fully susceptible to camben lazole (CBZ) (Kates et al., 1973; Colglazier et al., 1975). The R strain used in this thesis was isolated from a flock of sheep in upper New York state (USA) following lack of response to TBZ (Dr. R. S. Rew, personal communications). After isolation in the mid-70's the R strain had been maintained by passage in sheep without exposure to any drug. I performed an egg hatch assay as described previously (Lacey et al., 1987a), to verify the status of the S and R strains. The effective concentration of TBZ causing 50% egg hatch inhibition (EC_{50}) was 3.5 μM for the R strain and 0.8 μM for the S strain. The resistance factor (RF) defined (Lacey and Prichard, 1986) as the ratio of the EC_{50} of the R strain to that of the S strain was 4.4. Similar egg hatch assays confirmed that the R strain was resistant to TBZ while the S strain was susceptible (Maingi et al., 1990).

2.2 ANIMALS

The parasites were maintained in penned worm-free cross-bred sheep at Macdonald college farm. The sheep were fed with high quality hay ad libitum and a grain and mineral concentrate (0.5 kg/sheep/day) throughout the experiment. They were also given a coccidiostat, decoquinate (Decox*, Mayer and Baker, Canada), pre-mixed in feed (dose rate = 0.1 mg/kg body weight/day) throughout the experiment. The sheep (6-12 months old) were were treated with ivermectin one month before infection. They were then infected orally with 10-15 thousand third stage larvae of either strain. The sheep infected with different strains were effectively isolated from each other and cross-infection was prevented by a thorough cleaning of the pens every 7 days.

2.3 COLLECTION OF PARASITES

2.3.1 Unembryonated eggs

Unembryonated eggs were recovered between 1-3 months post-infection from faeces by a modification of the flotation, sedimentation and centrifugation procedure of Weston et al. (1984). Canvas bags were tied over the anus of infected sheep and faeces obtained from these bags every 6 h and transferred to a refrigerator. About 10 kg of faeces were blended with 10 volumes of water at 4°C and the resultant faecal slurry sieved sequentially through metal meshes of apertures 2, 1, 0.4, 0.15 and 0.09 mm, discarding the residue at each step. The 0.09 mm filtrate was left to stand for 2-3 h to sediment the eggs. The supernatant was then decanted off and the sediment was poured onto a 38 μm metal sieve to remove the excess water. Eggs were retained in the residue on the 38 μm sieve. The residue was then dispersed in cold sucrose solution (specific gravity 1.2)

in a large metal dish (30 x 15 x 6 cm) to allow the eggs to float. A fitted plastic plate with an upright handle was then floated on the sucrose solution to allow the eggs to adhere onto it. After 15 min the eggs were washed off the plastic plate using a fine spray of water. The flotation procedure was repeated many times until no more eggs could be seen in the harvest upon microscopic examination. The harvested eggs were diluted with water to lower the specific gravity to 1 so that they could be sedimented by centrifugation (700g, 2 min). The eggs were then re-suspended in sucrose solution and re-centrifuged (3000 g, 10 min) so that they could float at top of the solution from where they could be recovered with a spatula. The recovered eggs were again diluted with water and the whole process of sedimentation and flotation by centrifugation repeated until the eggs were completely free of faecal debris, as judged by microscopic examination. All operations were conducted at 4°C. The clean concentrated eggs were stored in liquid N_2 or briefly on ice before use.

2.3.2 Infective third stage larvae (L_3)

Infective third stage larvae (L_3) were recovered by modification of the procedure described in the Manual of Veterinary Parasitological Techniques (Ministry of Agriculture Fisheries and Food, London, 1986). Faecal pellets obtained as described in the preceding section were placed, together with several petri-dishes half-filled with water, into covered plastic containers (20 x 10 x 5 cm) and kept at 27° C for seven days. The larvae migrated into and onto the petri-dishes from where they were recovered using a fine spray of water. More larvae were recovered by carefully washing them off the sides and top of the plastic containers. To further clean and separate the L_3 from free-living nematodes, the recovered

larval suspension was poured onto a large filter paper (Whatman no. 1) and blotted completely dry using paper-towels. Free-living nematodes died of dehydration within 12 h and the L_3 were recovered after placing the dry filter paper face down onto a Baermann funnel. Dead larvae and faecal debris remained adhering to the filter paper, while the clean and motile H. contortus larvae migrated to the base of the funnel from where they were recovered 30 min later. The motile and infective L_3 could be stored, without deterioration, in tap water at 10° C for up to 6 months.

2.3.3 Adult worms

Sheep were killed by captive bolt 2-3 months post-infection. The adult parasites were quickly recovered from the abomasal mucosa with forceps and bathed in bicarbonate saline (0.8% NaCl, 0.15% $NaHCO_3$) at 37°C. The parasites were homogenized or transferred to liquid N_2 while still alive.

2.4 CHEMICALS AND SOLUTIONS

Pure samples of unlabelled BZs were gifts from the indicated suppliers: fenbendazole (FBZ)-Hoechst, AG, Frankfurt am Main, W. Germany; mebendazole (MBZ)-Janssen Pharmaceutica, Mississauga, ON; oxfendazole (OFZ)-Syntex Inc., Palo Alto, CA; thiabendazole (TBZ)-Merck Frosst, Kirkland, QC.; oxibendazole (OBZ), albendazole (ABZ), rycobendazole (albendazole sulphoxide, ABZSO) and albendazole sulphone ($ABZSO_2$)-Smith Kline Animal Health, Philadelphia, PA).

Tritiated BZs, [^{3}H]OBZ, [^{3}H]MBZ, [^{3}H]OFZ and [^{3}H]ABZ were donated by Dr. E. Lacey (CSIRO, Australia). All other chemicals were reagent grade. MES (2[N-morpholino]ethanesulphonic acid) buffer (pH. 6.5) consisting of

0.025 M MES, 1 mM EGTA (ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid), 0.5 mM $MgSO_4$ and 2 mM GTP (guanosine-5'-triphosphate) was routinely used in preparation of parasite extracts and drug solutions.

Stock concentrations of [^{3}H]BZs were prepared in 20% (v/v) dimethyl sulphoxide (DMSO) in MES buffer. The concentrations (in μM or picomoles (pmol)) of [^{3}H]BZs were determined by high performance liquid chromatography (HPLC). The disintegrations per minute (dpm) in 10 μl of the stock concentrations were determined on a beta counter (Rack, beta, LKB) and the specific activities (dpm/pmol) calculated. The specific activities were routinely determined every 30 days to ensure that their values did not significantly change during storage Stock concentrations of unlabelled BZs were prepared in pure DMSO. Stock concentrations used are stated in the appropriate Materials and Methods sections but calculated final concentrations are reported in Results and in the Table/Figure legends throughout the thesis.

2.5 PREPARATION OF WHOLE PARASITE EXTRACTS (SUPERNATANTS)

Eggs, L_3 or adult worms were washed several times with cold distilled water and cold MES buffer and homogenised on ice with a polytron cell disrupter (PT 10, OD, Kinematica GMBH Luzernschweiz) in MES buffer (1 ml/200 mg parasites) until seen by microscopic examination to be disrupted completely. Eggs , L_3 and adult worms required 2 x 5, 2 x 10 and 0.03 min homogenisation time respectively. The homogenate was centrifuged (100, 000 g, 4° C, 1 h) to produce a clear supernatant that was used in binding and electrophoretic studies. The supernatant was also used for tubulin purification by poly-L-lysine (PLL) chromatography (Lacey and Snowdon,

1990) or taxol-induced polymerisation method (Vallee, 1986; Collins and Vallee, 1987). The PLL-purified tubulin was used in binding and electrophoretic studies. The taxol-purified tubulin was used in electrophoretic and ELISA studies.

2.6 DETERMINATION OF PROTEIN CONCENTRATIONS

Protein concentrations of various preparations were determined by the Coomassie blue method (Bradford, 1976) using bovine serum albumin (BSA, Biorad) as standard.

2.7 BINDING ASSAYS

2.7.1 Principal and general procedure.

The binding assay was a modification of that described previously (Sherline et al., 1974; Lacey and Prichard, 1986). For this thesis, [^{3}H]BZ binding of parasite supernatants (total binding (TB)) was separated into high-affinity binding (HAB) and low-affinity binding (LAB). Two μl of pure DMSO or 2 μl of 500 μM unlabelled BZ were preincubated in 2 or 3 replicates with 90 μl of either buffer (blank) or S or R parasite supernatants for 30 min at 37°C using 1.5-ml epperndoff tubes in a shaking water bath. Then 10 μl of [^{3}H]BZ (0.156-20 μM) were added to each tube and incubated for a further 30 min. It was assumed that the high concentration of the unlabelled BZ blocked all the high-affinity receptors for BZ. Consequently, in tubes containing the unlabelled drug, the [^{3}H]BZ bound to the low-affinity receptors while in the tubes lacking the unlabelled BZ both LAB and HAB of the [^{3}H]BZ occurred. The assay was terminated by addition of 0.5 ml charcoal suspension to make a final volume of 0.6 ml. The 1% (w/v) animal charcoal (Fisher) suspension containing 1% (w/v) BSA

in MES buffer was routinely prepared at the beginning of the experiment and stirred constantly during use. The unbound drug was adsorbed onto the charcoal (Sherline et al., 1974) during a further 5 min incubation at 37°C. The charcoal (and the charcoal-adsorbed drug) was sedimented by centrifugation at 13,000g for 5 min at room temperature and the bound drug (dpm) in 0.45 ml of the supernatant quantified by scintillation counting on a beta-counter in 5 ml PCS (Amersham) diluted 2 to 1 with toluene (Fisher).

The raw data, corrected to dpm, were mathematically analyzed using computer programs (McPherson, 1987; Unnerstall, 1990) to obtain binding constants (see below) or were transformed into pmol/mg protein, for graphical presentation, as follows:

$$\text{TB (pmol/mg)} = \frac{dpm_{ls} - dpm_{lb}}{S.A. \times [\text{protein}]} \times 1.3$$

$$\text{LAB (pmol/mg)} = \frac{dpm_{us} - dpm_{ub}}{S.A. \times [\text{protein}]} \times 1.3$$

where: dpm_{ls} and dpm_{lb} = dpm of sample and blank, respectively, in the presence of the labelled ligand only; dpm_{us} and dpm_{ub} = dpm of sample and blank, respectively, in presence of the labelled and unlabelled ligands; S.A. = specific activity (dpm/pmol) of the labelled ligand and 1.3 is the correction factor for the volume counted (0.45 ml out of 0.6 ml were actually counted).

During the computer analysis or manual transformation, HAB was derived as the difference between TB and LAB. The procedure outlined above was appropriately modified as described for each specific study.

2.7.2 The saturation assay

For the saturation assay, the procedure was similar to the general one outlined above except that the concentration of the [^{3}H]BZ was varied while the protein and the unlabelled BZ concentrations remained fixed. The labelled and unlabelled ligands were homologous BZs. The purpose of the saturation assay is to increase the concentration of the [^{3}H]BZ until saturation of the drug-receptors can be achieved and then to determine the binding constants (K_a and B_{max}) for the saturable receptors.

2.7.3 The displacement (inhibition) assay

For the displacement (inhibition) assay, the unlabelled BZ concentration is varied while the protein and the [^{3}H]BZ concentrations remain fixed. The unlabelled BZ concentration is gradually raised from zero until no more displacement of the labelled BZ occurs. Unlike the saturation assay, the labelled and unlabelled ligand were not necessarily homologous. The displacement method was used to study the binding of those BZs which could not be obtained in the labelled form. The purpose of the displacement assay was to determine whether all of the BZs bind to the same receptor(s) and to compare the affinities of the unlabelled BZs for the high-affinity receptor(s) recognised by the labelled BZs.

2.8 ANALYSIS OF BINDING DATA

In this study of BZ resistance, the computerised programs EBDA and LIGAND (compiled by McPherson, 1987) have been used to analyze the binding data. I have used a combination (see Unnerstall, 1990) of Scatchard (1949) or Hofstee (1952) analysis (EBDA), iterative curve fitting (LIGAND) and graphical presentation strategy to analyze saturation or displacement drug

binding data and obtain K_a and B_{max} values of specific binding.

LIGAND (Munson and Rodbard, 1980) is a computerised program for analyzing saturation and displacement data if initial estimates of parameters are available. Initial estimates may be approximated by the user based on past experience or may be obtained from a sister program, EBDA (McPherson, 1983). LIGAND uses an exact mathematical model (see below) of ligand-receptor interaction and a statistically valid, appropriately weighted non-linear least square curve fitting method with objective measurement of the goodness of fit. It uses total ligand concentration rather than free ligand concentration, since the former is accurately known and, therefore, provides a more accurate estimate of parameters and their standard errors. However, LIGAND subsequently presents the data in Scatchard form (for saturation data) or sigmoid curves (for displacement data) since more information can be deduced by visual inspection of these type of graphs. In the first program EBDA (McPherson, 1983), Scatchard (for the saturation study) or Hofstee (for the displacement study) analyses are performed to determine the binding model (single or multiple sites), and to obtain initial estimates of K_a and B_{max} values that are fed into the next program, LIGAND (Munson and Rodbard, 1980) to obtain final estimates. Even if the binding model and initial estimates are already known or can be safely guessed, EBDA must be used to transform the raw data into appropriate form and prepare a file for LIGAND. LIGAND will not read raw data directly.

The mathematical theory adopted by LIGAND to analyze saturation or displacement data was described by Feldman (1972) and Munson and Rodbard (1980) and is summarized below.

The mathematical model describes a reversible reaction,

$$P + Q \rightleftharpoons PQ,$$

where:

P is the ligand reacting with a receptor Q to form a complex PQ (first consider a one ligand-one receptor interaction).

By the law of mass action, at equilibrium,

$$\frac{[PQ]}{[P][Q]} = \text{a constant}$$

From this one may write,

$$K = \frac{B}{FE} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where:

K = association constant; B = bound ligand concentration; F = free ligand concentration; and E = free receptor concentration.

By the law of conservation of matter

$$R = E + B \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

$$L = F + B \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

where:

R and L are the respective total receptor (i.e., B_{max}) and total ligand concentrations.

Solving equation (1) for B and substituting for B into equations (2) and (3) yields:

$$R = E + KEF. \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

$$L = F + KEF. \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (5)$$

Solving equation (4) for E yields:

$$E = \frac{R}{(1 + KF)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (6)$$

which may be substituted into equation (5) to give:

$$L = F + \frac{KRF}{(1 + KF)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (7)$$

Now given a known value of L and initial estimates of R and K, equation (7) can be solved for F. In turn, the solution for F is substituted into equation (6) to find E and then using equation (1) B is calculated. Using LIGAND, this sequence of calculations is done to predict B for every point of L entered so that a predicted curve of total ligand concentration (L) versus bound (B) is constructed. Then, iteratively, the values of K and R (i.e the initial estimates) are adjusted (see equation 7) to get a curve of best fit to the actual data. The values of K and R that result in a curve of best fit to the data, as measured by the non-linear least square method, are the final estimates of the association constant (K_a) and B_{max} respectively.

To account for NSB, another expression may be added to equation (7):

$$L = F + \frac{KRF}{(1 + KF)} + NF \quad \ldots\ldots\ldots\ldots\ldots\ldots (8)$$

where:

N = the ratio of bound/free at infinite ligand concentration and effectively represents the gradient of the line relating NSB and free ligand concentration.

Similarly, the model may be extended to situations where any number of ligands (which is the situation for displacement data) are simultaneously reacting with a single or multiple classes of receptor(s) (see Munson and Rodbard, 1980). Therefore, for multiple reactants equation (8) is complex but can be solved using the computerised program, LIGAND.

The data analysis is done in two major steps (see Unnerstall, 1990). In the first step, using EBDA, initial estimates are obtained, a model (1 site versus 2 or more sites) is chosen and a file is prepared for LIGAND. In the second step, using LIGAND, final estimates are calculated and the model hypothesis (1 site versus 2 or more sites) can be tested.

The statistical techniques that are employed, using EBDA/LIGAND, to test the reliability of the data and the parameters derived from it were described by Munson and Rodbard (1980). The data are weighted using the model,

$$\text{Weight} = 1/(\text{calculated bound})^2 \quad \text{i.e., } 1/Y^2$$

which gives more weight to points with lower variance. The error estimates are calculated assuming that the model is linear and that the errors are normally and independently distributed. A statistic used to test the goodness of fit is the "runs test" which determines whether points are randomly distributed around the predicted line or curve. The F test is used to test model hypothesis.

2.9 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was performed in slab gels on whole parasite supernatants or purified preparations pre-boiled for 2 min in reducing buffer containing 0.0625 M Tris-HCl (pH. 6.8), 5 M urea, 4% (w/v) SDS, 10%

(v/v) mercaptoethanol and 10% (v/v) glycerol, utilising the mini-protean II PAGE cell (Biorad). The sample was routinely diluted 1:1 with reducing buffer. Four % and 7.5 or 12% polyacrylamide gels were used as stacking and separating gels respectively. Electrophoresis was run at 100 V for 10 min and then 150 V for 1 h. The conducting buffer was 0 025 M Tris-HCl (pH. 8.3) containing 0.192 M glycine and 2% (w/v) SDS. Protein standards (Biorad) and purified H. contortus egg or sheep brain tubulins were used as standard markers to identify tubulin in crude preparations. After electrophoresis, the gel was stained with Coomassie blue/silver stain or the proteins were electrophoretically transferred onto nitrocellulose paper (Biorad) for immunoblotting.

2.10 IMMUNOBLOTTING (WESTERN BLOTTING)

SDS-PAGE proteins were transferred to nitrocellulose paper (Biorad) utilising the mini-Transblot cell (Biorad) and the protocol for the apparatus. The electroblot transfer was performed at 100 V for 1 h. After protein transfer, the nitrocellulose paper was rinsed with phosphate buffered saline (PBS) (pH. 7.3) and fixed with 0.2% glutaraldehyde (Sigma) to improve the retention of tubulin during washing (Van Eldik and Wolcohok, 1984). After rinsing in PBS, the transblots were incubated for 2 h at room temperature in 10% (w/v) BSA in PBS to block non-specific binding. The primary antibody was a monoclonal anti-chicken α- or β-tubulin IgG (Amersham). The secondary antibody was peroxidase-conjugated anti-mouse IgG (BioCan) which was used in preference to biotinylated IgG (Amersham) which cross-reacted with non-tubulin proteins in H. contortus. The diluted primary (1:10,000) and secondary (1:1,000) antibody contained 1% (w/v) BSA and 0.1% (v/v) Tween-20. The blots were washed with PBS

containing 0.4% Tween-20, for 3 x 15 min following 1 h incubation with the primary or secondary antibody. The substrate was diaminobenzidine (Sigma).

Chapter III

OBZ AND MBZ: SATURATION ASSAY

3.1 INTRODUCTION

Previous studies on BZ resistance (Sangster et al., 1985; Lacey and Prichard, 1986; Lacey et al., 1987b) have reported that whole soluble-extracts (high speed supernatants) prepared from BZ-resistant nematodes bind less BZ drug than those prepared from BZ-susceptible nematodes. However, in these studies BZ binding was handled as a single receptor ligand interaction and the data were analyzed using manual graphical methods. The specificity of the reduced BZ binding was not substantiated since the binding data were not corrected for non-specific binding (NSB). A ligand is unlikely to be 100% specific (Unnerstall, 1990; Bylund and Yamamura, 1990) and several types of specific receptors may exist (Molinoff et al., 1981). Even the so called specific ligands are usually more or less partially selective (see Yamamura et al., 1985, 1990; Cattabeni and Nicosia, 1984). Burt (1985) adds 'the experimenter attempting binding study for the first time soon learns that **everything binds everything** and there is useful and useless binding'. To decide between useful and useless requires a very long study following basic principles (Creese, 1985). To be pharmacologically useful a ligand should be sufficiently selective to elicit activity specific to a known receptor, at a relatively low concentration, without altering activities of other proteins at the same concentration. Usually, the reason for this selectivity is that the selective ligand has greater affinity for this receptor compared to other receptors. However, as a rule, specificity is

inversely related to ligand concentration and decreases (i.e other types of receptors with progressively less affinity get recruited) as the concentration of the pharmacological agent is increased; i.e, in vivo, overdosage may cause toxicity or side effects. The specific or high affinity receptor should be biologically or pharmacologically relevant (Enna, 1984; Burt, 1985; Creese, 1985). Thus, in the case of an anthelmintic, the interaction of the ligand with the specific receptor should cause a cascade of events leading to death or elimination of a parasite from the body and alteration of the receptor could lead to drug resistance. There are at least four basic criteria (Enna, 1984; Burt, 1985; Kinnier, 1990) necessary to establish that the binding site represents a biologically relevant receptor: a) **saturability**; b) **distribution**; c) **substrate specificity**, and d) **high-affinity**. In reference to saturability, the number of binding sites should be limited in number. Therefore, to eliminate all doubts, it should be possible to readily saturate the number of displaceable sites. Regarding distribution, the displaceable sites should only be found in those tissues or preparations known to contain the biologically active receptors. Regarding substrate specificity, if displaceable binding represents attachment to the biologically relevant receptor, then only those substances known or expected to interact with that receptor should demonstrate appreciable potency as inhibitors of specific radioligand binding. An appreciably potent inhibitor may be roughly defined as that capable of causing 50% radioligand inhibition at concentrations known to elicit a pharmacological response in vivo (Creese, 1985). Regarding high-affinity, the ligand must show selectivity so that the binding-affinity for this receptor is appreciable compared to other receptors. It has not been shown before that

tubulin binds BZs with high affinity compared to other proteins i.e selectivity (specificity) of BZs for tubulin in parasite homogenate has not been substantiated.

This Chapter focuses on high-affinity binding (HAB) of BZs to whole parasite supernatants in order to determine whether resistance involves a specific type or group of receptors. [^{3}H]BZ binding of parasite supernatants was separated into HAB and low-affinity binding (LAB) as described in Chapter II. In general, specific binding is taken as the difference between total binding (TB) and binding that occurs in the presence of an excess concentration of unlabelled ligand, the non-specific binding (NSB) (see Bylund and Yamamura, 1990; Unnerstall, 1990). NSB may be composed of several components: a) true nonspecific binding to the tissue under study; b) free ligand not effectively removed; and c) nonspecific binding to separation materials (test tubes, etc). It is important to realise that NSB of the tissue under study is actually the unsaturable **low-affinity** specific binding which is biologically irrelevant or in which the investigator is not interested (Johnson and Frasier, 1984). The magnitude of binding affinity necessary to make an interaction pharmacologically relevant is impossible to generalise. Therefore in this thesis the term low-affinity binding and high-affinity binding are used in preference to non-specific binding and specific binding, respectively. It must be kept in mind, however, that the classical definition of specific binding assumes biological relevancy, saturability, high-affinity binding and selectivity. To determine the specific binding (i.e HAB) parasite supernatants may be pre-incubated with an unlabelled ligand before adding the labelled ligand. It is hoped that the unlabelled ligand blocks the high-affinity receptors so that subsequent labelled-ligand binding occurs

to the low-affinity receptors only. Therefore HAB is determined by subtracting LAB from TB. One major feature of this approach, in this study, is that the labelled and unlabelled ligands were homologous to ensure specificity.

B_{max} is the maximum drug binding at infinite ligand concentration and represents the total number of receptors in the system. Previously, B_{max} was calculated using TB of parasite supernatants (Sangster et al., 1985; Lacey et al., 1987b). However specific binding quickly reaches a plateau while NSB increases indefinitely with ligand concentration. Therefore at infinite ligand concentration, most of the binding may be non-specific. In this study B_{max} is calculated for the HAB to determine whether resistance is associated with alteration in the number of high-affinity receptors. Similarly the apparent association constant (K_a) is calculated for the HAB to see if it is affected by resistance. Unlike previous studies in which manual graphical methods were used, a computerised approach is used to calculated the K_a and B_{max} values.

The drug receptor interaction, at equilibrium, obeys the law of mass action from which Bennett and Yamamura (1985) derived the relationship:

$$B = \frac{B_{max} \cdot L}{(K_d + L)}$$

where:

B = drug bound at any given drug concentration, B_{max} = maximum drug bound at infinite drug concentration, L = free drug concentration, and K_d = the dissociation constant = $1/K_a$.

In order to determine the K_a and B_{max}, the routine has been to linearise

the hyperbolic saturation curve. One such manipulation is the double reciprocal plot of Lineweaver and Burk employed previously by Sangster et al., 1985, but the most popular way was described by Scatchard (1949) according to the following equation:

$$B = B_{max} - K_d \cdot B/L$$

so that a plot of B/L versus B is a straight line with a slope = $1/K_d$ and an x-intercept = B_{max}. The Scatchard plot has the advantage that receptor subpopulations may be graphically visualised (see Klotz, 1982; Nørby et al., 1980). Linear Scatchard plots suggest that one class of binding sites may be present. Non-linear plots suggest that non-specific binding or multiple classes of binding sites with dissimilar binding affinities (K_a) may be present. However, non-linear Scatchard plots may be caused by other factors which must be eliminated by independent means before making final conclusions (see Molinoff et al., 1981). For mathematical analysis of ligand binding data using any method, accurate assumptions must be made in order to determine correct K_a and/or B_{max} values (reviewed by Bennett and Yamamura, 1985 and Unnerstall, 1990). First, the number of classes of binding sites should be correctly determined so that the calculated binding constants describe each class of binding sites separately. To accurately determine binding constants using linear transformations such as the Scatchard or Lineweaver and Burk plots, it must be proved that the ligand is pure and the receptor population is homogeneous i.e., one ligand one receptor interaction (Klotz, 1982). Whereas it may be easy to have a pure ligand, a homogeneous receptor population cannot easily be claimed. Second, the binding process should obey simple reversible mass action laws i.e the receptor-ligand interaction should not involve covalent bonding and the data must be obtained under steady state conditions (i.e., after

equilibrium and saturation). The prequisite of saturation in analysis of binding data has been emphasized by several writers (see Johnson and Frasier, 1984, 1985; Klotz, 1982; Nørby et al., 1980; Bennett and Yamamura, 1985). Thirdly, the binding of ligand to the receptor site should not affect subsequent ligand receptor interaction (i.e., no cooperativity). Negative or positive cooperativity (Molinoff et al., 1981) which can be explained by several molecular events (Levitzki and Koshland, 1969) are difficult to circumvent once they occur to a significant extent (Nørby et al., 1980). Lastly, the concentration of free ligand must be accurately measured and non-specific binding properly defined. Most preferably, total ligand concentration should be used directly in the analysis (Munson and Rodbard, 1980; Johnson and Frasier, 1984, 1985). The program, LIGAND (Munson and Rodbard, 1980), takes into account the above points and circumvents some of the limitations of simple linear transformation or regression of ligand binding data. For example, LIGAND uses an exact mathematical model of ligand-receptor interaction and a statistically valid, appropriately weighted non-linear least square curve fitting method with objective measurement of the goodness of fit (see Feldman, 1972; Munson and Rodbard, 1980 or Chapter II). The level of NSB can be regarded as an unknown parameter and estimated simultaneously with other parameters. The program uses total ligand concentration rather than free, since the former is accurately known and, therefore, provides a more accurate estimate of parameters and their standard errors.

Displacement or inhibition of radioligand binding by the unlabelled ligands has been used to determine the K_a values of the latter and/or resolve receptor subpopulations (reviewed by Molinoff et al., 1981). LIGAND can be used to analyze saturation or displacement data. I have used

a combination of Scatchard analysis (EBDA), iterative curve fitting (LIGAND) and graphical presentation strategy to analyze saturation or displacement binding data.

In this Chapter saturation assays (see definition in Chapter II) were performed to resolve the [^{3}H]OBZ or [^{3}H]MBZ TB of egg, L_3 or adult-worm whole-supernatants into LAB and HAB and the EBDA/LIGAND program was used to determine the binding constants (K_a and B_{max}) for the HAB. It is shown in Chapter VI that HAB is associated with tubulin containing preparations only. Displacement assays are described in subsequent Chapters.

3.2 MATERIALS AND METHODS

3.2.1 Standardisation of binding conditions

Conditions reported previously (Lacey and Snowdon, 1988) to influence total binding were standardised.

3.2.1.1 Effect of sample preparation

Glass-glass homogenisation by hand or Potter-Elvejehm and homogenisation by the polytron cell disrupter were compared. The use of fresh versus frozen material was compared and the maximum concentration of DMSO that had no detrimental effect on binding was determined.

3.2.1.2 Effect of protein concentration

Protein concentrations of egg, L_3 or adult worm supernatants were adjusted between 10 and 300 μg/90 μl. The binding assay was performed as outlined previously (Chapter II) using an increasing protein concentration and fixed stock concentrations of [^{3}H]BZ (0.5, 2.0, 8.0 or 16.0 μM) to

determine optimal protein concentrations for the assay.

3.2.1.3 Effect of BZ solubility

Since BZs may come out of solution when a stock concentration in DMSO is diluted with incubation buffer, it was necessary to determine that this did not occur during the assay. The experiment was carried out simulating the standard binding procedure (see Chapter II) but omitting the charcoal step. It was expected that the dpm obtained in absence or presence of a reasonably high unlabelled [BZ] would be similar if no drug precipitation occurred. Therefore, 2 μl of DMSO or 2 μl of 500, 1000 or 1500 μM unlabelled stock BZ in DMSO were preincubated in triplicate with 90 μl of MES buffer for 30 min at 37° C. Then 10 μl of 0.3125 to 50 μM stock [^{3}H]BZ were added and incubated for a further 30 min. The tubes were left to stand at room temperature for 1 h. Then 0.5 ml of 1% BSA in MES buffer were added to each tube, incubated for 5 min at 37° C and centrifuged at 13,000 x g for 5 min. The dpm present in 0.45 ml of the supernatant were then determined.

3.2.2 Binding of whole parasite supernatants

Saturation assays were performed to resolve the [^{3}H]OBZ or [^{3}H]MBZ total binding (TB) of egg, L_3 or adult worm whole superratants into low-affinity (LAB) and high-affinity binding (HAB) and then determine the binding constants (K_a and B_{max}) by mathematical analysis.

3.2.3 Mathematical analysis

The mathematical theory adopted by LIGAND and EBDA has already been outlined (Chapter II). EBDA was used to perform Scatchard plots of the

ligand saturation data, so that initial estimates of binding constants could be obtained and the binding model (one or two sites) predicted before final analysis using LIGAND. The Scatchard transformed data was presented graphically for visual inspection and, based on this inspection, the site model was selected. The initial estimates and the predicted model were used in LIGAND to obtain final estimates of K_a and B_{max} values.

3.2.4. Statistical analysis

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

3.3 RESULTS

3.3.1 Effect of sample preparation on binding.

Initial studies showed that the brief homogenisation with the polytron cell disrupter gave comparable binding to glass-glass homogenisation with Potter-Elvejehm. Similarly, it was found that supernatant fractions prepared from parasites isolated and stored, immediately after isolation, in liquid nitrogen, and supernatant fractions from unfrozen freshly isolated parasites gave comparable results. The presence of < 5 % (v/v) final DMSO concentration had negligible effect on binding. Higher DMSO concentrations had a detrimental effect on binding. Therefore final DMSO concentrations of < 5% were routinely used.

3.3.2 Optimal protein concentrations for the binding assay.

In a series of studies, it was found that standardized binding (pmoles/mg protein) at fixed final label concentrations was uniform for

protein concentrations above 10, 50 and 100 μg protein/assay for eggs, larvae and adult worms, respectively. Below these limits, binding was irregular. Subsequent experiments were done using protein concentrations above these limits. The reproducibility of replicate binding determinations was routinely within less than ± 10 % while between experiment variation of binding was within less than ± 25 % of the mean value. The between experiment variation was probably due to subtle differences in sample preparation since the same degree of difference between S and R was always detected when S and R samples were prepared and assayed simultaneously. Therefore, S and R samples were routinely analyzed at the same time.

3.3.3 Effect of the solubility of BZs on binding.

The presence or absence of 10 μM final unlabelled BZ concentration did not affect solubility of [^{3}H]OBZ up to 5 μM final labelled ligand concentration (Fig. 3.1). That is, OBZ was soluble in assay buffer up to 15 μM final total concentration. When 20 μM final unlabelled concentration was used, some precipitation was observed above 2 μM final [^{3}H]OBZ concentration. When 30 μM final unlabelled concentration was used significant precipitation of the drug occurred (Fig. 3.1). Similar results were obtained for MBZ. Preliminary results showed that, following preincubation with the unlabelled drug, the level of LAB was independent of the unlabelled ligand concentration in the range 5 - 10 μM final unlabelled drug concentration. Based on these results a final unlabelled ligand concentration of 10 μM was used to define LAB in the range 0.0156 - 2.0 μM final concentration of the labelled ligand.

3.3.4. Graphical analysis

The relationships between TB, LAB and HAB are presented in Figure 3.2 and 3.3 for OBZ and MBZ respectively. There was a marked difference in HAB of OBZ or MBZ between the S and R strains while LAB was relatively similar for both strains. The LAB of the S and R strains was not significantly different for both drugs and all developmental stages. The HAB of the R strain was significantly lower ($P < 0.01$) than that of the S strain for both drugs and all stages.

The drug binding profiles of the larvae and adult worms were similar but dissimilar to those of eggs for both OBZ (Fig. 3.2) and MBZ (Fig. 3.3). TB for larvae and adult worms was not saturable, within the range of drug concentration used, due to the presence of a considerable amount of LAB. However, TB for the eggs was saturable because they contained relatively little LAB. The LAB of adults and larvae was nearly linear for both drugs but that of the eggs increased curvilinearly with the drug concentration.

The Scatchard plots generated by LIGAND after fitting the respective curves of total ligand concentration versus ligand bound are shown in Figures 3.4-3.6. The Scatchard plots for TB of adult worm (Fig. 3.4) and larval (Fig. 3.5) supernatants were curvilinear suggesting the presence of two receptors that differ significantly in affinity. The 'runs test' for the Scatchard plots showed ($P > 0.05$) that TB of larvae and adult worms cannot be described by a single site model. Similarly, the F-test indicated ($P = 1$) that a two site model was better than a single site one for TB of larval and adult worm supernatants but subtraction of LAB was more appropriate. When LAB was treated as NSB and subtracted from TB, the Scatchard plots became linear with correlation coefficients close to

unity. However, the Scatchard plots of TB of the eggs were linear and similar in form and slope (but different in x-intercept) to those of HAB (Fig. 3.6), indicating that there is little LAB in the eggs (and can be ignored) and the TB can be described as a single site model.

The Scatchard plots of HAB of the S and R strains were linear and had similar slopes but differed in their x-intercepts indicating that the K_a values of the S and R strains were similar but their B_{max} values were different for all stages and both drugs.

3.3.5 Mathematical and statistical analyses

The K_a and B_{max} estimates of HAB are shown in Table 3.1. Also shown in Table 3.1 are the susceptibility factor (SF) values. The SF is the ratio of the B_{max} of the S strain to that of the R strain (Lacey and Prichard, 1986; Lacey and Snowdon, 1988). The SF was calculated and chosen in preference to its reciprocal (the resistance factor) as a measure of the degree of resistance.

(i) 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 than for the other stages.

(ii) 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. For the S strain and both drugs, B_{max} values were significantly different

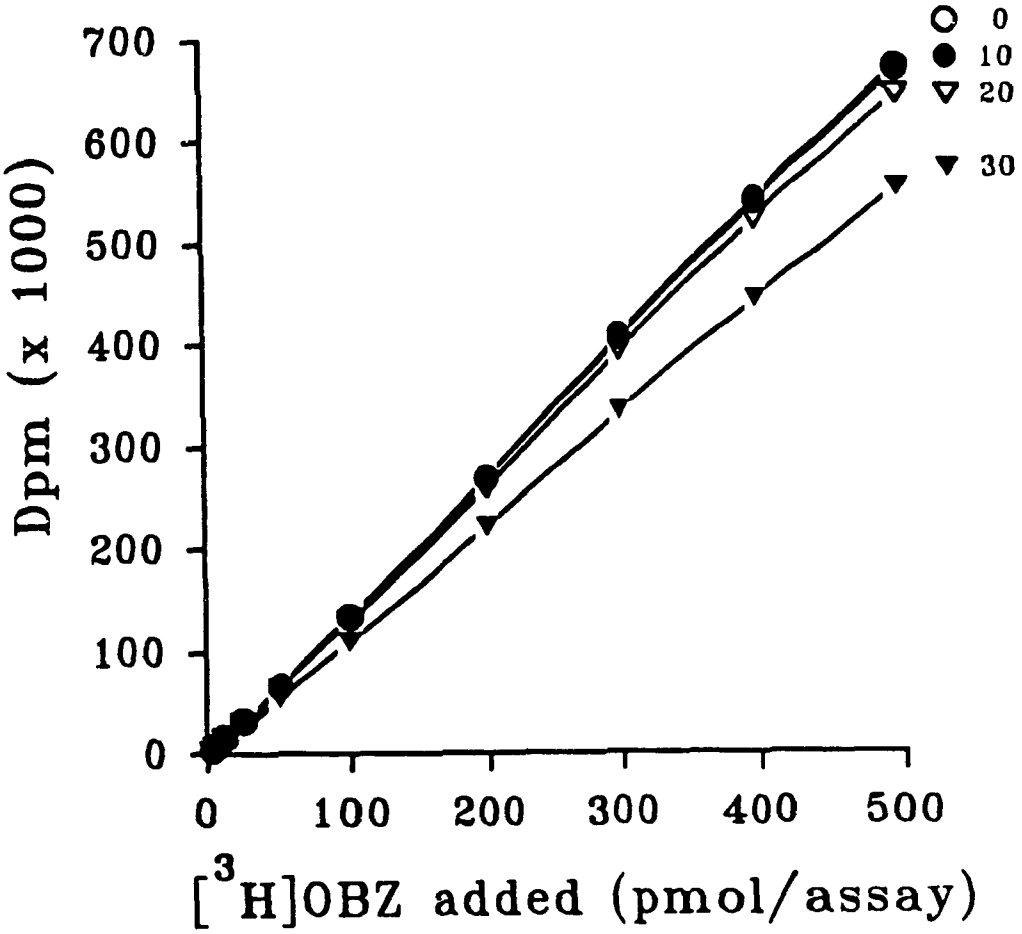


Fig. 3.1. Solubility of OBZ and the binding assay. Two μl of DMSO or 2 μl of 500, 1000 or 1500 μM unlabelled OBZ in DMSO in MES buffer were added to 90 μl MES buffer to make a final unlabelled concentration of 0, 10, 20 and 30 μM, respectively. The tubes were preincubated in triplicate for 30 min at 37°C. Then 10 μl of 0.3-50 μM stock [^{3}H]OBZ were added to each assay and incubated for a futher 30 min. 0.5 ml of 1% (w/v) BSA in MES buffer were added, incubated, centrifuged and the dpm present determined as described in Materials and Methods. Each point is a mean of triplicate determinations.

Fig. 3.2. **TB and LAB and HAB of OBZ in S or R egg, larval and adult worm supernatants.** Each point is a mean ± SE of 4-8 experiments conducted in duplicate as described in Materials and Methods.

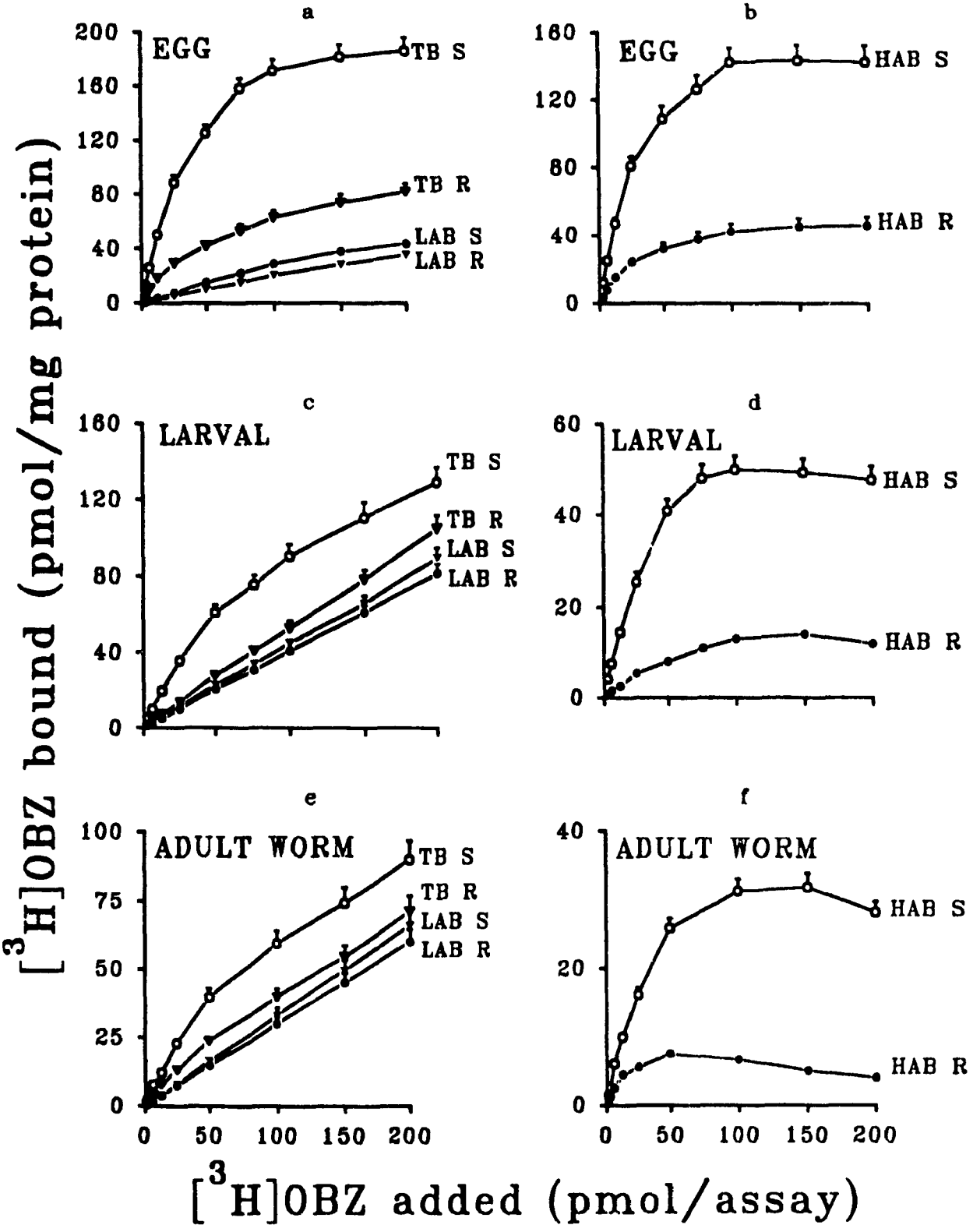

a
EGG
TB S
TB R
LAB S
LAB R
b
EGG
HAB S
HAB R
c
LARVAL
TB S
TB R
LAB S
LAB R
d
LARVAL
HAB S
HAB R
e
ADULT WORM
TB S
TB R
LAB S
LAB R
f
ADULT WORM
HAB S
HAB R
[3H]OBZ bound (pmol/mg protein)
[3H]OBZ added (pmol/assay)

Fig. 3.3. **TB and LAB and HAB of MBZ in S or R egg, larval and adult worm supernatants.** Each point is a mean ± SE of 4-8 experiments conducted in duplicate as described in Materials and Methods.

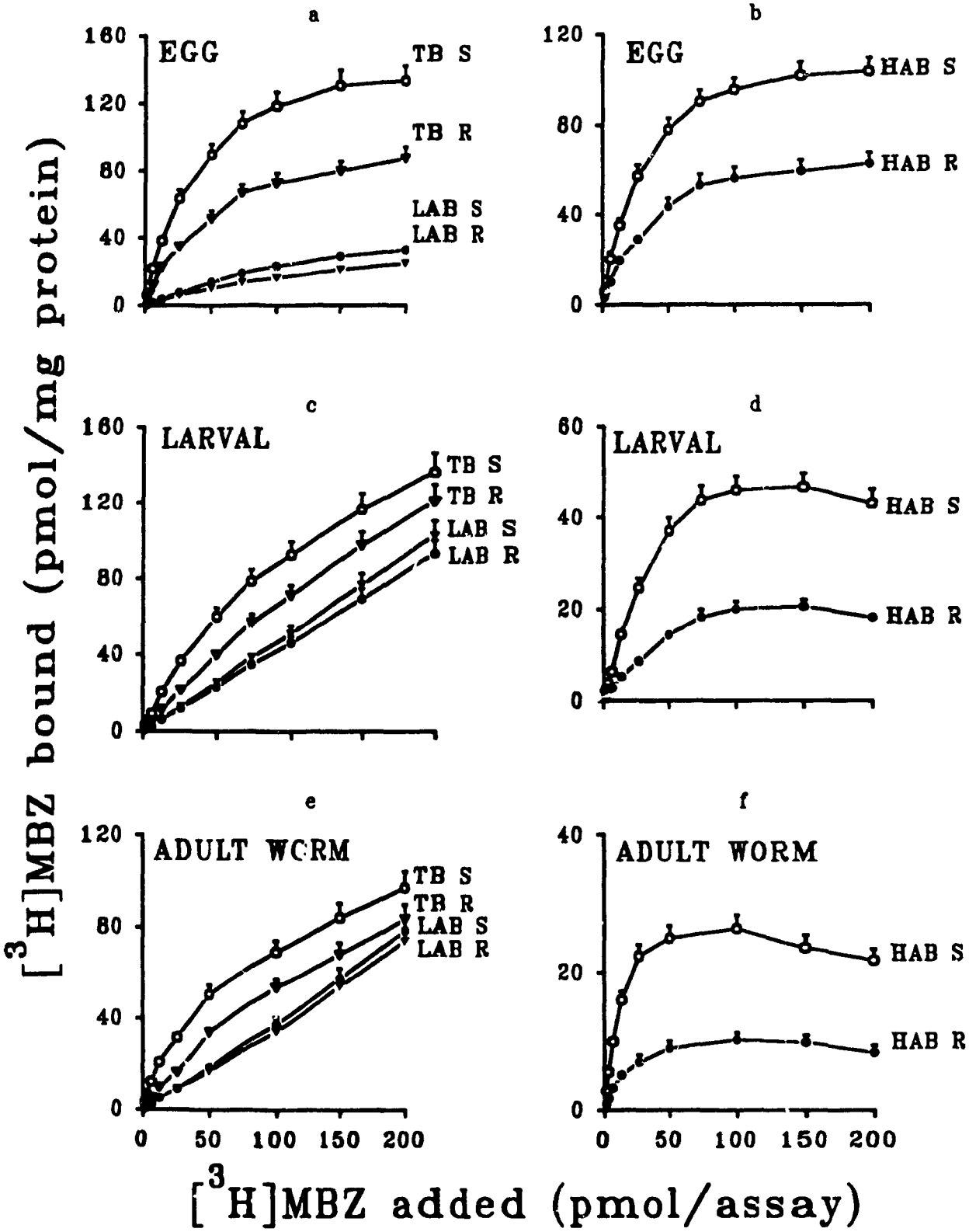
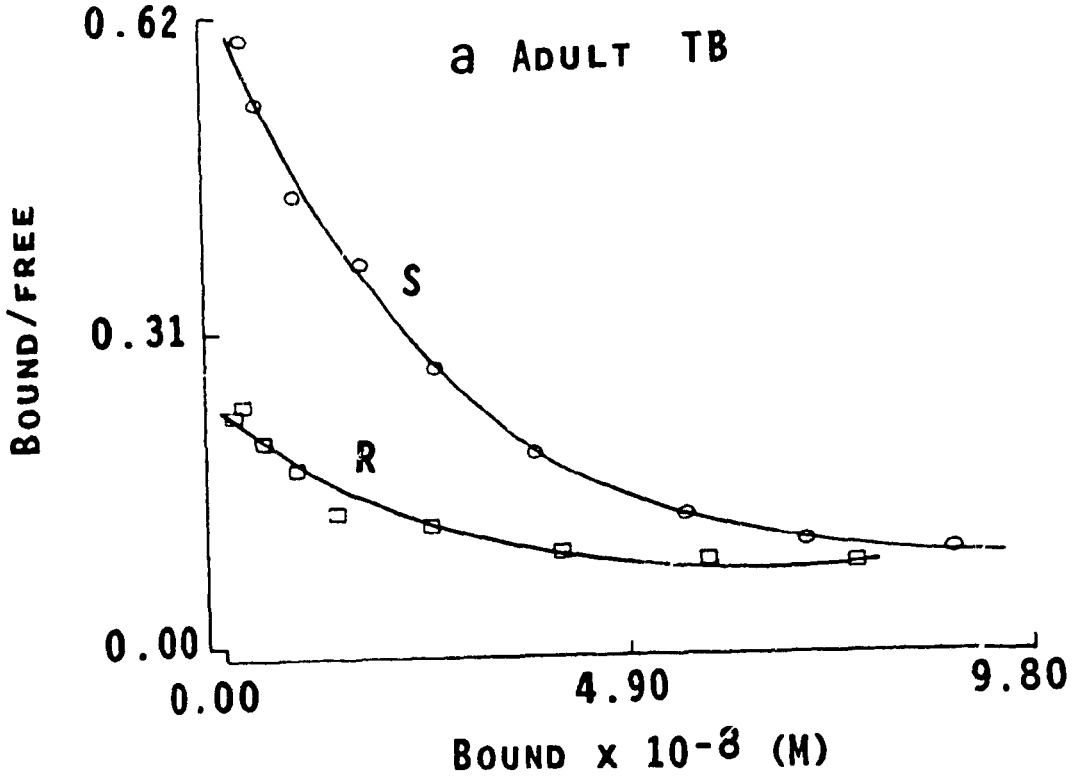

a
EGG
TB S
TB R
LAB S
LAB R
b
EGG
HAB S
HAB R
c
LARVAL
TB S
TB R
LAB S
LAB R
d
LARVAL
HAB S
HAB R
e
ADULT WORM
TB S
TB R
LAB S
LAB R
f
ADULT WORM
HAB S
HAB R
[3H]MBZ bound (pmol/mg protein)
[3H]MBZ added (pmol/assay)
0
40
80
120
160
20
60
50
100
150
200

Fig. 3.4. Scatchard plots of TB and HAB of MBZ in S or R adult worms supernatants. For TB (a), the curved lines were drawn by hand through the points plotted by LIGAND to demonstrate that TB is not consistent with a single site model and that B_{max} values derived by linear regression of TB data are only speculative. For HAB (b), the plots and the line of best fit were generated using LIGAND and show that HAB is consistent with a single site model. The plot of S is parallel to that of R so that the two have equal slopes (i.e equal K_a values) but different x-intercepts (i.e different B_{max} values). The data are presented as Scatchard plots for visual inspection only. A plot of total ligand added versus bound was iteratively fitted using LIGAND and Scatchard plots were then generated.


a ADULT TB
0.62
0.31
0.00
BOUND/FREE
S
R
0.00
4.90
9.80
BOUND x 10-8 (M)

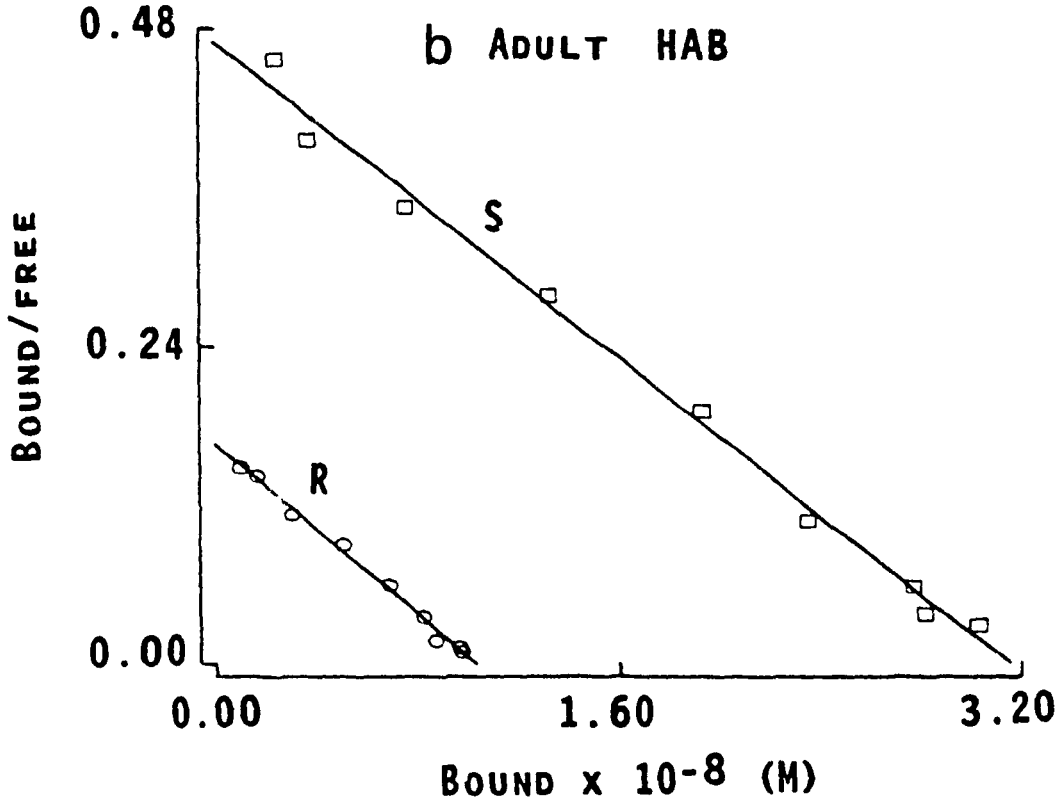
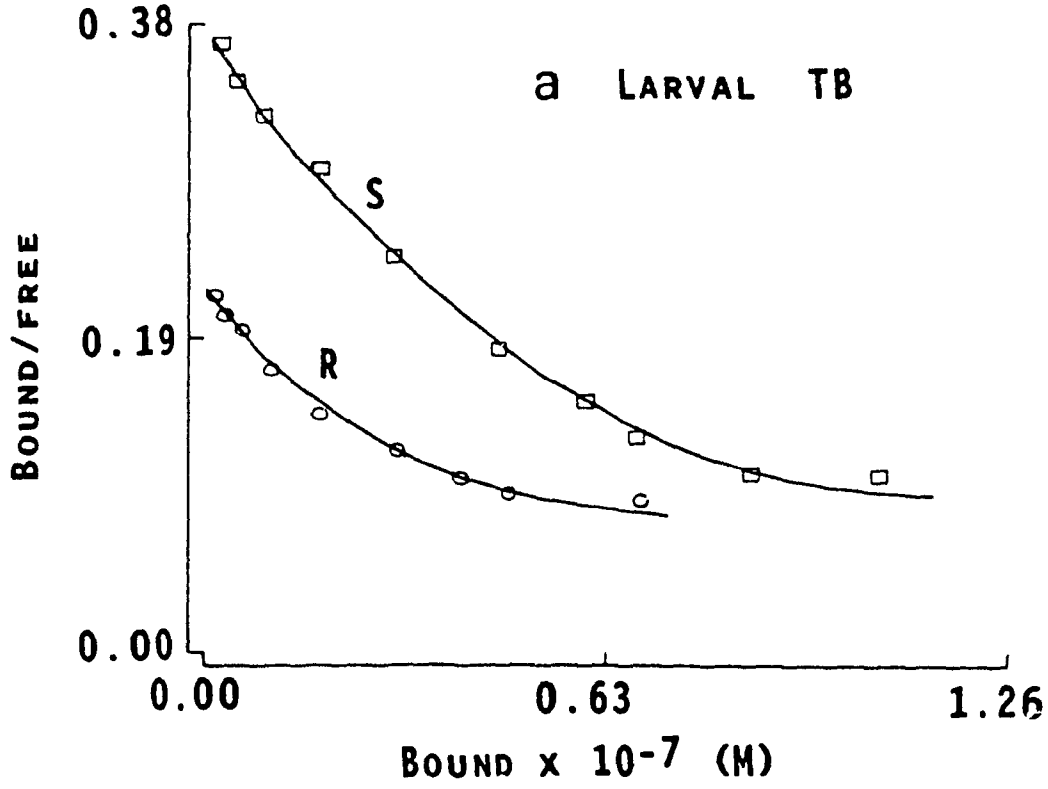

b ADULT HAB
0.48
0.24
0.00
BOUND/FREE
S
R
0.00
1.60
3.20
BOUND x 10-8 (M)

Fig. 3.5. **Scatchard plots of TB and HAB of MBZ in S or R larval supernatants.** Procedures and conclusions are the same as for the adult worms desribed in Fig. 3.4.


a LARVAL TB
S
R
0.38
0.19
0.00
BOUND/FREE
0.00
0.63
1.26
BOUND x 10-7 (M)

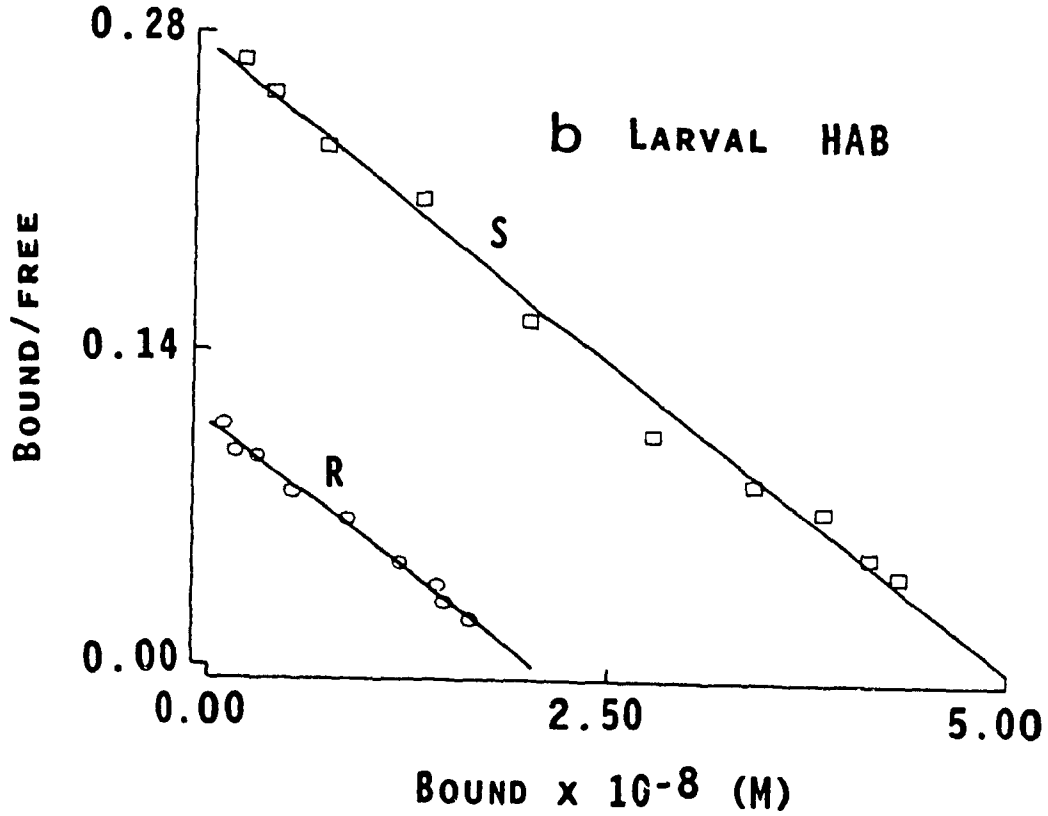
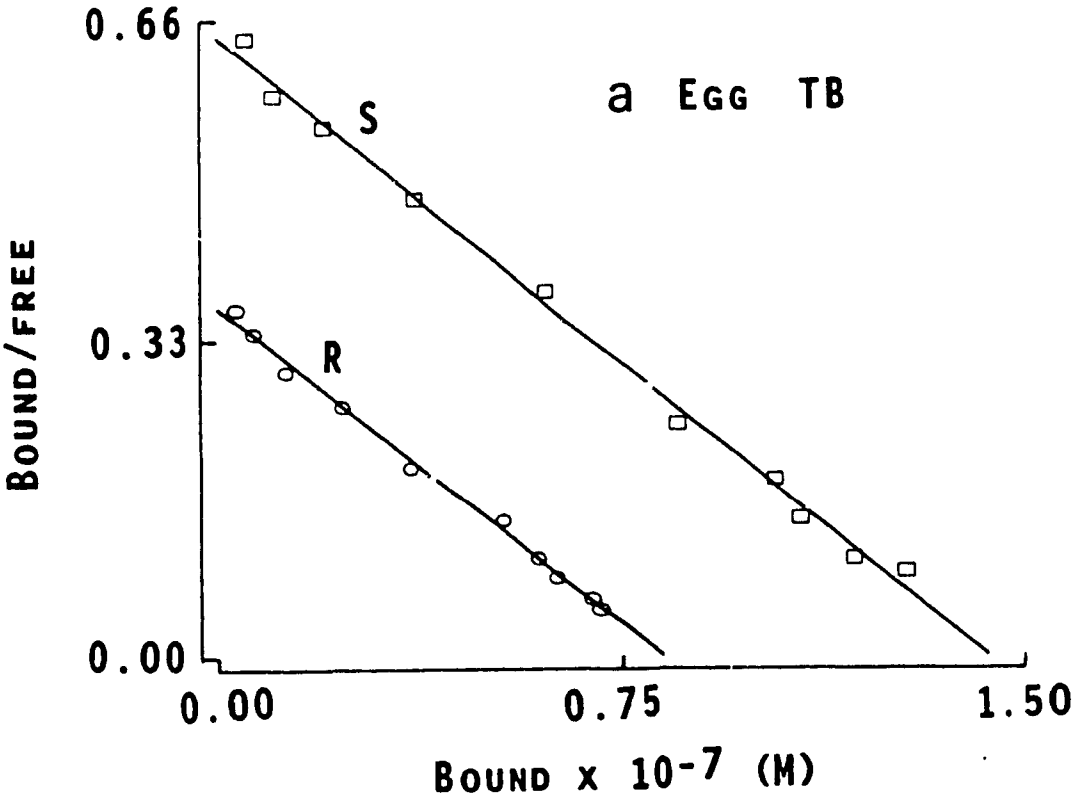

b LARVAL HAB
S
R
0.28
0.14
0.00
BOUND/FREE
0.00
2.50
5.00
BOUND x 10-8 (M)

Fig. 3.6. **Scatchard plots of TB and HAB of MBZ in S or R egg supernatants.** Unlike the adult worms (Fig. 3.4) or larvae (Fig. 3.5), the TB plots (a) are linear suggesting that TB of eggs can be approximated to a single site model. Similar to adult worms or larvae, the HAB plot (b) of S is parallel to that of R. Procedures and conclusions are otherwise the same as for the adult worms (see Fig. 3.4).


a EGG TB
S
R
0.66
0.33
0.00
BOUND/FREE
0.00
0.75
1.50
BOUND x 10-7 (M)

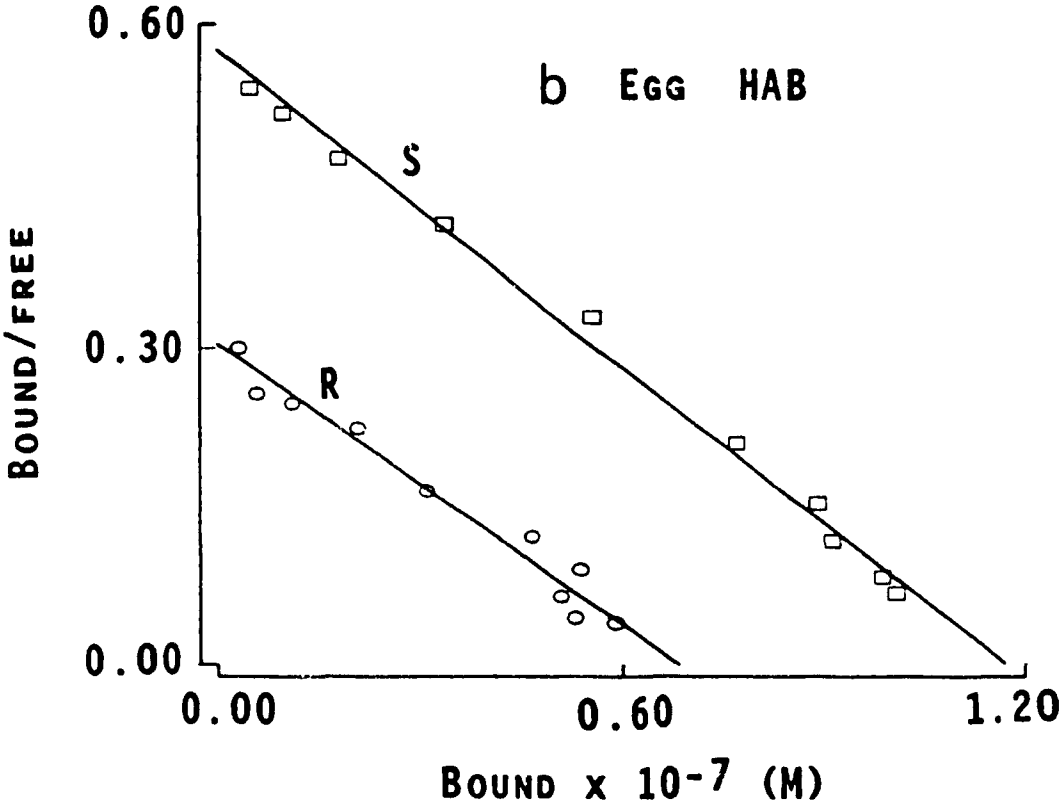

b EGG HAB
S
R
0.60
0.30
0.00
BOUND/FREE
0.00
0.60
1.20
BOUND x 10-7 (M)

TABLE 3.1. Constants for OBZ and MBZ specific binding (HAB) in S or R egg (E), larval (L) or adult worm (A) supernatants, obtained by saturation assay.

Drug	Stage	Strain	K_a (x 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[a]	147 ± 8[c,d]	3.5[f]
		R	6.6 ± 0.6[a]	42 ± 3[a]	
	L	S	5.0 ± 1.2[a]	59 ± 4[c]	7.4[f]
		R	4.8 ± 1.3[a]	8 ± 2[b]	
	A	S	7.0 ± 1.0[a]	44 ± 4[c]	4.9[f]
		R	5.3 ± 1.0[a]	9 ± 2[b]	

* mean ± S.E of 4-8 experiments, values calculated using LIGAND

\+ SF = ratio of B_{max} of S to that of R.

a,b Within the column and drug, values sharing the same letter are not significantly different (K_a, $P > 0.05$; B_{max}, $P > 0.01$); values with different letters are significantly different (K_a, $P < 0.05$; B_{max}, $P < 0.01$)

c B_{max} of S significantly higher than that of 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 are not significantly different ($P > 0.05$), values with different letters are significantly different ($P < 0.01$)

($P < 0.01$) between developmental stages (i.e, B_{max} values decreased from egg through larva to adult stage). 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 there was no significant difference between larvae and adults.

(iii) **SF values**

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

3.4 DISCUSSION

It has been postulated that the mode of action of BZs is through tubulin binding and the mechanism of BZ-resistance is alteration in tubulin binding (Lacey, 1988). Therefore this study focused on tubulin. It is shown in Chapter VI that HAB is associated with tubulin. The data in this Chapter show that the S and R strains of H. contortus differ in levels of HAB. Thus, resistance is associated with a loss of the high-affinity receptors. The differences in HAB between S and R strains can be demonstrated using eggs, infective larvae (L_3), as well as adult worms. This suggests that the receptor(s) for the BZs may be similar at each stage of development. The concentration of the receptor(s) per mg 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 strain. For the R strain, however, B_{max} values were higher in the eggs than larvae or adults but there was no significant difference between the latter two stages. This might be due to the inherently diminished HAB in the R strain. Compared

with the eggs, more of the binding in the larvae or adult worms was LAB. On the other hand, compared with the larvae and adult worms more of the binding in the eggs was HAB. Resistance was associated with a reduction in amount (pmol/mg protein) of HAB. BZ-resistance was not associated with LAB. Most of the LAB of the larvae and adult worms was probably NSB since it was nearly linear when binding was plotted against ligand concentration. There is little LAB of the egg supernatants and the plot of this LAB versus ligand concentration was curvilinear indicating a tendency to saturation. Some or all of the LAB in the eggs may represent specific LAB to tubulin. The source of the greater amount of LAB in the larval and adult worm supernatants than in those of eggs is obscure. It may involve tubulin and/or other proteins which are not present in the eggs.

OBZ is more affected by the reduction in HAB than MBZ. The susceptibility factor (SF) values for eggs, larvae and adult worms are not significantly different although the SF values of OBZ are significantly higher ($P < 0.01$) than those for MBZ. The results suggest that BZ-resistance can accurately be diagnosed using either 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 I) were in good agreement with the egg hatch assay data (Chapter II) indicating the validity of both methods for diagnosing BZ-resistance.

BZ-resistance did not affect the K_a values. Also the K_a for egg, 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 those for the other supernatants (Table 3.1). This unique behaviour in affinity of [^{3}H]MBZ for adult worm supernatants cannot easily be explained. 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 principle protein of the mitotic spindle (Dustin, 1984) and may be abundant in rapidly dividing cells, such as eggs. HAB may depend on tubulin content which may in turn depend on the mitotic requirement of a developmental stage. Tubulin content (per mg protein) was found to decrease from egg through larva to adult stage (see Chapter VII). However, HAB may also depend on the type(s) of tubulin present. The tubulin isoform patterns of the S strain were different from those of the R strain (see Chapter VIII). The R population may consist primarily of the resistant worms but with a proportion of the susceptible worms. 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.

Chapter IV

OFZ AND ABZ: SATURATION STUDY

4.1 INTRODUCTION

It has been reported before (Colglazier et al., 1975; Berger, 1976; Waller and Prichard, 1986) that nematode strains resistant to one BZ may be automatically resistant to other BZs. This suggests that BZs may have a common mode of action and the mechanism of resistance may be the same for all BZs. On the other hand, BZs with strong inhibitory activity against mammalian tubulin were potent inhibitors of nematode egg-hatch and non-inhibitors failed to prevent egg-hatch. However, some BZs such as OFZ and ABZSO with strong anthelmintic efficacy, in vivo, did not inhibit mammalian tubulin-polymerisation nor egg-hatch (Lacey et al., 1987a). These studies suggest that the effects of some BZs in vitro may not always predict effects in vivo. Nevertheless, Lacey and Prichard (1986) reported that MBZ, OBZ, ABZ, OFZ and FBZ were similar in that their TB to whole parasite extracts derived from the BZ-resistant strain was reduced compared to those derived from the BZ-susceptible strain. However, the specificity of this reduced binding was not addressed. In Chapter III it was shown that the cause of the reduced binding by the BZ-resistant supernatant is the loss of the high-affinity receptors. In this Chapter, saturation assays were performed using ABZ and OFZ to determine whether the same explanation also holds for these ligands. Labelled ABZ was not available in sufficient quantity to look at each stage of development. Therefore only the binding to egg supernatants is reported for this ligand. The binding of OFZ to egg, larval and adult worm supernatants is

described.

4.2 MATERIALS AND METHODS

[^{3}H]OFZ and [^{3}H]ABZ binding to S or R strains of *H. contortus* was examined in a saturation study at 37°C using egg, larval or adult worm supernatants as described for [^{3}H]MBZ and [^{3}H]OBZ (chapter III).

4.3 RESULTS

4.3.1 Oxfendazole (OFZ)

The binding of [^{3}H]OFZ to S and R strains at 37° was compared using egg, larval and adult worm supernatants. The binding was not saturable (Fig. 4.1) and could not be well resolved into HAB and LAB. For adult worm supernatants (Fig. 4.1 e) [^{3}H]OFZ binding could hardly be inhibited using excess unlabelled OFZ. Using larval supernatants (Fig. 4.1 c& d) some [^{3}H]OFZ binding was inhibited by excess OFZ. For egg supernatants (Fig. 4.1 a & b), unlabelled OFZ inhibited [^{3}H]OFZ binding to a greater extent than for adult worm or larval supernatants. In all three instances TB for the S strain was greater than that of the R strain and, where inhibition was achieved, apparent HAB of the S strain was greater than that of the R strain.

4.3.2 Albendazole (ABZ)

ABZ binding using S or R egg supernatants is shown in Fig. 4.2. As for [^{3}H]OBZ and [^{3}H]MBZ (Fig. 3.1 and 3.2) or [^{3}H]OFZ (Fig. 4.1) the S strain bound more drug than the R strain. Unlike [^{3}H]OFZ, HAB of ABZ was

Fig. 4.1. TB and apparent LAB and apparent HAB of OFZ in S or R egg, larval and adult worm supernatants. Each point is a mean ± SE of 5 separate experiments conducted in duplicate as described in Materials and Methods.

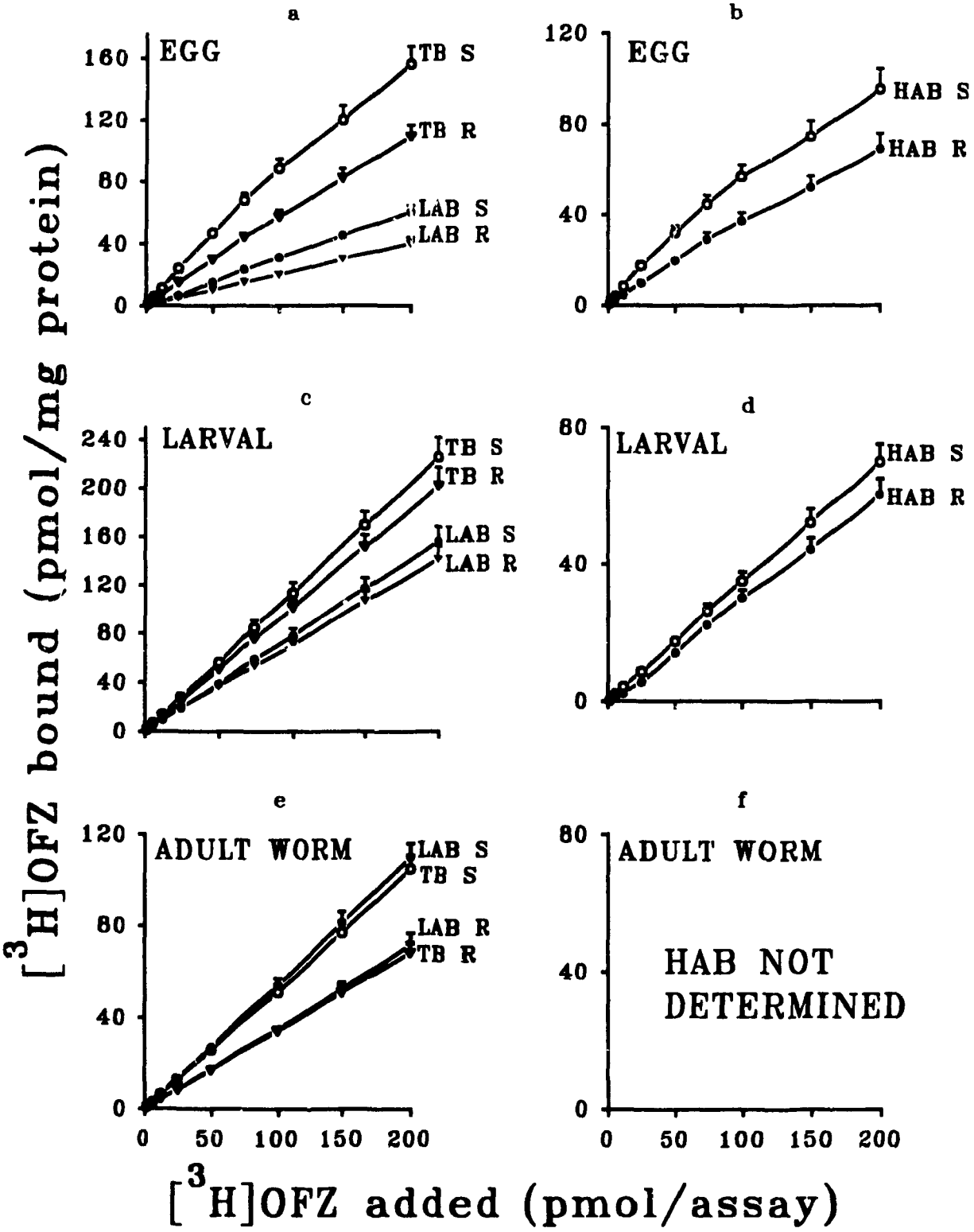
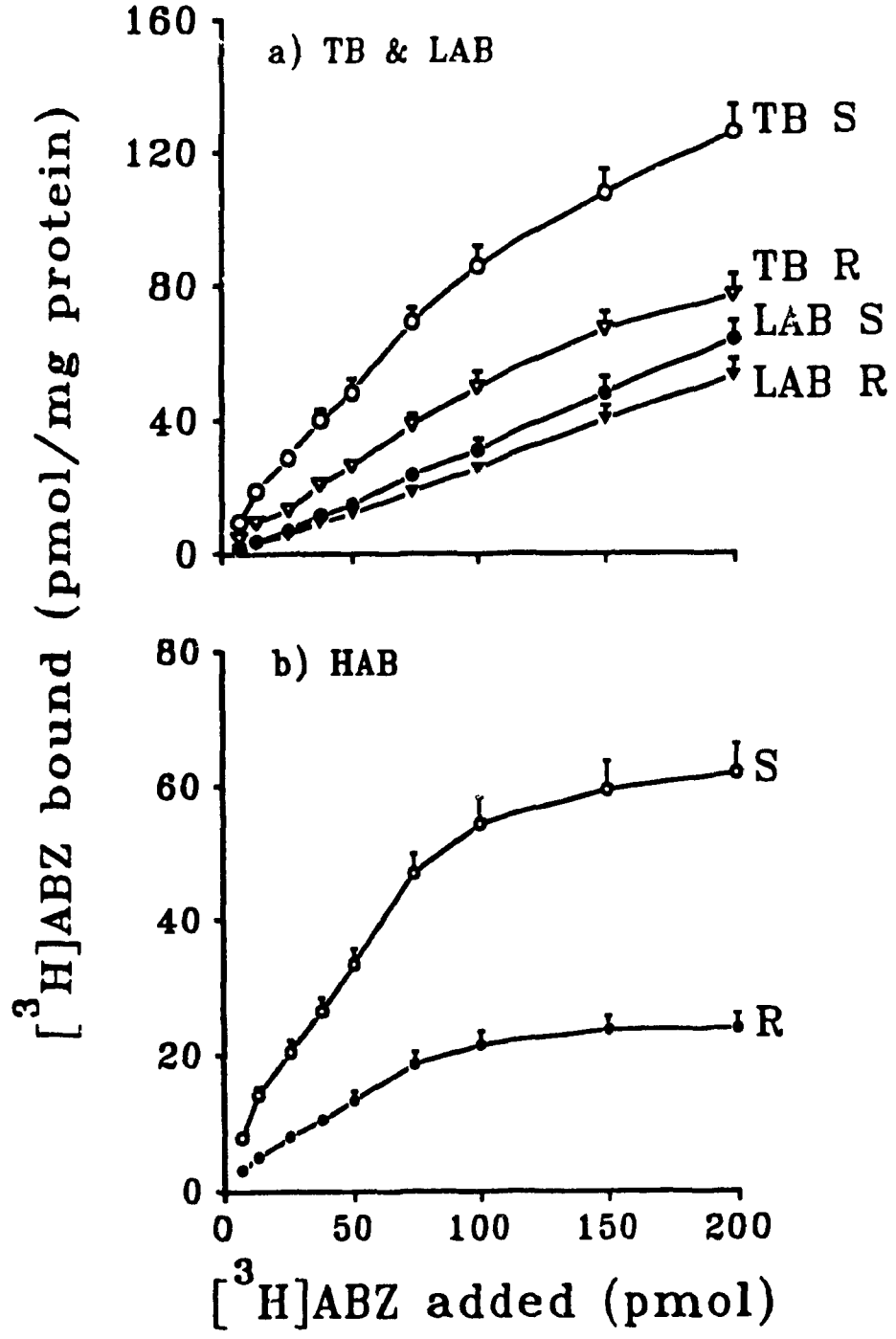

a
EGG
TB S
TB R
LAB S
LAB R
160
120
80
40
0
b
EGG
HAB S
HAB R
120
80
40
0
c
LARVAL
TB S
TB R
LAB S
LAB R
240
200
160
120
80
40
0
d
LARVAL
HAB S
HAB R
80
40
0
e
ADULT WORM
LAB S
TB S
LAB R
TB R
120
80
40
0
f
ADULT WORM
HAB NOT
DETERMINED
80
40
0
0
50
100
150
200
[3H]OFZ bound (pmol/mg protein)
[3H]OFZ added (pmol/assay)

Fig. 4.2. **TB and apparent LAB and apparent HAB of ABZ in S or R egg supernatants.** Each point is a mean ± SE of 3 separate experiments conducted in duplicate as described in Materials and Methods.

EGG


a) TB & LAB
TB S
TB R
LAB S
LAB R
160
120
80
40
0
b) HAB
S
R
80
60
40
20
0
0
50
100
150
200
[3H]ABZ bound (pmol/mg protein)
[3H]ABZ added (pmol)

saturable within the [drug] used. However, unlike [^{3}H]OBZ or [^{3}H]MBZ in which both TB and HAB of egg supernatants are readily saturable, TB of [^{3}H]ABZ was not saturated within the range of [drug] used.

4.4 DISCUSSION

TB of [^{3}H]OBZ and [^{3}H]MBZ at 37°C is readily resolved into high-affinity binding (HAB) and low-affinity binding (LAB) (Chapter III). The HAB of [^{3}H]MBZ and [^{3}H]OBZ to supernatants of *H. contortus* was reduced for the R strain compared with the S strain, while the LAB was not affected. Similar results were obtained using [^{3}H]ABZ (Fig. 4.2). However, compared to [^{3}H]OBZ or [^{3}H]MBZ, the binding of [^{3}H]ABZ to egg supernatants was more difficult to saturate. Although the binding of [^{3}H]OFZ was not saturable and could not be easily resolved into LAB and HAB, the ability of [^{3}H]OFZ to bind to the supernatants of the R strain was reduced (Fig.4.1). Readily saturable binding indicates strong affinity for a specific receptor (Burt, 1985; Unnerstall, 1990). In general, OFZ binding was of low-affinity type (non-displaceable) and non-saturable. OFZ, thus, fails to meet some of the principle criterion for identification of a specific receptor (Burt, 1985). However, exactly what constitutes 'high-affinity' depends on context. It has been suggested as a general guide that a ligand capable of causing 50% radioligand inhibition (cf. IC_{50} value) at concentrations known to elicit pharmacological response <u>in vivo</u>, has high-affinity for the receptor (Creese, 1985). The IC_{50} for OFZ was determined by displacement (inhibition) assay (Chapter V). Based on the saturation criterian, the data in this Chapter suggest that the tubulin binding affinity of [^{3}H]ABZ is intermediate between that of [^{3}H]OBZ/MBZ and [^{3}H]OFZ. When saturation

has not been achieved within the range of [ligand] used, mathematical analysis will be inaccurate and meaningless (Bennett and Yamamura, 1985; Unnerstall, 1990). The binding affinities of ABZ and OFZ were compared with other unlabelled BZs using the displacement assay (Chapter V). Meanwhile it can be concluded that resistance affects the binding of OBZ, MBZ, ABZ and OFZ and possibly other BZs.

Chapter V

UNLABELLED BZS: DISPLACEMENT ASSAY

5.1 INTRODUCTION

Cross-displacement (inhibition) studies were performed 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 the high-affinity receptors in supernatants derived from S and R strains. There are four basic criteria for selection of a radioligand for use in displacement studies (Enna, 1984; Munson, 1984; Bylund and Yamamura, 1990): a) **selectivity**: While, ideally it is best to use a ligand known to interact with only a single site , such specificity is exceptional. Accordingly, efforts must be directed towards finding a ligand that is relatively selective for the receptor under investigation; b) **high affinity**: If the affinity of the ligand for the receptor is too low, then the concentration that may be used will also result in binding to a significant number of nonspecific sites, making it difficult to study the receptor; c) **stability**: The radioligand must be chemically stable and free from metabolic degradation; and d) **specific activity**: Because of the extremely small quantities of radioligand used in a binding assay, a high specific activity is necessary to maintain a ratio of specific to nonspecific binding high enough to quantify the displaceable component. It can be deduced from Chapter III that [^{3}H]OBZ and [^{3}H]MBZ satisfy these criteria.

Displacement of radioligand binding by unlabelled ligands can be used to determine the K_a values of the latter, provided well characterised

labelled ligands are available (see Molinoff et al., 1981 for review). In some instances the displacement approach is more applicable than the saturation approach. It is particulary useful if the unlabelled ligands of interest cannot be obtained in the labelled form. It requires less parasite material because non-specific binding (NSB) is estimated at the single labelled-drug concentration used. In the saturation approach NSB has to be determined over the entire range of [label] used especially when NSB is non-linear. However if NSB is linear, it may be determined by linear regression using a few points. It was shown in Chapter III that NSB (LAB) was not necessarily linear. Using the displacement approach several ligands may easily be characterised and compared in a single run, thereby limiting between experiment variations. If saturation cannot be achieved using the labelled form of a drug, then the displacement approach becomes more appropriate. Since a very low concentration of the labelled ligand (against a range of unlabelled drug concentrations) must be used, possible interferences from NSB, encountered at high concentrations of the labelled ligand, are avoided.

As outlined in Chapter II, LIGAND analyses binding data by fitting curves of total ligand (labelled plus unlabelled, in case of displacement data) versus bound ligand. However, for convenience LIGAND presents displacement data as a plot of log [displacer] versus % bound which is a sigmoid curve and provides quick visual comparison of ligands by their IC_{50} values (see Fig. 5.1). The data can be linearized by a Hofstee plot (Hofstee, 1952) of bound versus bound/[displacer] in order to obtain initial estimates of the binding constants for the displacer. LIGAND utilises iterative curve fitting and a non-linear least square method to obtain accurate estimates of K_a and B_{max} values from displacement data.

However to do so, LIGAND must be supplied, by the user, with initial estimates of K_a and B_{max} of the unlabelled ligands and an accurate K_a value of the radioligand. An accurate K_a for the radioligand must be determined by the saturation method. Initial estimates for the unlabelled ligand may be deduced by experience or calculated from a Hofstee plot of the displacement data. LIGAND can provide accurate estimates starting with preliminary ones that are within a range of 10-100 times more or less the real values (see McPherson, 1987). However, the closer the initial estimates are to the true values, the greater are the chances for a successful iteration using LIGAND. It is shown in this Chapter that unlabelled BZs (FBZ, OBZ, MBZ, ABZ, OFZ and TBZ) may be similarly affected by resistance and may bind to similar high-affinity receptors as [^{3}H]OBZ and [^{3}H]MBZ.

5.2 MATERIALS AND METHODS

5.2.1 Egg supernatants

Labelled OBZ or MBZ whose binding constants were previously characterised (see Chapter III) were used to monitor the binding of unlabelled OBZ, MBZ, FBZ, ABZ, OFZ or TBZ. Final concentrations (in 100 μl assay volume) of the unlabelled BZs necessary to produce zero to maximum displacement of 1 μM [^{3}H]BZ were empirically determined for each BZ before the final assay. Stock concentrations of the unlabelled BZs required to produce the necessary final concentrations were freshly prepared. Two μl of pure DMSO or various stock concentrations of unlabelled BZ in pure DMSO were added to 90 μl of either buffer (blank) or S or R samples and incubated in triplicate for 30 min at 37°C. Then 10 μl of 1 μM [^{3}H]OBZ or

[^{3}H]MBZ were added and the incubation continued for a further 30 min at 37°. The assay was completed as outlined in Chapter II 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 the labelled ligand only; dpm_{ls} and dpm_{lb} = dpm of sample and blank, respectively, in the presence of the labelled ligand only; dpm_{us} and dpm_{ub} = dpm of sample and blank, respectively, in the presence of the labelled and unlabelled ligands and B_i = dpm bound in presence of a given concentration, i, of the unlabelled ligand and the labelled ligand. Note that all tubes contained a fixed final labelled-BZ concentration of 0.1 μM.

The values of B_m and B_i were entered into EBDA/LIGAND (McPherson, 1987) to determine the low-affinity binding, the IC_{50} and the K_a of the unlabelled ligand and the high affinity receptor concentration (B_{max}). B_i in the presence of excess homologous unlabelled ligand was supplied as the initial estimate of LAB. However, the final level of LAB was determined (and subsequently subtracted) simultaneously with the estimation of the other parameters using EBDA or LIGAND.

5.2.2 Adult worm supernatants

Because of the lower content of HAB and the higher content of LAB in

the adult worm supernatants compared to the egg supernatants, a slightly different protocol was followed to compare the ability of unlabelled BZs to bind the high-affinity receptors in S or R derived adult worm supernatants. Final concentrations of the unlabelled BZs necessary to produce zero to maximum displacement of 0.2 μM [^{3}H]OBZ or [^{3}H]MBZ were determined for each BZ before the final experiment. Stock concentrations of the unlabelled BZs required to produce the necessary final concentrations were fresh preparations. Two μl pure DMSO in MES buffer or 2 μl of stock unlabelled BZ in pure DMSO were added in triplicate to 90 μl of either buffer (blank) or parasite supernatants and preincubated for 30 min at 37° C. Then 10 μl of 2 μM [^{3}H]MBZ or [^{3}H]OBZ were added to each tube and incubated for a further 30 min at 37° C. The assay was continued as described in chapter II to determine the bound dpm in each tube. Counts (dpm) were calculated for B_m and B_i as outlined above.

5.2.3 Mathematical analysis

For analysis of displacement data, the EBDA programme was used to transform the data into log(% bound/(100 - % bound)) versus log [displacer] in order to obtain initial estimates of the slope factor, P, and IC_{50}. The latter were then automatically introduced into the iterative portion of EBDA to obtain final estimates of P and IC_{50} by fitting the curve described by the following equation (Rodbard, 1984):

$$\text{Bound (dpm)} = \frac{(B_m - \text{NSB})}{(1 + ([\text{displacer}] / IC_{50})^P)} + \text{NSB}$$

where:

B_m = maximum dpm bound in absence of displacer, NSB = dpm non-specifically bound (LAB in this case), P = the slope factor (known

as the Hill coefficient), IC_{50} = concentration of displacer inhibiting 50 % specific (HAB in this case).

The EBDA programme could then be used to calculate the association constant, K_i, of the inhibitor (displacer) by relating IC_{50} to the association constant, K_a, of the radioligand as described by Cheng and Prusoff (1973):

$$\frac{1}{K_i} = \frac{IC_{50}}{(1 + H^*K_a)}$$

where:

H^* = concentration of radioligand.

This equation assumes that drugs are interacting competitively at the receptor site. However, the association constant values of the displacers could also be calculated, together with B_{max}, using LIGAND. For analysis using LIGAND the K_a of [^{3}H]OBZ and [^{3}H]MBZ were fixed at 10^7 M^{-1} in order to directly compare the affinities of the unlabelled ligands for the S and R receptors. The K_a of [^{3}H]OBZ and [^{3}H]MBZ were found by saturation study to be of the order of 10^7 M^{-1} (see Chapter III).

5.3 RESULTS

5.3.1 Egg supernatants

The results are summarized in Table 5.1 and refer to high-affinity binding. It was estimated, by LIGAND analysis, that the amount of low-affinity binding was insignificant at the 0.1 μM final label concentration

used and was, therefore, ignored (fixed to 0) during the analysis. The B_{max} values of the R strain were lower than those of 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. The B_{max} values were independent of the unlabelled ligand used indicating that the latter displaced the labelled ligands to a similar extent and that the displacement was always complete. (In the displacement study, B_{max} values represent the concentration of the high affinity receptors recognised by the radioligand that are also recognised by the unlabelled ligand).

By inspection of K_a or IC_{50} values (Table 5.1) the ability of BZs to bind the high-affinity receptors can be ranked. 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 :

$$FBZ \geq MBZ \geq OBZ > ABZ > OFZ > TBZ$$

However, the K_a values derived from the displacement of [^{3}H]OBZ were generally higher than those derived from [^{3}H]MBZ displacement. The K_a values calculated based on Cheng-Prosoff's equation for competitive interactions, using EBDA, and those calculated based on the direct ligand-receptor interaction model, using LIGAND were not significantly different ($P > 0.05$).

5.3.2 Adult worm

Parameter estimates from the displacement studies, involving preincubation at 37°C, are shown in Tables 5.2 from which the rank order of the affinities and IC_{50} values of the BZs may be inferred. The rank order of IC_{50} values of the various BZs can also be inferred graphically in Fig. 5.1 from which it may also be noted that all the curves converge

Table 5.1 Inhibition of [^{3}H]BZ[a] binding to tubulin in egg supernatants[b] of S or R strains.

[^{3}H]BZ	BZ[c]	S strain[d]			R strain[d]		
		IC_{50}[e]	K_a[f]	B_{max}[g]	IC_{50}[e]	K_a[f]	B_{max}[g]
[^{3}H]OBZ	FBZ	98	36	102	42	40	22
	OBZ	165	25	95	87	28	22
	MBZ	127	32	109	106	32	22
	ABZ	320	7	97	207	8	19
	OFZ	1212	3.2	106	1006	3.6	21
	TBZ	3080	0.9	89	2600	1.2	21
[^{3}H]MBZ	FBZ	125	34	107	129	27	41
	OBZ	229	15	104	325	7	42
	MBZ	161	24	105	105	29	39
	ABZ	346	8	98	208	9	37
	OFZ	1520	2.8	106	1070	3.3	40
	TBZ	3344	1	97	617	1	35

a Final [(^{3}H)BZ] was 0.1 μM.

b Final [protein] was 100-200 μg/assay.

c Final concentration: 0-20 μM for FBZ, MBZ & OBZ; 0-50 μM for ABZ and 0-200 μM for OFZ & TBZ.

d Mean of 3 experiments each done in triplicate.

e IC_{50} = [BZ] inhibiting 50% of radioligand binding (nM).

f K_a = apparent association constant of the unlabelled BZ (x 10^6 M^{-1}).

g B_{max} = [High-affinity receptor] (pmol/mg protein).

Table 5.2 Inhibition of [^{3}H]BZ[a] binding to tubulin in adult worm supernatants[b] of S or R strains.

[^{3}H]BZ	BZ[c]	S strain[d]			R strain[d]		
		IC_{50}[e]	K_a[f]	B_{max}[g]	IC_{50}[e]	K_a[f]	B_{max}[g]
[^{3}H]OBZ	FBZ	159	17.6	27	nd	nd	nd
	OBZ	124	22.2	22	nd	nd	nd
	MBZ	197	15.3	28	nd	nd	nd
	ABZ	648	2.7	27	nd	nd	nd
	OFZ	1223	1.7	23	nd	nd	nd
	TBZ	3863	0.54	26	nd	nd	nd
[^{3}H]MBZ	FBZ	226	16.5	28	100	26.8	9.5
	OBZ	176	18.8	25	109	25.4	7.7
	MBZ	187	23.4	30	223	15.8	11.0
	ABZ	874	3.3	28	1580	2.0	7.9
	OFZ	1603	1.7	27	779	4.3	9.2
	TBZ	5624	0.42	26	7033	1.6	7.8

a Final [(^{3}H)BZ] was 0.2 μM.

b Final [protein] was 100–200 μg/assay.

c Final concentrations: 0–20 μM for FBZ, MBZ & OBZ; 0–50 μM for ABZ and 0–200 μM for OFZ & TBZ.

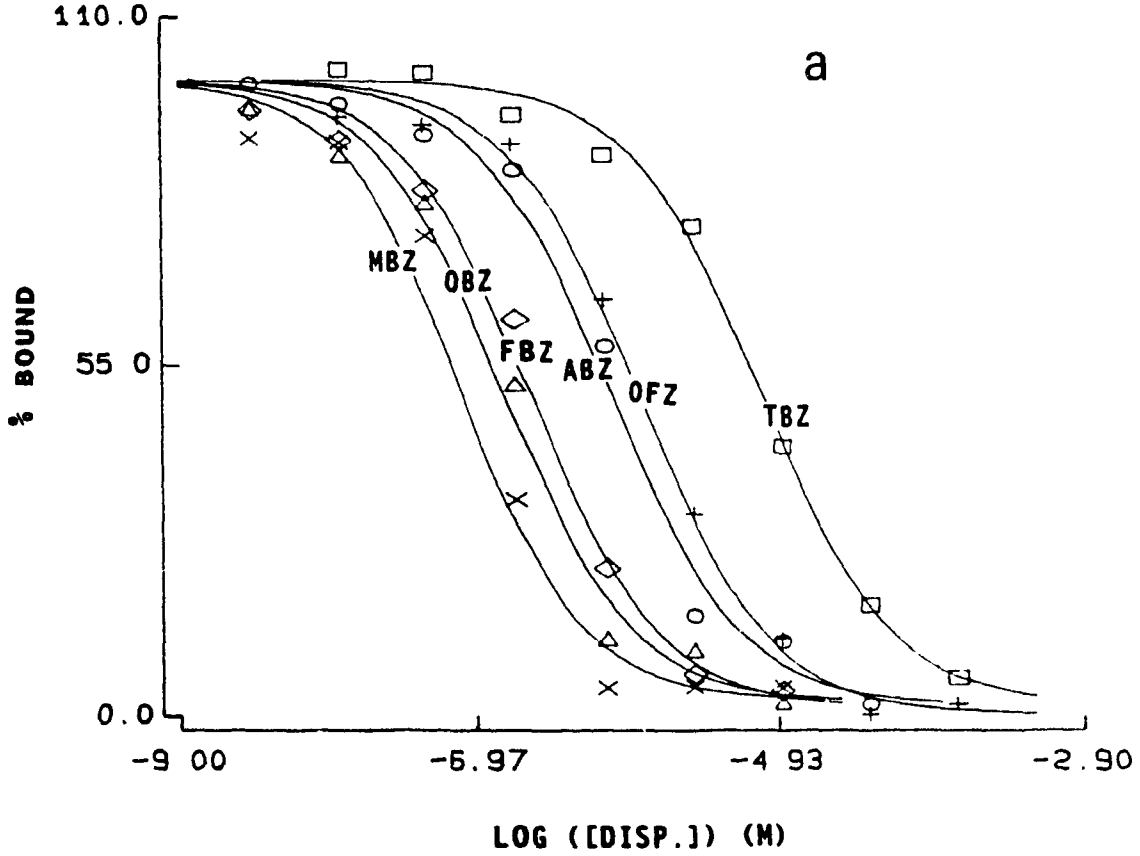
d Mean of 3 experiments each done in triplicate.

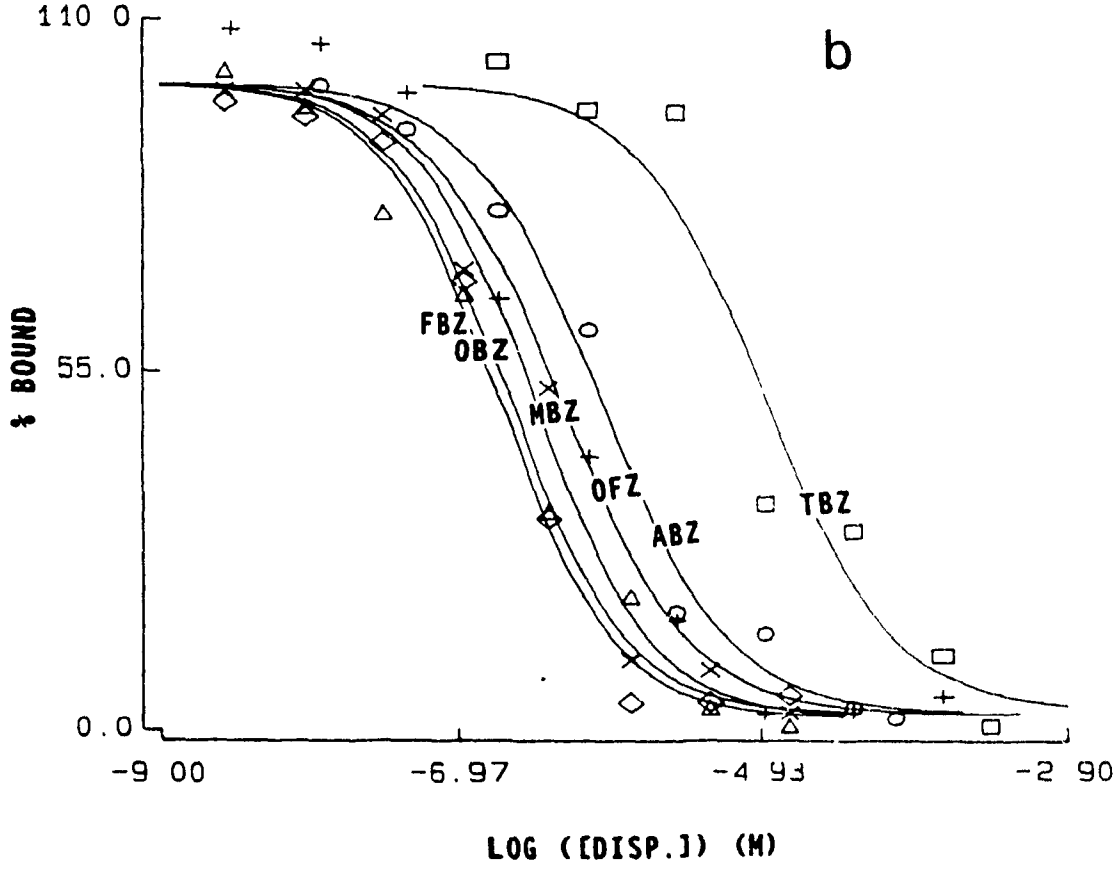
e IC_{50} = [BZ] inhibiting 50% of radioligand binding (nM).

f K_a = apparent association constant of unlabelled BZs (x 10^6 M^{-1}).

g B_{max} = [High-affinity receptor] (pmol/mg protein).

Fig. 5.1. LIGAND generated displacement curves for the specific displacement (inhibition) of MBZ by unlabelled BZs in S (a) or R (b) supernatants. Adult worm supernatants were incubated in triplicate with unlabelled BZs at 37°C for 30 min. Then the [^{3}H]MBZ was added and incubated for a futher 30 min. The analyses were performed by fitting curves of total ligand concentration versus bound ligand, using LIGAND. The data refer to specific binding (HAB) and are presented in this co-ordinate system, after the analysis, for visual inspection. Note that all curves converge to a similar minimum suggesting that all the BZs bind to the same receptors to a similar extent. The concentration required to inhibit the radioligand binding by 50% (IC_{50}) is a measure of the affinity of the BZs for the high-affinity receptors and can be read from the graph. The curves of the lower affinity ligands are displaced towards the right.


110.0
55 0
0.0
% BOUND
a
MBZ
OBZ
FBZ
ABZ
OFZ
TBZ
-9 00
-6.97
-4 93
-2.90
LOG ([DISP.]) (M)


110 0
55.0
0.0
% BOUND
b
FBZ
OBZ
MBZ
OFZ
ABZ
TBZ
-9 00
-6.97
-4 93
-2 90
LOG ([DISP.]) (M)

to a common minimum.

With the S strain, the displacement of [^{3}H]OBZ gave the following rank order of affinity:

OBZ $\geq$ FBZ $\geq$ MBZ >> ABZ > OFZ >> TBZ

which was broadly similar to that obtained for the displacement of [^{3}H]MBZ from the S strain:

MBZ $\geq$ OBZ $\geq$ FBZ >> ABZ > OFZ >>TBZ

or for the displacement of [^{3}H]MBZ from the R strain:

FBZ $\geq$ OBZ > MBZ >> OFZ > ABZ >> TBZ

The same rank order of strength is obtained whether IC_{50} or K_a values were considered (Table 5.2). The B_{max} values obtained for the S strain were higher than those obtained for the R strain. [^{3}H]OBZ was more affected by resistance than was [^{3}H]MBZ. The displacement of [^{3}H]OBZ from the R strain was not analyzed because of insufficient HAB.

5.4 DISCUSSION

It has been shown using the displacement approach that the B_{max} values for the HAB of the R strain were lower than those of the S strain. The B_{max} values of the R strain, and not the S strain, depended on the radioligand displaced with [^{3}H]OBZ showing lower values than [^{3}H]MBZ (Table 5.1 & 5.2). It could not be determined whether MBZ is able to see some receptors not seen by OBZ or whether ligand-receptor interaction is different between OBZ and MBZ due to conformational barriers (Lacey and Watson, 1985) or due to co-operative effects (see Yamamura et al., 1985). Positive co-operativity, whereby previous ligand binding may enhance subsequent ligand binding, can affect one BZ more strongly than another.

While the reason for this is not known, it suggests that, compared to MBZ, OBZ is more affected by resistance and may perform more poorly against resistant H. contortus in vivo.

It can be seen in Fig. 5.1 that all the displacement curves tend to converge to a common minimum (see also Chapter IX & X) indicating that all BZs displace to the same extent albeit at widely different concentrations. Cross-displacement analysis indicated that B_{max} values were independent of the unlabelled ligand used indicating that all the BZs studied could displace the [^{3}H]BZs to the same extent. This suggests (see Enna, 1984) that all the BZs were able to bind to the same high-affinity receptors defined by OBZ or MBZ. The observation that the BZs can displace each other suggests that the high-affinity receptors they recognise are similar and there may be a single class of sites for all the BZs. It could not be determined if they recognise similar low-affinity receptors.

According to the IC_{50} and K_a values the BZs may be ranked as follows:

$$FBZ \geq MBZ \geq OBZ \gg ABZ > OFZ \gg TBZ$$

The rank order is largely independent of the radioligand displaced or the strain used. This further suggests that all the BZs studied bind to the same class of sites, albeit with different affinity. While B_{max} values are consistently and significantly reduced for the R strain, the differences between the K_a values of the S and R strains do not follow a consistent pattern. Generally the K_a values of the S and R strains are not significantly different. Similar observations were made using the saturation assay (Chapter III). Thus resistance is associated with a loss of high-affinity receptors but the K_a is not affected. The displacement data suggest that all the BZ anthelmintics studied (FBZ, MBZ, OBZ, ABZ, OFZ and TBZ) are affected by resistance; they all bind the same high-

affinity receptors and the high-affinity receptors of the S and R strains may be similar although reduced for the R strain.

Chapter VI

THE ROLE OF TUBULIN IN THE HIGH-AFFINITY BINDING OF BZS

6.1 INTRODUCTION

The evidence for the involvement of parasite tubulin in the binding and mode of action of BZ anthelmintics is largely indirect (see Lacey, 1988 for review). Borgers and De Nollin (1975) first reported the *in vivo* disintegration of the normal microtubule matrix in intestinal cells of *Ascaris suum* treated with MBZ. Subsequent studies confirmed this observation in other BZ-sensitive parasites (Atkinson *et al.*, 1980, Sangster *et al.*, 1985). Suspicion that BZs might bind parasite tubulin is strengthened by *in vitro* observations that BZs inhibit the polymerization of tubulin purified from mammalian (Friedman and Platzer, 1978; Ireland *et al.*, 1979; Laclette *et al.*, 1980) or parasite (Barrowman *et al.*, 1984; Dawson *et al.*, 1984) tissues. However, BZs are selectively toxic to parasites while being non-toxic to mammals. Demonstration of the basis of this selectivity would provide a fundamental argument for tubulin as the site of BZ action. It is still necessary to substantiate the role of parasite tubulin in BZ binding. Colchicine is known to bind tubulin and inhibit its polymerisation to microtubules (Dustin, 1984). BZs have been shown to inhibit the binding of [^{3}H]colchicine to purified mammalian tubulin (Friedman and Platzer, 1978; Havercroft *et al.*, 1981) or parasite extracts (Friedman and Platzer, 1980; Köhler and Bachmann, 1981). Reports of total [^{3}H]BZ binding to whole parasite extracts are available (Lacey, 1988). There are a number of reports in which purified mammalian tubulin has been used to study BZ-tubulin interactions (see Lacey, 1988 for

review). However, there are limited reports in which pure parasite tubulin has been used in such studies. Dawson et al. (1984) and Barrowman et al. (1984) have reported the inhibition of polymerization of tubulin purified from the large nematodes, Ascaridia galli and Ascaris suum, respectively. A major limitation for the demonstration of direct [^{3}H]BZ binding to parasite tubulin is the difficulty encountered in purifying parasite tubulin. Unlike mammalian brain, the concentration of tubulin in most parasites seems to be below the critical level necessary for purification using the classical procedures (Lacey and Snowdon, 1990). Therefore, most of the recent evidence which incriminates tubulin in BZ-binding is based on the comparative binding of [^{3}H]BZs to purified mammalian tubulin (see Lacey, 1988) or parasite whole supernatants or tubulin enriched parasite extracts (Lacev and Prichard, 1986; Tang and Prichard, 1988, 1989). In Chapter III binding specificity has been based on the inhibition of [^{3}H]BZ binding by the homologous unlabelled BZs. Accordingly two types of binding, the high-affinity and low-affinity, were identified. In this chapter I have used poly-L-lysine purified fractions to demonstrate that tubulin may be the high-affinity binding molecule.

6.2 MATERIALS AND METHODS

6.2.1 Poly-l-lysine (PLL) linked agarose chromatography

Parasite supernatants from eggs, larvae or adult worms were enriched for tubulin using poly-L-lysine (PLL) agarose chromatography (Lacey and Snowdon, 1990). Ten to 25 mg of the crude protein extracts in 5 ml of 0.025 M MES buffer were applied to a C16 column (Pharmacia) packed with a 10 ml bed of PLL agarose (Sigma) at 4° C and eluted sequentially with 2 x 10 ml 0.025 M MES buffer, 3 x 10 ml 1.25 % (w/v) $(NH_4)_2SO_4$, 3 x

10 ml 2.5% (w/v) $(NH_4)_2SO_4$ and 5% (w/v) $(NH_4)_2SO_4$ in MES buffer and collected in 1 ml fractions. Protein concentrations were measured for each fraction and 3 major protein peaks corresponding to 0.025 M MES (peak 1), 1.25 % and 2.5 % $(NH_4)_2SO_4$ (peak 2) and 5 % $(NH_4)_2SO_4$ (peak 3) were identified. Preliminary studies showed that drug binding may not be detectable using dilute column fractions and high $(NH_4)_2SO_4$ salt content may inhibit binding. Therefore, fractions corresponding to each protein peak were pooled and concentrated (reduced to < 3% of the original volume) and desalted by ultrafiltration using membrane cones (Amicon). All operations were conducted at 4° C. The concentrate was reconstituted in MES buffer and stored in liquid N_2 or briefly on ice before use. Each protein peak was subjected to BZ binding, ELISA and electrophoretic analysis.

6.2.2 Binding of PLL purified fractions

Assays were performed on the PLL purified preparations in order to determine those fractions which contained HAB. In order to compare all fractions for both strains at the same time, the fractions were compared immediately after purification and concentration using a stock [^{3}H]BZ concentration of 2 μM. Then a range of [^{3}H]BZ concentrations was used to determine those fractions which contained saturable binding.

6.2.3 SDS-PAGE and immunoblotting of PLL purified fractions

SDS-PAGE and Western blotting using highly specific mouse anti-α- or anti-ß-chicken tubulin monoclonal antibodies (Amersham) and peroxidase conjugated anti-mouse IgG (BioCan) were performed on PLL-agarose chromatography fractions in order to determine those fractions which contained tubulin. Between 0.2 and 1 μg purified egg tubulin or 5 and 30

μg crude protein per well were routinely loaded on SDS-PAGE gels. For comparison of different samples and fractions equal protein loads were used. However, the protein load was increased several fold for fractions which appeared negative for tubulin content, to ensure that these fractions did not contain tubulin.

6.3 RESULTS

6.3.1 Binding and western blot of PLL purified egg fractions

The results of PLL chromatography and the subsequent [^{3}H]OBZ binding of egg proteins are shown in Fig. 6.1 and 6.2. The results were similar to those described below for adult worms and larvae except that all the binding was recovered in peak 3. There was no binding in peak 1 and 2 which together accounted for > 90% of the total protein recovered from the column (see Fig. 6.1a). Binding increased with [drug] for peak 3 but not for peaks 1 and 2. The HAB of [^{3}H]OBZ in peak 3 was saturable (Fig. 6.2) indicating that it was specific binding. LAB in the third peak was relatively low and apparently saturable. Most of the binding in peak 3 was HAB and the R strain demonstrated less HAB than the S strain. Similar results were obtained using [^{3}H]MBZ.

On the stained SDS-PAGE gel, tubulin was the major band in the third peak (Fig. 6.3a). By Western blot (Fig. 6.3b) and ELISA tubulin was found in peak 3 only.

6.3.2 Binding and western blot of PLL purified adult worm fractions

The results of PLL chromatography and the subsequent [^{3}H]OBZ binding of adult worm proteins obtained, by PLL chromatography are shown in Fig.

6.4 and 6.5. Similar results were obtained using larval proteins (not shown). LAB binding was present in all the 3 peaks but HAB was restricted to the third peak (Fig. 6.4). The HAB of [^{3}H]OBZ in peak 3 was saturable (Fig. 6.5). Unlike the egg fractions, LAB of all the peaks of adult or larval preparations, increased linearly with [drug] and was not saturable within the range of [drug] tested, indicating chat it may have been non-specific binding. LAB of the S and R strains were similar but the HAB of the R strain was lower than that of the S strain. Similar results were obtained using [^{3}H]MBZ. The third peak accounted for < 15 % of the total protein eluted from the column (see Fig. 6.4a).

Western blot (Fig. 6.6b) of the 3 peaks for larvae and adult worm supernatants showed that tubulin was present in the third peak only. On the stained gel (Fig. 6.6a), the purity of tubulin in the third peak was low and the tubulin band could not be identified precisely with Coomassie stain. The eluents were also analyzed by ELISA and there was no tubulin reaction in protein peaks devoid of HAB.

6.4 DISCUSSION

The data suggest that OBZ and MBZ bind tubulin with high-affinity while other proteins bind these ligands with low-affinity. By PLL-agarose chromatography, the HAB was found in tubulin-containing fractions only. However, LAB was also present in tubulin-rich fractions. It could not be determined whether some LAB occurs to some tubulin isoforms with low reactivity for BZs or whether the LAB that was found in the tubulin-rich fractions was due to non-tubulin proteins. Egg supernatants contained no LAB in peak 1 and 2 although there was LAB in peak 3 (Fig. 6.1). This LAB

Fig. 6.1. **OBZ binding of fractions obtained by PLL chromatography of S or R egg supernatants.** Ten to 25 mg of crude protein were applied to a column packed with PLL-linked agarose at 4°C and eluted with a concentration gradient of $(NH_4)_2SO_4$ in MES buffer to obtain protein peak 1 (P1), peak 2 (P2) and peak 3 (P3). Proteins corresponding to each peak were pooled, concentrated and quantitated (a). TB, LAB and HAB of the three peaks and whole supernatants (W) were compared using 20 pmol (0.2 μM final concentration) of [^{3}H]OBZ (b). HAB and tubulin were found in P3 only (see Fig. 6.3).

EGG

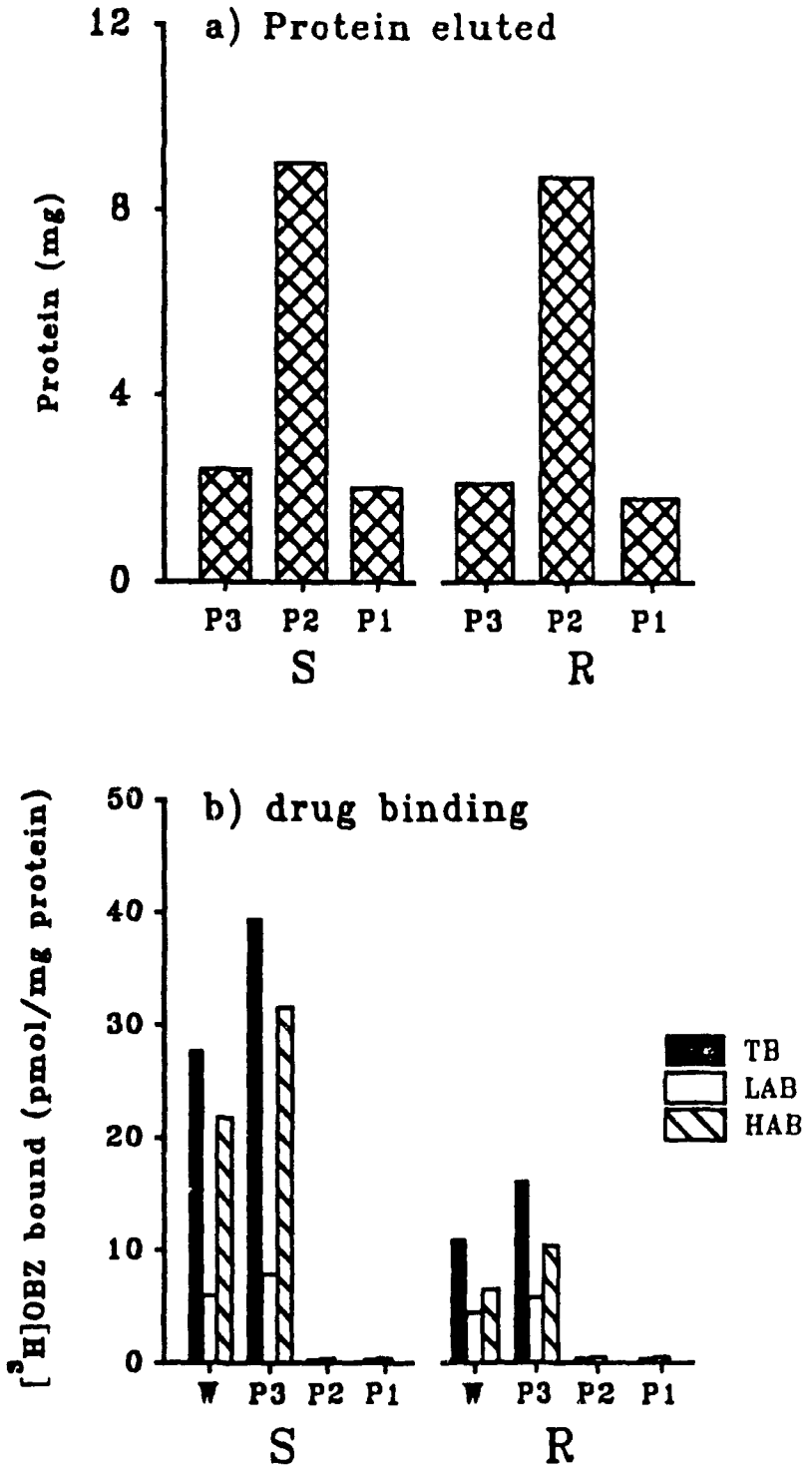

a) Protein eluted
Protein (mg)
12
8
4
0
P3
P2
P1
P3
P2
P1
S
R
b) drug binding
[3H]OBZ bound (pmol/mg protein)
50
40
30
20
10
0
W
P3
P2
P1
W
P3
P2
P1
S
R
TB
LAB
HAB

Fig. 6.2. Saturation analysis of OBZ binding of protein in peak 3 obtained by PLL chromatography of S or R egg supernatants. See Fig. 6.1 or Materials and Methods for details. There was no binding in peak 1 and 2 even when the drug concentration was increased. Peak 3 contained HAB (b) and tubulin (Fig. 6.3) but not peak 1 and 2. The data are the mean ± SE of three experiments each determined in triplicate.

EGG

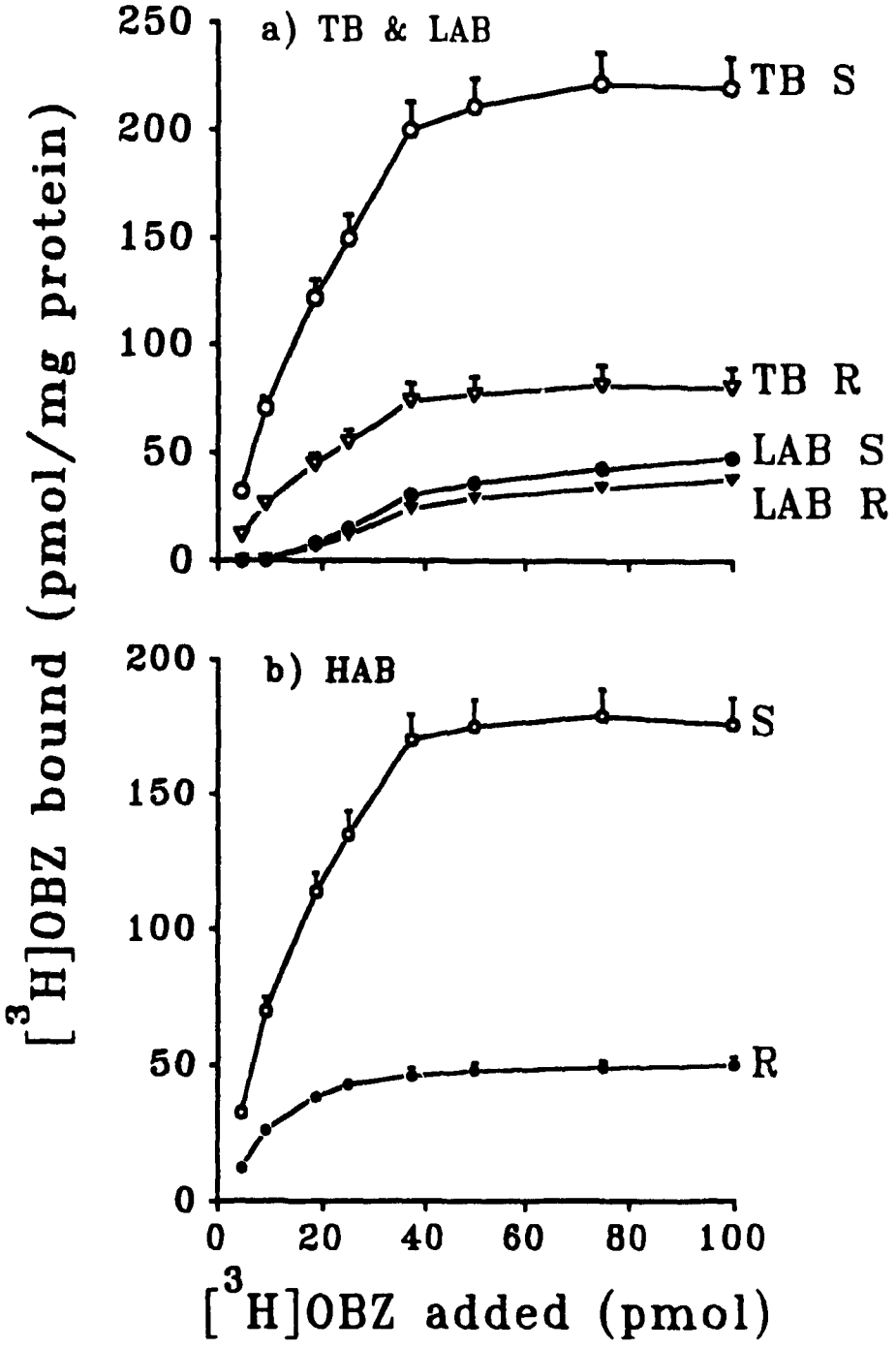
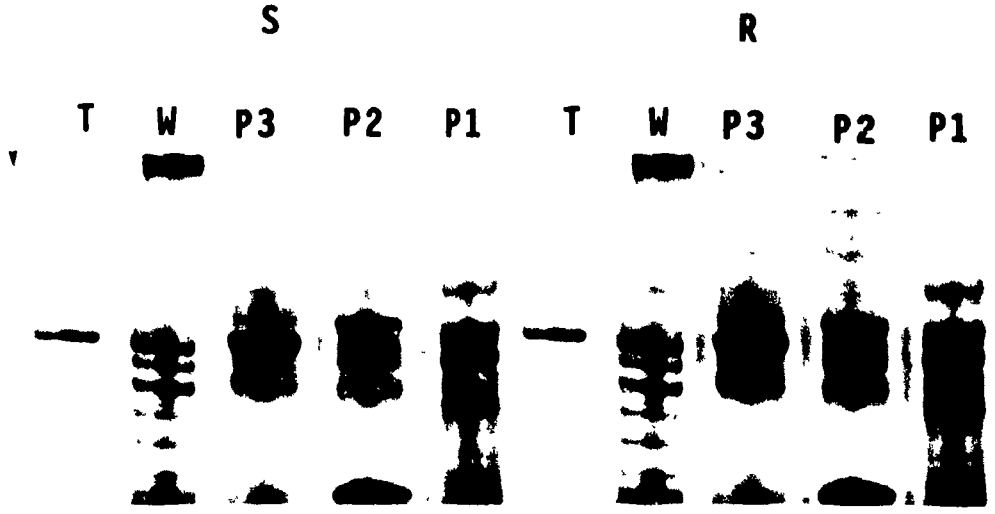
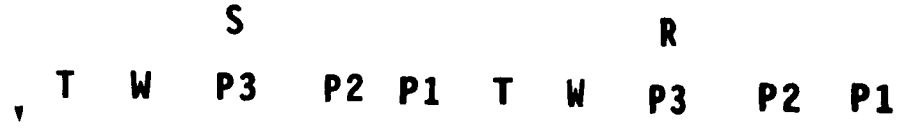

a) TB & LAB
TB S
TB R
LAB S
LAB R
b) HAB
S
R
250
200
150
100
50
0
0
20
40
60
80
100
[3H]OBZ bound (pmol/mg protein)
[3H]OBZ added (pmol)

Fig. 6.3. **SDS-PAGE and Western blot of protein peaks (P1, P2 & P3) obtained by PLL chromatography of S or R egg supernatants.** See Fig. 6.1 or Materials and Methods for details. The SDS-PAGE gel was stained with Coomassie blue and enhanced with silver (a). Tubulin was immunoblotted with anti-β-tubulin monoclonal antibodies (b). Western bolt of α-tubulin gave similar results. T = tubulin purified from H. contortus eggs using taxol.





tubulin

Fig. 6.4. **OBZ binding of protein peaks (P1, P2 & P3) obtained by PLL chromatography of S or R adult worm supernatants.** See Fig. 6.1 or Materials and Methods for details. Proteins from each peak were pooled, concentrated and quantitated (a). TB, LAB and HAB of the three peaks and the whole supernatants (W) were compared using 20 pmol (0.2 μM final concentration) of [^{3}H]OBZ (b). Binding increased with drug concentration for all peaks but HAB (Fig. 6.5) and tubulin (Fig. 6.6) were restricted to P3.

ADULT WORM

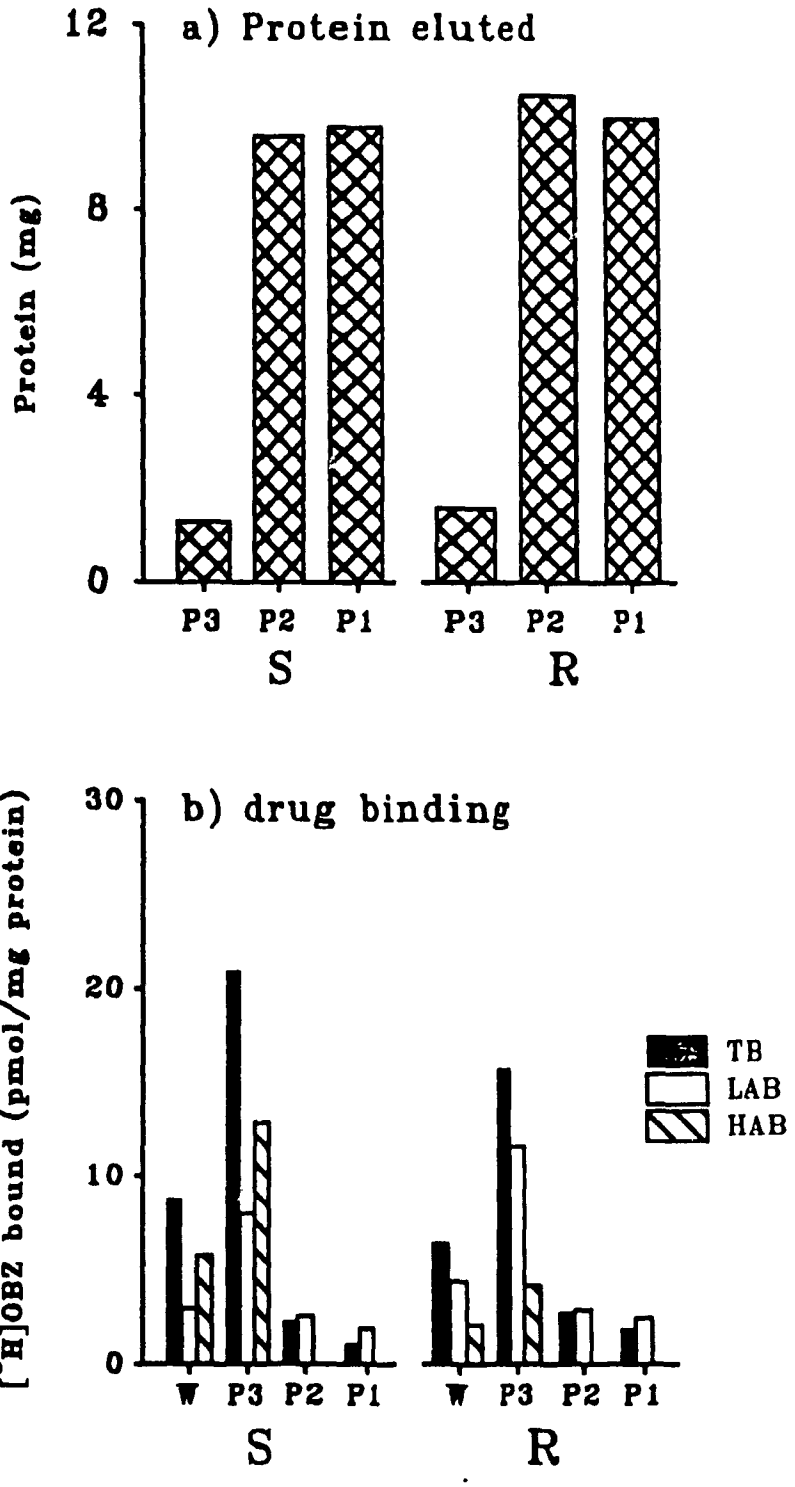

a) Protein eluted
Protein (mg)
12
8
4
0
P3
P2
P1
P3
P2
P1
S
R
b) drug binding
[3H]OBZ bound (pmol/mg protein)
30
20
10
0
TB
LAB
HAB
W
P3
P2
P1
W
P3
P2
P1
S
R

Fig. 6.5. **Saturation analysis of OBZ binding of protein peaks, P3 (a and b) and P1 and P2 (c), obtained by PLL chromatography of S or R adult worm supernatants.** See Fig. 6.1 or Materials and Methods for details. HAB of P3 (b) was saturable. There was no HAB in P2 and P1 but TB and LAB of P3 (a), and P1 and P2 (c) increased linearly. P3 contained tubulin but not P1 and P2 (Fig. 6.6). The data are the mean ± SE of three experiments each determined in triplicate.

ADULT WORM

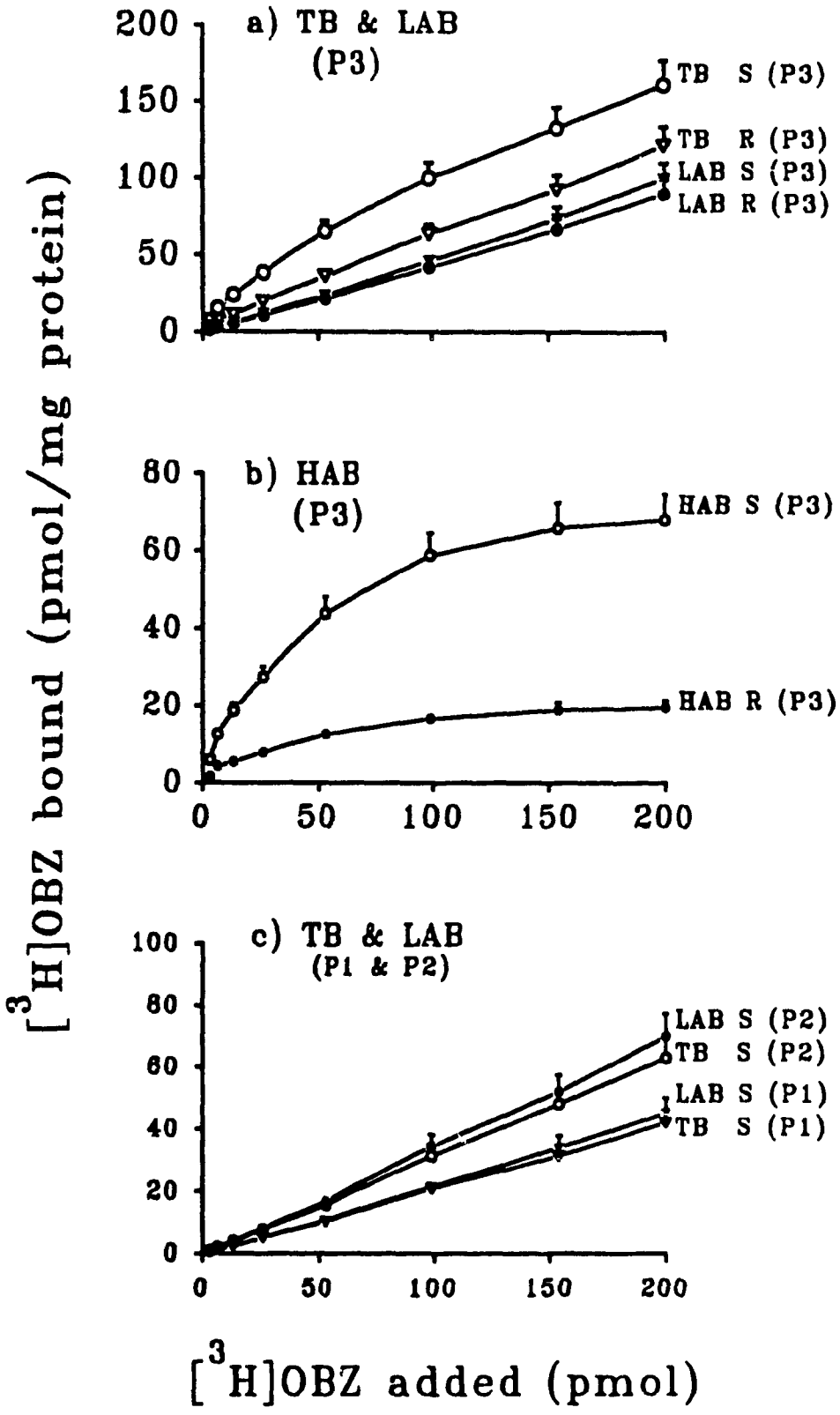
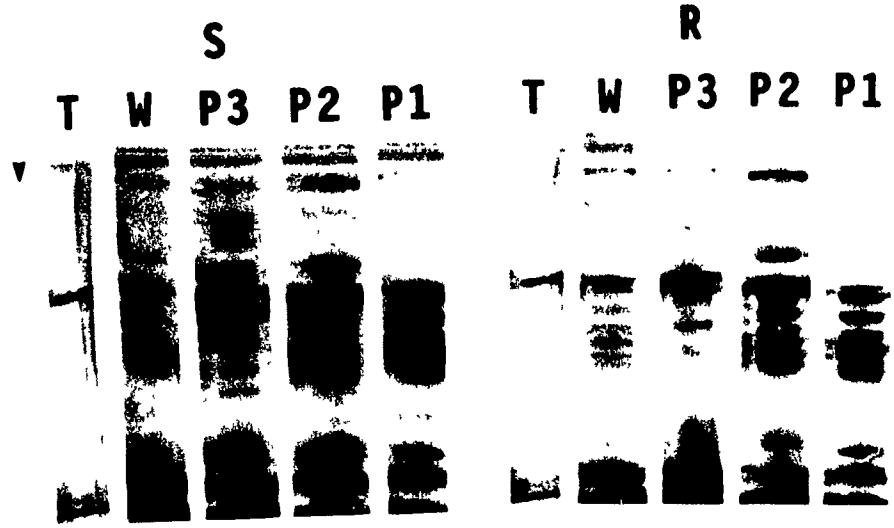
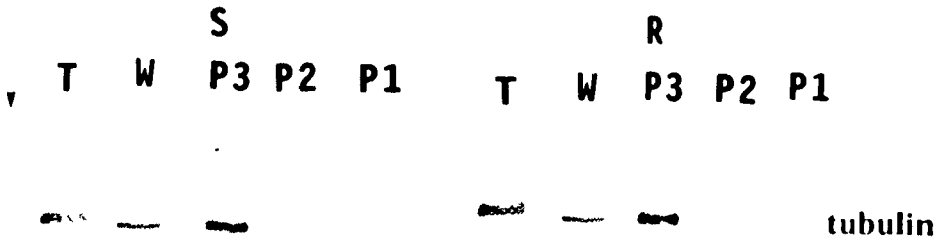

a) TB & LAB
(P3)
TB S (P3)
TB R (P3)
LAB S (P3)
LAB R (P3)
200
150
100
50
0
b) HAB
(P3)
HAB S (P3)
HAB R (P3)
80
60
40
20
0
0
50
100
150
200
c) TB & LAB
(P1 & P2)
LAB S (P2)
TB S (P2)
LAB S (P1)
TB S (P1)
100
80
60
40
20
0
0
50
100
150
200
[3H]OBZ bound (pmol/mg protein)
[3H]OBZ added (pmol)

Fig. 6.6. SDS-PAGE and Western blot of protein peaks (P1, P2 & P3) obtained by PLL chromatography of S or R adult worm supernatants. See Materials and Methods for details. The SDS-PAGE gel was stained with Coomassie blue and enhanced with silver stain (a). Tubulin was immunoblotted with anti-β-tubulin monoclonal antibodies (b). Western blot of α-tubulin gave similar results. T = tubulin purified from H. contortus eggs using taxol.


S
R
T W P3 P2 P1
T W P3 P2 P1


S
R
T W P3 P2 P1
T W P3 P2 P1
tubulin

in peak 3 of eggs was apparently saturable, suggesting specificity and possible tubulin involvement. However, adult worm and larval supernatants contained non-saturable (Fig. 6.4 & 6.5) low-affinity binding in peak 1, 2 and 3 indicating that LAB may be largely non-specific binding for these developmental stages. Figure 6.5 and previous studies in which whole larval or adult worm supernatants (Chapter III) were used suggest that LAB would be far greater than HAB at infinite ligand concentration. The first studies which described kinetics, including K_a (the apparent association constant) and B_{max} (maximum binding at infinite ligand concentration), of BZ-tubulin interaction using adult worm or larval supernatants of H. contortus were based on total binding. In chapter III, K_a and B_{max} values of BZ high-affinity (specific) binding to supernatants derived from BZ resistant and BZ susceptible H. contortus were reported. It was observed that the K_a of the S and R strains were similar while the B_{max} (maximum binding at infinite [ligand] = [receptor]) for R strain was reduced. Similar observations were made using tubulin-enriched extracts. It may be concluded that tubulin is the high-affinity binding molecule in parasite supernatants of H. contortus.

Chapter VII

TUBULIN CONTENT IN PARASITE SUPERNATANTS

7.1 INTRODUCTION

Tubulin content varies widely in different tissues and different organisms (Dustin, 1984). Brains of vertebrates are rich in tubulin. Embryonic chick brain tubulin can constitute as much as 42% of the soluble protein (Bamburg et al., 1973). However, in parasitic nematodes, tubulin content is very low. Friedman et al. (1980), for example, estimated tubulin to be only 0.3% of the soluble protein in eggs of Ascaris suum. Köhler and Bachmann (1981) estimated tubulin to be only 0.8% of the soluble protein in intestinal cells of A. suum. Tubulin was estimated to account for 0.25% of the soluble protein (Tang and Prichard, 1988) in N. brasiliensis and for about 3% (Tang and Prichard, 1989) in B. malayi and B. pahangi.

For estimation of the tubulin content in a tissue, the colchicine binding assay, using purified vertebrate tubulin as standard, is the most common method. However, the specificity of colchicine may not be 100% in all organisms or tissues. It is not clear, therefore, how much of the colchicine binds to non-tubulin proteins and the stoichiometry of colchicine-tubulin interaction of the standard tubulin may be different from that of the tubulin in crude preparations from different organisms. This method is also hampered by the rapid decay of colchicine-binding activity in cell homogenate (Wilson, 1970). Another method, called isotope-dilution vinblastine-precipitation method, has been reported by Burnside et al. (1973). In this method, isotope labelled tubulin is added

to a homogenate in vitro, and dilution by endogenous tubulin is estimated from the specific activity of tubulin which is recovered by vinblastine precipitation and further purified and quantitated by SDS-PAGE. After enriching for tubulin using methods such as poly-L-lysine (Lacey and Prichard, 1986) the tubulin band may become discernible on SDS-PAGE gels and the % tubulin content estimated by densitometry and extrapolation to original material (Tang and Prichard, 1988). However, in H. contortus, the tubulin band may not be clear and other protein contaminants may co-migrate with tubulin (see Chapter VI).

There seems to be no universal method for quantitation of tubulin for all organisms and tissues, making it very difficult to compare tubulin content in different organisms and tissues. One possible approach may be to isolate the tubulin and quantify it directly and extrapolate back to the original cell homogenate. This means that the isolation method must be proved to extract virtually all of the tubulin in cell homogenates. Isolation of tubulin from vertebrate sources has been relatively easy (Eipper, 1975; Lacey, 1988; Shelanski et al. 1973; Lacey and Snowdon, 1990). A potential limitation in applying these methods is that, depending on the organism or tissue used, other proteins co-purify with tubulin, necessitating further purification steps (Luduena, 1979). They become even more unsuccessful for parasite nematodes which contain very little tubulin. A critical concentration of tubulin is necessary for the most commonly used assembly/disassembly method of Shelanski et al. (1973). However, it has been reported that this critical concentration may be sufficiently lowered by taxol (Vallee, 1986) to allow assembly and purification of tubulin from parasite nematodes (Tang and Prichard, 1988, 1989). However, for H. contortus, the tubulin yield was low and the method

was unsuccessful for larvae and adult worms because other proteins, apparently, co-purified with tubulin and not all the tubulin could be extracted. This Chapter reports SDS-PAGE and Western blot, and ELISA methods used to compare the tubulin content in S and R eggs, larvae and adult worms of H. contortus.

7.2 MATERIALS AND METHODS

7.2.1 SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting (Western blot) 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 analyzed by SDS-PAGE utilising the discontinuous system in slabs (Mini-protean II, Biorad) as described previously (see Chapter II). Four percent and 12% 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. After protein transfer, the paper was incubated in 10% (w/v) BSA in PBS for 2 h at room temperature to block nonspecific sites.

Monoclonal anti-chicken α- or β-tubulin mouse IgG (Amersham) were used in Western blot assays as primary antibody for the visualization of tubulin bands. The second antibody was peroxidase-conjugated anti-mouse IgG (BioCan) or biotinylated anti-mouse IgG (Amersham). 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 incubations with the first and second antibodies were each 1 h long. After each incubation the

nitrocellulose paper was washed (3 x 15 min) with phosphate-buffered saline (PBS) (pH 7.4) containing 0.4 % (v/v) Tween-20. In experiments in which the second antibody was peroxidase-conjugated, the substrate was added after the secondary incubation to visualise the reaction. In the experiments in which biotinylated anti-mouse IgG (Amersham) was used as second antibody, the paper was incubated for 30 min with streptavidin-peroxidase followed by washing (2 x 15 min) before adding the substrate. The substrate was diaminobenzidine (Sigma). 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. Equal protein loads of S or R egg-, larval- and adult worm-derived supernatants in 10 μl of reducing buffer were loaded onto a 1.5 mm thick SDS-gel. Parasite materials freshly prepared or stored in liquid nitrogen before use were compared.

7.2.2 Enzyme-linked immuno-absorbent assay (ELISA).

Previous studies (Das et al., 1989) have shown that treatment of ELISA plates with PLL improves the binding of tubulin to the plate. A series of experiments using PLL-treated or non-treated plates showed that the absorbency was affected by the non-tubulin protein content, as well as the amount of tubulin. In order to compare the tubulin content in the S and R supernatants, tubulin was removed from adult worm derived supernatants by poly-L-lysine affinity chromatography to produce tubulin-free supernatants (TFS) that were included in the incubation buffer to produce the same total protein content in all assays. The TFS were examined by Western blotting to ensure that tubulin was completely

removed. Parasite supernatants prepared from S or R eggs, larvae or adult worms were serially diluted with 0.025 M MES buffer (pH 6.5) containing 5 M urea and 0.2 μg/μl TFS protein, such that each well contained the same amount of total protein, and applied to PLL-treated plates. Standards containing known amounts of purified egg tubulin diluted in TFS protein buffer, to maintain the same total protein content were included. Two hundred and fifty μl of 0.05 % (w/v) poly-L-lysine (PLL) solution (Sigma) were added to each well of the ELISA plate (Falcon 3912, Canalab) and incubated for 1 h at 37°C. The PLL solution was discarded. Then 250 μl of 0.2% (v/v) glutaraldehyde were added to the wells and incubated for 30 min at room temperature to achieve complete immobilization of the PLL. The wells were washed with 3 x 250 μl of MES buffer and 100 μl of various dilutions of parasite supernatants or standard were put in each well. After incubation for 1 h at 37°C the wells were rinsed once with 250 μl of MES buffer. Then 250 μl of 5% (w/v) BSA prepared with low ionic-strength buffer (0.025 M PBS, pH 7.3) were put in each well and incubated for 1 h at 37°C to block nonspecific sites. Buffer of higher ionic strength was avoided because it washed away the tubulin from the PLL (see also Das et al, 1989). Nonspecific binding was completely eliminated by preparing the primary and secondary antibodies in 0.025 M PBS containing 5% (w/v) BSA and 0.2 % (v/v) Tween-20. One hundred μl of mouse anti-α- or anti-β-chicken tubulin monoclonal antibodies (Amersham) diluted at a ratio of 1:20,000 and 1:10,000 respectively were put in each well and incubated for 30 min at room temperature. The plate was washed with PBS (3 x 250 μl/well) containing 0.4% (v/v) Tween-20 before adding the second antibody. The second antibody was anti-mouse peroxidase-conjugated IgG (BioCan) diluted 1: 20,000. One hundred μl of the latter were put in each well and

incubated for 30 min at room temperature followed by 3 x 250 µl PBS/well for washing. The substrate (100 µl/well) was 0.4 mg/ml ortho-phenylene diamine (Fisher) in phosphate-citrate buffer (pH 5.0) containing 0.01% (v/v) H_2O_2. The reaction was stopped after 2 min with 50 µl/well 2.5 M H_2SO_4. The plates were read at 492 nm.

7.3 RESULTS

7.3.1 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. 7.1. Developmental stages of the S strain were compared using fresh samples prepared on the same day (Fig. 7.1a). Similarly the eggs (Fig. 7.1b) or larvae or adult worms (not shown) of the S and R strains were compared using fresh material. Subsequently, the developmental stages and both strains were compared in a single run (Fig. 7.1c & d) using material prepared at various times and stored in liquid N_2. 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. Apparently, anti-α tubulin antibodies responded more sharply to changes in tubulin content. 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 and larval supernatants (Fig. 7.2). These extra bands were weak or absent in the egg supernatants. It was shown that these extra bands were due to this particular second

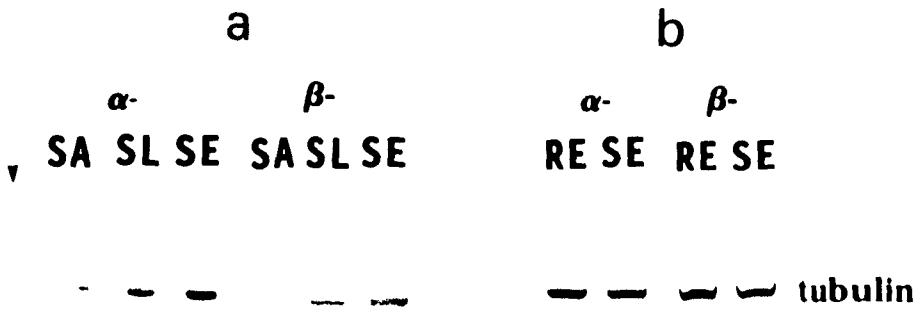
antibody (Amersham) which was not used further.

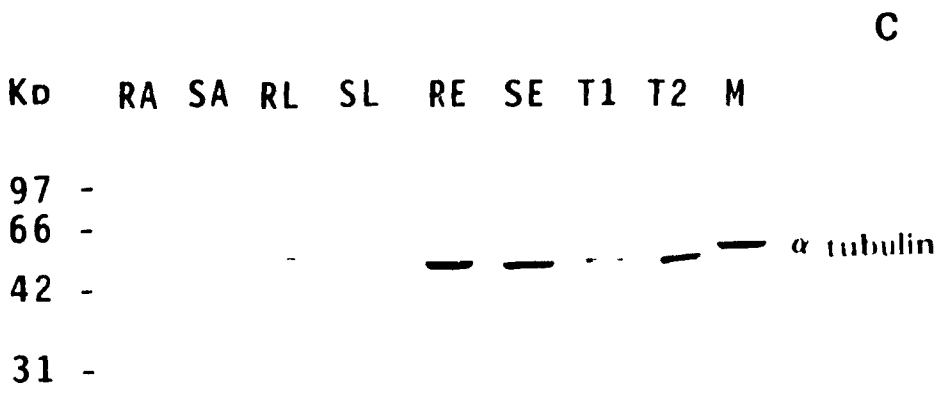
7.3.2 ELISA

When pure tubulin diluted with buffer was applied to PLL-treated or non-treated plates a saturable curve could be obtained, but when the same amount of tubulin was diluted with buffer containing other H. contortus proteins, the curve was displaced downward (Fig. 7.3). In other experiments the effect of varying the amount of TFS-protein or the ratio of pure tubulin to TFS-protein was examined. It was observed that the absorbance values obtained depended on the total amount of both tubulin and other proteins and on the ratio of tubulin to other proteins. Whereas the total amount of tubulin could be corrected for, it was not possible to control the ratio of tubulin to other proteins in the parasite samples. Therefore, it would be inappropriate to depend on absorbance values obtained using pure tubulin alone to estimate the absolute tubulin content in crude preparations. In order to compare the relative tubulin content in S and R supernatants, the latter were serially diluted with buffer containing TFS proteins and a graph of [protein] versus absorbance constructed. Thus all the wells contained equal total amounts of protein (TFS protein + whole-supernatant protein).

From Fig. 7.4 it can be seen that the S and R derived supernatants contained similar total amounts of alpha or beta tubulin at each stage of development but that the eggs contained more tubulin than the larvae which in turn contained more tubulin than the adult worms. Anti-α tubulin monoclonal antibodies were more reactive than the anti-ß ones and the antibodies were, therefore, diluted at a rate of 1:20,000 and 1:10,000, respectively.

Fig. 7.1. **Western blots using anti-α or anti-β-tubulin monoclonal antibodies (Amersham) and peroxidase-conjugated IgG (Biocan).** Developmental stages of the S strain were compared in one run using fresh samples prepared on the same day (a). Similarly, the eggs (b) or larvae or adult worms (not shown) were compared using fresh material. Developmental stages of both strains were also compared in a single run using samples prepared at various times and stored in liquid N_2 (c & d). Pure tubulin from H. contortus eggs, 0.07 μg (T1) or 0.14 μg (T2) or sheep brain, 0.17 μg (M) and protein, 5 μg, in parasite supernatants of each sample were loaded on the SDS gel. Key: RA = resistant adult; SA = susceptible adult; RL = resistant larval; SL = susceptible larval; RE = resistant egg; SE = susceptible egg.


a
b
α-
β-
α-
β-
SA SL SE SA SL SE
RE SE RE SE
tubulin


c
KD RA SA RL SL RE SE T1 T2 M
97 -
66 -
42 -
31 -
α tubulin

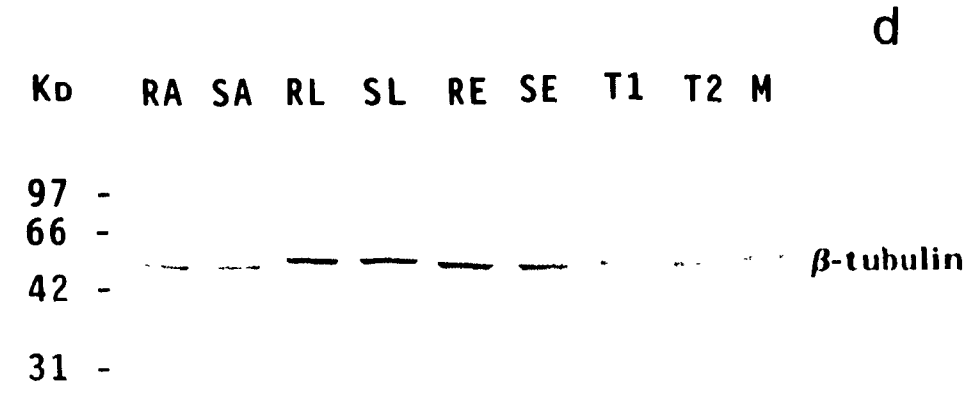
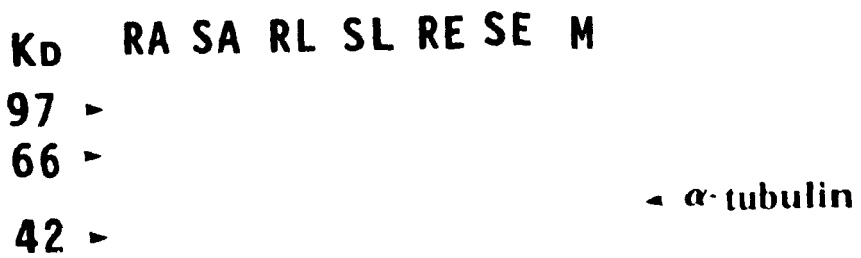

d
KD RA SA RL SL RE SE T1 T2 M
97 -
66 -
42 -
31 -
β-tubulin

Fig. 7.2. **Western blots using anti-α or anti-β-tubulin monoclonal antibodies (Amersham) and biotinylated IgG (Amersham) and streptavidin-peroxidase (Amersham).** Five μg protein of each parasite sample and 0.2 μg pure tubulin (M) from sheep brain were loaded on the SDS gel. Key: RA = resistant adult; SA = susceptible adult; RL = resistant larval; SL = susceptible larval; RE = resistant egg; SE = susceptible egg; M = pure mammalian tubulin.


KD RA SA RL SL RE SE M
97
66
42
α-tubulin


KD RA SA RL SL RE SE M
97
66
42
β-tubulin

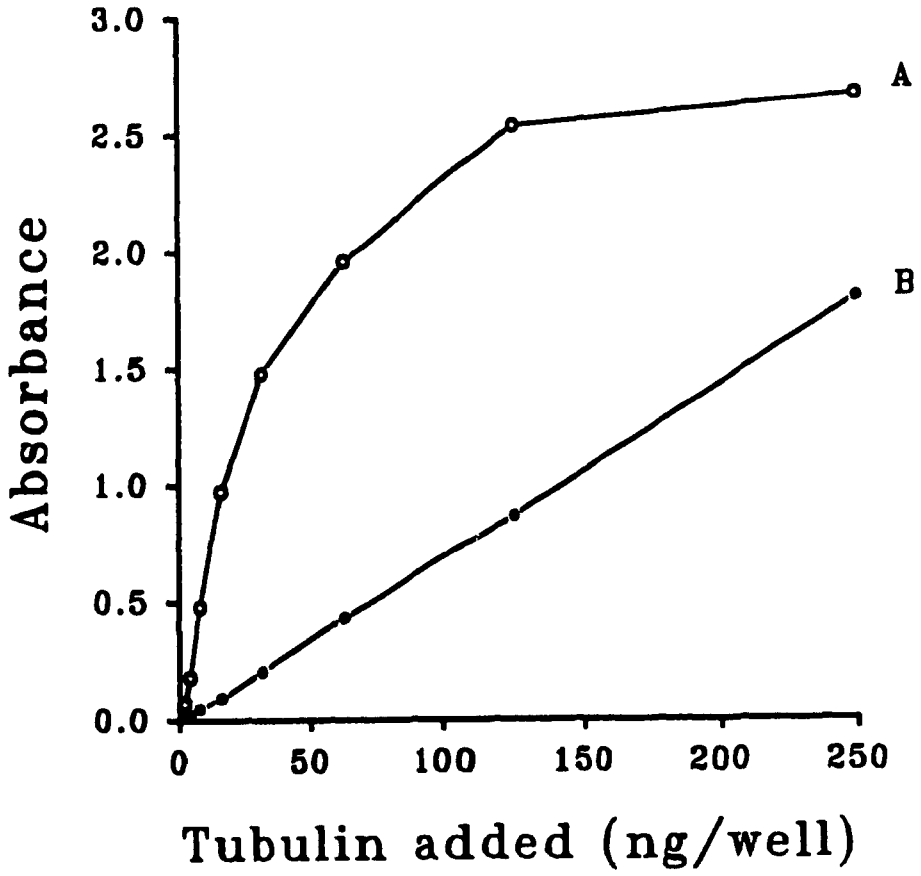
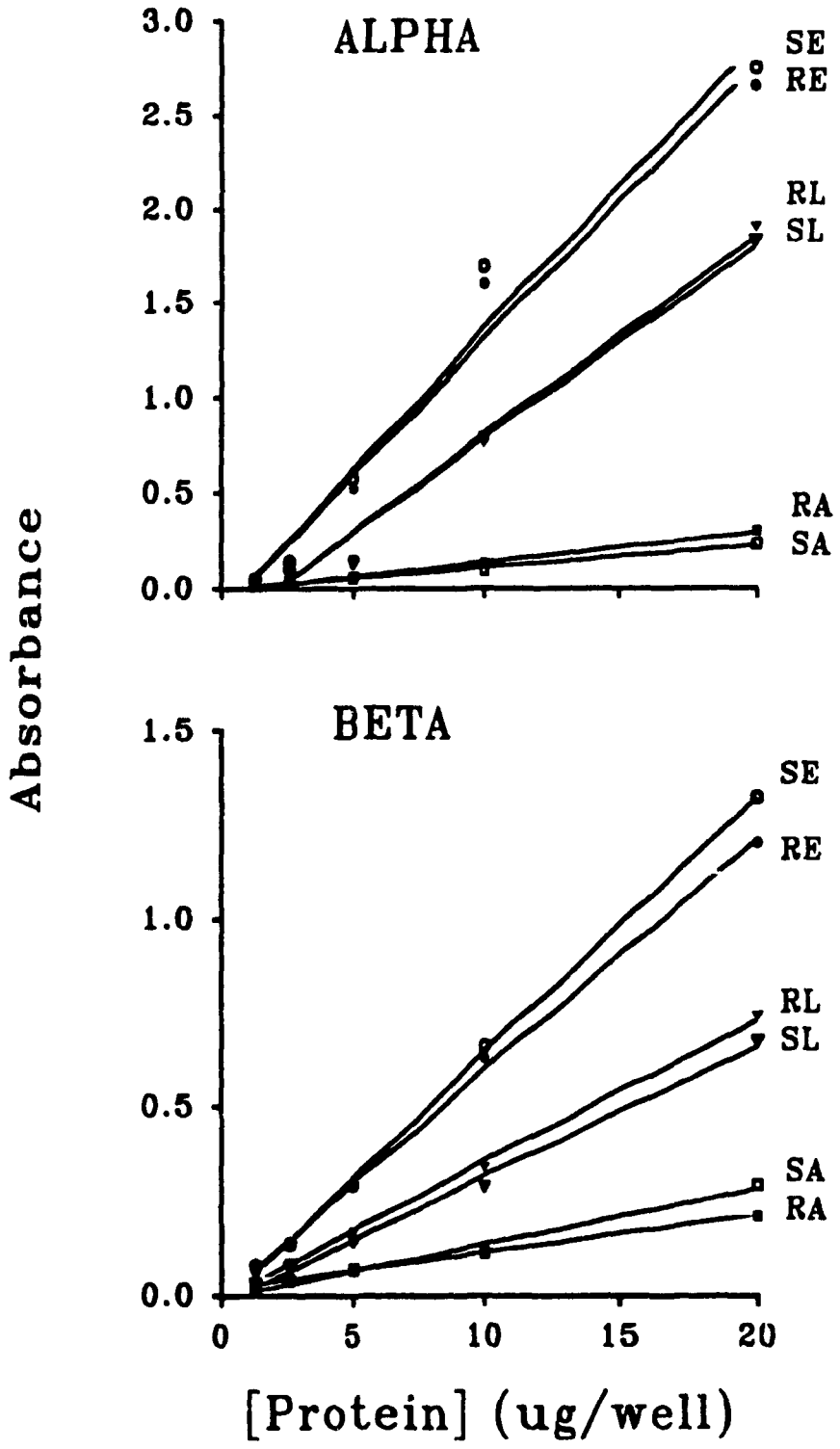


Fig. 7.3. ELISA of purified tubulin using anti-β-tubulin monoclonal antibodies (Amersham) and peroxidase-conjugated IgG (BioCan). Tubulin purified from H. contortus eggs was diluted with MES buffer (A) or MES buffer containing 0.2 μg/μl tubulin-free protein (B).

Fig. 7.4. ELISA of tubulin in egg, larval or adult worm supernatants of the S or R strain. Supernatants were assayed with anti-α- or anti-β-tubulin monoclonal antibodies (Amersham) and peroxidase-conjugated IgG (BioCan) as described in Materials and Methods.


ALPHA
BETA
SE
RE
RL
SL
RA
SA
Absorbance
[Protein] (ug/well)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20

17.4 DISCUSSION

Western blot analyses indicated that supernatants prepared from unembryonated eggs contained more tubulin per unit weight of protein than the supernatants derived from larvae or adult worms The larvae were intermediate between eggs and adult worms in tubulin content and HAB. This was confirmed using the Enzyme-Linked-Immunosorbent-Assay (ELISA). Therefore, HAB may depend on tubulin content which may in turn depend on the mitotic requirement of a developmental stage. Tubulin is the principle protein of the mitotic spindle (Dustin, 1984) and may be abundant in rapidly dividing cells, such as eggs. It has been observed, in previous chapters, that 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. The data are consistent with several *in vitro* studies (Kirsch and Schleich, 1982; Rapson *et al.*, 1985; Rew *et al.*, 1986) which suggest that developing stages (eggs or larvae) of parasites may be more susceptible to BZs than are non-developing stages (adult worms). However, tubulin content may not explain the difference in HAB between the S and R strains. HAB may also depend on the type(s) of tubulin present. The tubulin isoform pattern of the S strain is different from that of the R strain (see Chapter VIII).

Compared with the eggs, more of the binding in the larvae and adult worms was LAB (see Chapter III). On the other hand, compared with the larvae and adult worms more of the binding in the eggs was high-affinity binding. Benzimidazole-resistance was not associated with LAB. Most of the LAB of the larvae and adult worms was probably non-specific binding. LAB of larvae and adult worms was detectable in both tubulin-enriched and

tubulin-free supernatants. There was no LAB in egg-derived fractions devoid of tubulin. The source of the greater amount of LAB in the larval and adult worm supernatants than in those of eggs is obscure. It may involve other proteins which are not present in the eggs. It has been observed by Western blot (Fig. 7.2) that larvae and adult worms may contain proteins that are absent or present in eggs at lower levels.

Because of the scarcity of parasite material and contamination by non-tubulin proteins upon purification, determination of the absolute tubulin content in H. contortus tissues was not completed. The measurement of tubulin content in H. contortus homogenate needs to be re-addressed, in the future, in order to determine the stoichiometry (i.e moles of BZ bound per mole of tubulin) of the BZ-tubulin interaction in this nematode. However, the stoichiometry of the BZ-tubulin interaction would be more accurately addressed using pure tubulin in binding studies. For pure mammalian tubulin the stoichiometry of colchicine binding is a range of 0.5-0.8 (Hains et al., 1978) while that of MBZ is 0.73 (Laclette et al., 1980). No one has reported the stoichiometry of these drugs using drug-active pure-tubulin from nematode sources. BZ binding is reduced for the R strain compared to the S strain, although the total tubulin content per mg protein of the two strains may be similar. At the same time the tubulin isoform patterns of the two strains are dissimilar (Chapter VIII). These findings suggest that some tubulin isoforms may not take part in binding and stoichiometric calculations can be misleading.

Chapter VIII

TUBULIN ISOFORMS OF THE S AND R STRAINS

8.1 INTRODUCTION

Genetic (Oakley, 1985), immunological (Morejohn et al., 1984) and electrophoretic (Wolff et al., 1982) studies in other eukaryotes demonstrate that within an organism the tubulin pools are structurally heterogeneous. Tang et al. (1987) have demonstrated this heterogeneity for mammalian brain, Brugia pahangi, B. malayi and Nippostrongylus brasiliensis. Some studies in fungi, yeast and the free-living nematode, C. elegans, suggest that variances in tubulin genes or gene products may account for BZ-resistance (Foster et al., 1987; Sheir-Neiss et al., 1978; Orbach et al., 1986; Thomas et al., 1985; Driscoll et al., 1989). In this chapter tubulin isoforms of the S and R strains were visualised using monoclonal antibodies known to be specific to α- or ß-tubulin (Blose et al., 1982). These anti-chicken tubulin monoclonal antibodies recognize sequences between amino acids 340 and 400 near the respective C terminal ends of the α- or ß-tubulin (Serrano et al., 1986). These sequences appear to be conserved for all α- or ß-tubulins, respectively, since these antibodies could recognise tubulin from a wide variety of organisms (Blose et al., 1982; Tang and Prichard, 1988, 1989).

8.2 MATERIALS AND METHODS

8.2.1 Tubulin purification

Tubulin purified from H. contortus eggs by poly-L-lysine (PLL) chromatography (see chapter VI) or by the taxol-induced polymerization

procedure (Vallee, 1986; Collins and Vallee, 1987) was used to compare the tubulin isoforms of the S and R strains.

Purification of tubulin by taxol

Parasites were homogenised in MES buffer containing 0.1 M MES, 1 mM EGTA, 1 mM $MgSO_4$, 1 mM GTP, 80 μg/ml pepstatin and 10 μg/ml 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK). The homogenate was centrifuged at 40,000 g for 30 min at 4°C to get a clear supernatant that was re-centrifuged at 140,000 g for 1 h. Taxol and NaCl were added to the supernatant to the final concentration of 20 μM and 0.4 M, respectively, and incubated at 37°C for 20 min to induce polymerisation of tubulin. An underlayer of 10% glycerol was then introduced and centrifuged at 100,000 g for 30 min at 37°. The microtubule pellet was rinsed twice with MES buffer and suspended in 5 ml of the MES buffer containing 2 mM $CaCl_2$ and kept on ice for 45 min to allow the depolymerization of tubulin and detachment of taxol (see Collins and Vallee, 1987). The tubulin could re-polymerize without adding more taxol and was therefore subjected to one cycle of temperature-dependent polymerization-depolymerization (Shelanski et al, 1973) to remove any remaining taxol. The purified tubulin was kept in liquid N_2 or briefly on ice before use. A sample of purified tubulin was run on SDS-PAGE before further use.

8.2.2 Two dimensional polyacrylamide gel electrophoresis (2-D PAGE).

Two-D PAGE (O'Farrell, 1975) involving isoelectric focusing (IEF) and SDS-PAGE was performed utilising the mini-protean II 2-D PAGE cell (Biorad). The sample was diluted 1:1 with buffer containing 9.5 M urea (Fisher), 2% Triton X-100 (LKB), 5% 2-mercaptoethanol (Sigma) and 4%

ampholyte (LKB) of pH 4-6, 5-7, 7-9 and 3-10 blended in the ratio 4:4:1:1, respectively. The tube gel (6 cm long) contained 6.0 M urea, 4% acrylamide, 0.1% Triton X-100 and 4% ampholyte blended as described above. Taxol-purified tubulin (0.5 μg) or PLL-purified tubulin (5 μg) or whole supernatants (20 μg protein) of S or R strains in a final volume of 20 μl were loaded onto the tube gels. The gels were focused at 750 V for 7 h. It was verified that the tube gels loaded with S or R samples were equal in length and the pH gradient was estimated using a surface pH electrode (Biorad) before running the second dimension to ensure that the gels for the S and R strain were identical in pH gradient. In order to measure the pH gradient more precisely, several focused tube gels were sliced into 3 mm pieces and the corresponding sections pooled and placed in 0.5 ml of 0.1% sodium chloride in 3 ml culture plates The pH of the pieces was measured 1 h later with a surface pH electrode (Biorad).

The tube gels for the S and R strains were equilibrated for 15 min in SDS reducing buffer containing 5 M urea and embedded into 7.5% acrylamide SDS slab gels using 1% agarose. SDS-PAGE was performed at 150 V for 45 min. The S and R strains were routinely compared in the same run.

8.2.3 Transfer of 2-D PAGE proteins to nitrocellulose paper

Electroblot transfer was performed (100 V, 1 h) utilising the mini-Transblot cell (Biorad) and the standard protocol for the apparatus. The transferred protein was fixed on the nitrocellulose paper for 30 min with 0.2% (v/v) glutaraldehyde to improve the retention of tubulin spots during washing (Van Eldik and Wolcohok, 1984).

8.2.4 Western blotting of 2-D PAGE proteins

Western blotting using highly specific mouse anti-α- or anti-β-chicken tubulin monoclonal antibodies (Amersham) and peroxidase conjugated anti-mouse IgG (BioCan) were performed as described previously (Chapter II).

8.2.5 Effect of anthelmintic treatment with BZs

The R strain was subjected, in alternating sequence, to 5 *in vitro* and 4 *in vivo* selections by treatment with TBZ (Maingi *et al.*, 1990). Isolates obtained after each combined *in vitro* and *in vivo* selection, designated as R1, R2, R3 and R4 respectively, were used in binding and electrophoretic studies to monitor the effect of selection pressure on resistance. The S strain had been subjected to selection by *in vivo* treatment with CBZ (Kates *et al.*, 1973; Colglazier *et al.*, 1974, 1975) to yield a CBZ-resistant strain. This CBZ-resistant strain was obtained from Dr. G.A. Conder (The Upjohn Co., Kalamazoo, MI) in the latter stages of this study and was used in binding studies for comparison with the S strain.

8.3 RESULTS

8.3.1 Western blot of tubulin separated by 2-D PAGE

Representative results of Western blotting of α- and ß-tubulin after 2-D PAGE are shown in Fig. 8.1. Comparable results were obtained using whole supernatants or PLL- or taxol-purified tubulin although higher resolution of isoforms was obtained using purified tubulin. At least 2 ß-tubulin isoforms (the small and large spots in Fig. 8.1) may be present in the S strain and at least 2 ß isoforms (2 large spots in Fig. 8.1) in the

R strain. However, the S and R ß-tubulin spots are dissimilar in isoelectric value. The large spot of ß-tubulin in the S strain may contain two very close spots. It is also possible that the small ß-tubulin spot seen in the S strain may also be present in the R strain at a very low concentration. For α-tubulin 2 clear spots could be distinguished for both the S and R strains. The α-tubulin spots had similar isoelectric points for the S and R strains. The pH gradient of the tube gel was 4.3 to 6.1. Protease inhibitors such as phenylmethyl-sulfonyl fluoride (PMSF) and Pepstatin did not affect the results.

8.3.2 Effect of anthelmintic treatment with BZs

There were marked differences in HAB of OBZ (Fig. 8.2) and MBZ (Fig. 8.3) between the S and the CBZ-resistant strain. Eggs from the first generations of R2, R3 or R4 were compared with those of the R strain in binding studies at the same time under similar conditions. Selection pressure on the R strain had some effect on HAB (Fig. 8 2). There was no significant difference in HAB from one selected strain to the next in the sequence but compared with the original strain (R), the HAB of R3 or R4 were significantly lower ($P < 0.05$). However, upon rexamination using the eggs from the third generations of the R2, R3 and R4 strains the differences were no longer significant.

The tubulin isoform patterns of the R4 strain were similar to those of the R strain shown in Fig. 8.1. Electrophoretic studies using the CBZ-resistant strain were not completed due to insufficient material.

8.4 DISCUSSION

The ß-tubulin isoform patterns for the S and R strains were

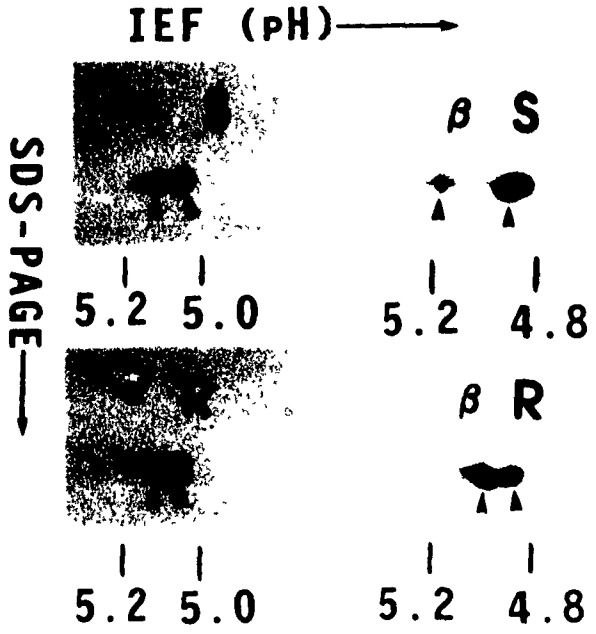


Fig. 8.1. Western blot of α- and ß-tubulin of the S and R strains after 2-dimensional electrophoresis. Taxol-purified tubulin derived from unembryonated eggs was subjected to isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second dimension. Mouse anti-α or anti-β chicken-tubulin monoclonal antibodies (Amersham) and peroxidase-conjugated anti-mouse IgG (BioCan) were used as outlined in Materials and Methods to identify the α- and β-tubulins. The data shown represent the same run of IEF. The other steps of the experiment were routinely performed simultaneously for the S and R strains and α- and β-tubulins.

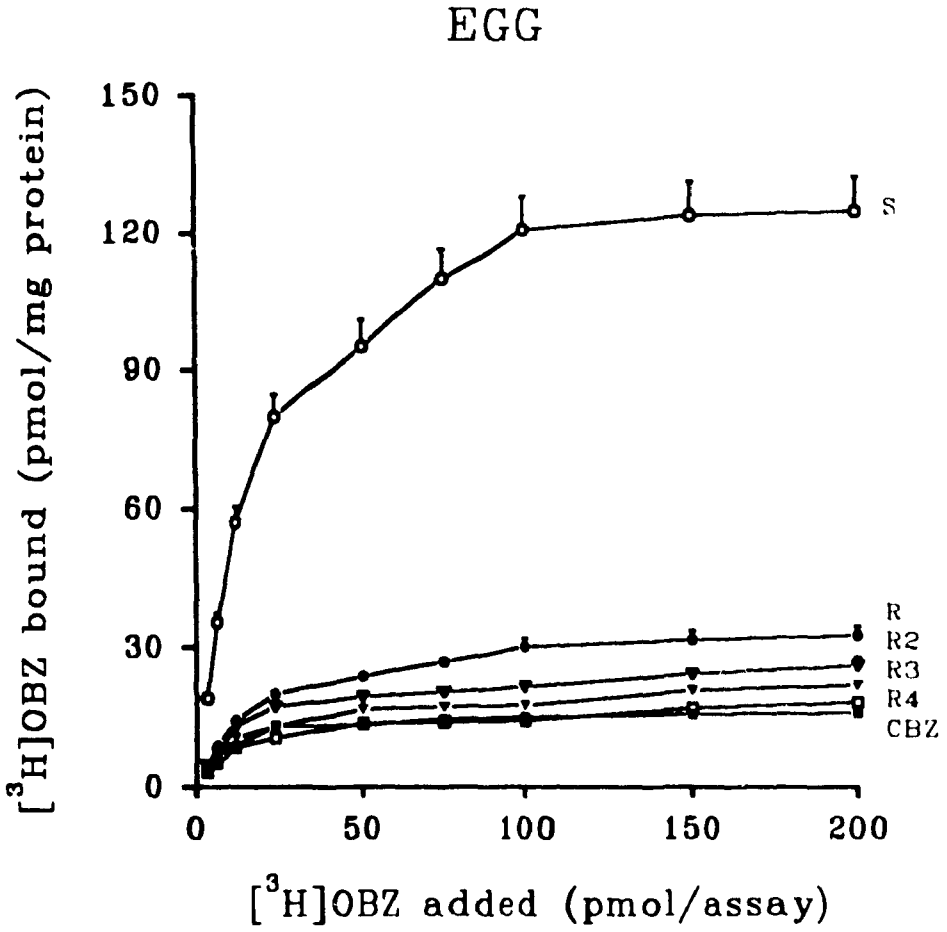


Fig. 8.2. Effect of selection pressure on HAB of OBZ. The R strain was subjected to selection pressure by treatment with TBZ to yield the R1, R2, R3 and R4 strains (see Maingi *et al.*, 1990). The S strain had been treated with CBZ to yield the CBZ-resistant strain (see Colglazier *et al*, 1974, 1975). The R2, R3, R4, S and CBZ-resistant (CBZ) strains were compared in BZ-binding assays using OBZ as outlined in Materials and Methods. The data are a mean ± SE of 3 experiments conducted in duplicate.

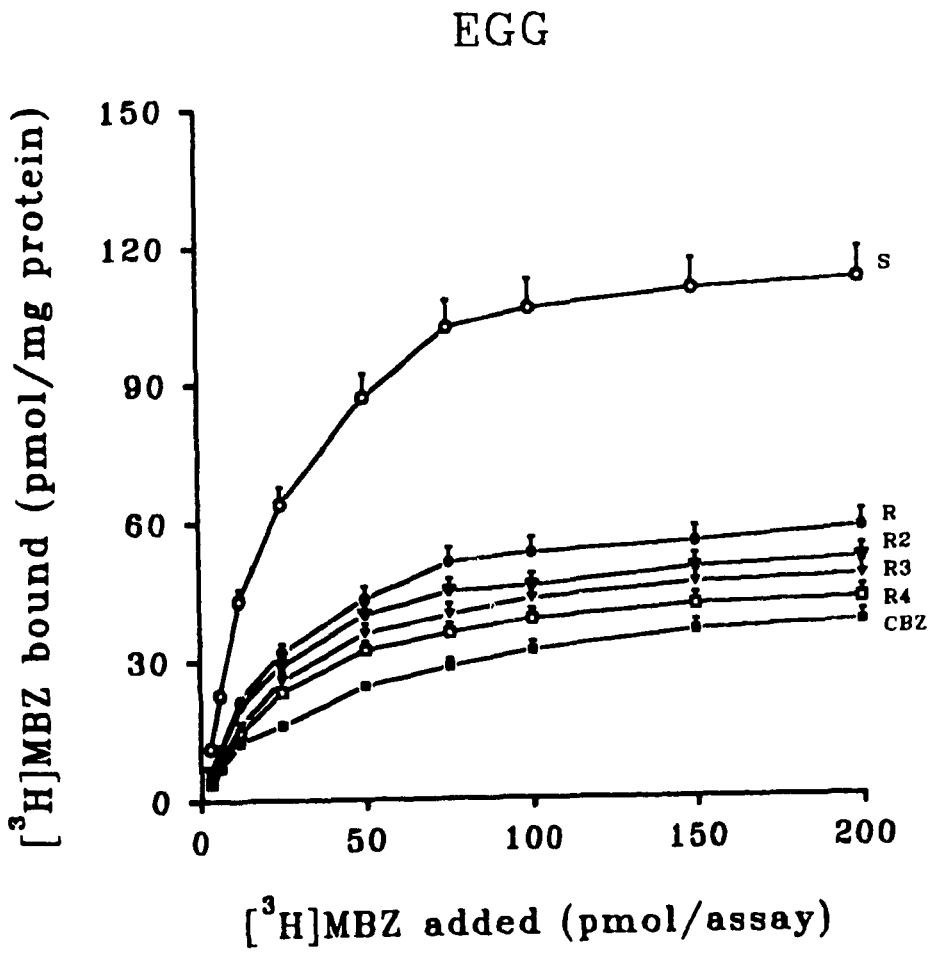


Fig. 8.3. Effect of selection pressure on HAB of MBZ. The R strain was subjected to selection pressure by treatment with TBZ to yield the R1, R2, R3 and R4 strains (see Maingi et al., 1990). The S strain had been treated with CBZ to yield the CBZ-resistant strain (see Colglazier et al., 1974, 1975). The R2, R3, R4, S and CBZ-resistant (CBZ) strains were compared in BZ-binding assays using MBZ as outlined in Materials and Methods. The data are a mean ± SE of 3 experiments conducted in duplicate.

dissimilar while those of the α-tubulin were similar. Alteration in ß-tubulin may account for BZ-resistance in this R strain of H. contortus. It could not be determined which spot(s) (isoform(s)) conferred BZ-susceptibility or resistance. It was observed previously (Chapter III) that the K_a (for high-affinity binding) of the S and R strains were similar while the B_{max} (maximum binding at infinite [ligand] = [receptor]) for R strain was reduced. If ß-tubulin isoforms were responsible for the high-affinity BZ-binding of the S strain, one could hypothesize that the R population is heterogeneous consisting of mainly individuals with the resistant isoform pattern shown here, which does not confer high-affinity binding, and a small proportion of individuals with a susceptible isoform pattern which confers some high-affinity binding. Under the conditions of the 2-D PAGE and Western blot analysis, low levels of the S-type isoform pattern may be difficult to discern in material from the R population used. This would explain why the S and R strains bind with similar K_a but dissimilar B_{max}. The reduced HAB of the CBZ-resistant strain compared to the parent S strain supports this hypothesis. Treatment of the R strain with TBZ caused temporary reduction in HAB. It is possible that if the R strain was subjected to prolonged selection pressure by treatment with BZs, the high-affinity binding may tend to decrease further. No change in isoform pattern was detected in egg tubulin of the R4 strain. The methods used in this study to visualize the tubulin isoforms may not be sensitive enough to reveal isoforms occuring at very low frequencies. The change in resistance from the S to the R strain may have already been large enough to conceal any further changes. The molecular changes upon selection may be more discernible starting with a susceptible rather than with the already resistant strain. Analysis of the DNA of individual male adult

worms of another TBZ-susceptible population indicated heterogeneity regarding the number of ß-tubulin DNA restriction fragments (Roos et al, 1990). The number and frequency of these fragments decreased upon selection for BZ-resistance using TBZ. Other studies on strains of fungi (including yeast) and the free-living nematode, C. elegans, resistant to BZs, suggest that differences in beta-tubulin genes may be responsible for resistance to BZs (Foster et al., 1987; Driscoll et al., 1989; Sheir-Neiss et al., 1978; Thomas et al., 1985; Orbach et al., 1986). These studies suggest that different mechanisms involving ß-tubulin may be responsible for resistance to BZs. Resistance to parbendazole by a fungus, Physarum polycephalum was associated with an alteration of a ß-tubulin isoform compared with the wild type (Foster et al., 1987) while BZ-resistance in the free-living nematode, C. elegans, was ascribed to deletion of a ß-tubulin gene (Driscoll et al., 1989). In this study it is shown that BZ-resistance in a parasitic nematode, H. contortus, may be associated with an alteration in the ß-tubulin isoform pattern and that this correlates with a decrease in BZ binding to tubulin. Although evidence is mounting that β-tubulin is involved in resistance to BZs, the binding site of BZs on tubulin remains unclear. The BZ-tubulin bond is non-covalent and unstable to strigent conditions (Lacey, 1988), so efforts to label the tubulin on SDS-PAGE or 2-D PAGE have been unsuccessful (Lubega, Tang and Prichard, unpublished observations). BZs are known to inhibit colchicine binding (see Lacey, 1988 for review) and yet the binding site of colchicine seems to be located on α-tubulin (Schmitt and Atlas, 1976; Williams et al., 1985; Serrano et al., 1984). It remains to be seen how the binding domains of BZs and colchicine overlap or how the binding of one ligand interferes with the binding of the other. It is possible that

BZs bind at an interface between α- and β-tubulin, while mutations in α-tubulin may be rare or lethal.

Chapter IX

EFFECT OF TEMPERATURE ON BZ BINDING

9.1 INTRODUCTION

Resistance to BZs in C. elegans is the result of the deletion of a specific ß-tubulin gene (ben-1) (Driscoll et al., 1989; Woods et al., 1989). However, the expression and extent of BZ-resistance in C. elegans has been observed to be temperature dependent whereby the mutations involved appear to be dominant or semi-dominant at 25° C while BZ-resistance is not observed in heterozygotes at 15° C (Driscoll et al., 1989). It has been postulated that either the synthesis of the ß-tubulin isotype involved in BZ-resistance in C. elegans is temperature dependent and the isotype itself is thermolabile (Woods et al., 1989) or that the BZ-tubulin interaction of the resistant strain is more stable at lower temperatures (Driscoll et al., 1989). I, therefore, compared the binding of the S and R strains at 4° and 37° to see if temperature would influence the binding of either or both of these strains.

Taxol has been confirmed to bind polymerized tubulin (Parness and Horwitz, 1981; Collins and Vallee, 1987) but it is not clear whether the other microtubule inhibitors can bind free or polymerized tubulin (see Lacey, 1988). The nature of the BZ-binding site is unknown, but seems to become inaccessible in polymerized tubulin (Tang, 1988). Mammalian microtubule protein is polymerized at 37° and depolymerized at 4° (Dustin, 1984). This change in tubulin state may influence BZ binding of mammalian tubulin (Tang, 1988) suggesting that BZs may bind free tubulin rather than polymeric tubulin. It is not known how much of parasite tubulin can be

polymeric at 37° in vitro. Generally, however, low tubulin content (which is the case in parasitic nematodes) does not favour polymerization of tubulin. In this chapter, the binding at 4° and 37°C were compared to see if the tubulin-microtubule dynamics would be important in BZ binding to parasite preparations in vitro. Several BZs were compared to see if temperature would selectively influence the binding of some BZs.

9.2 MATERIALS AND METHODS

9.2.1 Saturation assay

Saturation binding of whole or purified parasite preparations at 4° and 37°C were compared. The entire saturation experiment was carried out at 4° or 37°C using material from the same sample at the same time.

9.2.2 Displacement assay

The ability of unlabelled BZs to bind tubulin at 4° or 37° C was assessed by inhibition (displacement) of labelled OBZ or MBZ whose interaction with tubulin was previously characterised. Two μl of pure DMSO in MES buffer or 2 μl of stock unlabelled BZ in pure DMSO were added to 90 μl of either buffer (blank) or parasite supernatants and preincubated in triplicate for 30 min at 4° or 37° C. Then 10 μl of [^{3}H]MBZ or [^{3}H]OBZ were added to each tube and incubated for a further 30 min at 37° C. The assay was continued as described in Chapter II to determine the bound dpm. The data were mathematically analyzed as outlined in Chapter V.

9.3 RESULTS

9.3.1 Saturation assay

Generally, the binding at 4° was non-saturable and the excess

unlabelled BZs inhibited some of the binding of the tubulin-free supernatants at 4°. It is possible that the HAB of the whole supernatants at 4° includes the BZ-binding to tubulin and non-tubulin proteins. Therefore, in this Chapter LAB and HAB will be refered to as apparent LAB and apparent HAB.

9.3.1.1 Egg supernatants

TB, apparent LAB and apparent HAB of [^{3}H]OBZ using egg supernatants at 4° or 37° C are shown in Fig. 9.1. TB or apparent HAB reached maximum sooner at 37° than at 4°. There was more apparent LAB but less apparent HAB at 37° than at 4°. Results obtained using [^{3}H]OBZ or [^{3}H]MBZ were similar. However, TB, appparent LAB and apparent HAB of [^{3}H]ABZ (Fig. 9.2) at 4° were greater than those at 37°. For all ligands, similar results were obtained using S or R strains except that binding of the R strain was always lower than that of the S strain irrespective of temperature.

9.3.1.2 Adult worm supernatants

Maximum TB at 4° and 37° were similar but apparent LAB at 4° was less than that at 37° (Fig. 9.3a). Apparent HAB was greater at 4° than at 37° (Fig. 9.3b). Adult worm supernatants were fractionated by poly-L-lysine chromatography as described previously (Chapter VI) into tubulin-free and tubulin-rich fractions. Some binding by supernatants devoid of tubulin was inhibited by excess unlabelled drug at 4° but not at 37°. Therefore the radiolabelled-drug binding that was inhibited by excess homologous unlabelled drug is 'apparent HAB' and that which was not inhibited is 'apparent LAB'. [^{3}H]BZ binding by the tubulin-rich fraction was inhibited by excess unlabelled drug at 37° and, to an even greater extent, at 4°.

Results obtained using the S or R strain were similar except that TB or apparent HAB of the R strain was always lower than that of the S strain at either temperature.

9.3.2 Displacement assay

Saturation studies described above indicated that partition of [^{3}H]BZ binding of parasite supernatants into LAB and HAB may be influenced by temperature and the apparent HAB at 4° C may not be saturable. Therefore, the affinity of various BZs for tubulin at 4° and 37°C was compared by displacement assay (an indirect approach). The inhibition of [^{3}H]BZ binding by preincubation of the unlabelled drugs with the adult worm supernatants at 4° or 37° C is shown in Table 9.1. All drugs were more potent inhibitors (lower IC_{50} and greater K_a values) at 37° than at 4° except for ABZ which was apparently a more potent inhibitor at 4° than at 37°. B_{max} values (high-affinity receptor concentration) were similar for both types of experiments. Note that the preincubation was done at either 4° or 37°, whilst the subsequent incubation with the labelled ligand was always done at 37° so that only true HAB (tubulin binding) could be assessed in both cases. A similar rank order of performance of BZS was obtained using [^{3}H]OBZ (Fig. 9.4a) or [^{3}H]MBZ (Fig. 9.4b). Following preincubation at 4°, the relative potency of the drugs in terms of IC_{50} and K_a values could be ranked as follows:

$$FBZ \geq OBZ \geq MBZ \geq ABZ \gg OFZ > TBZ,$$

which was little altered following preincubation at 37°:

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

Fig. 9.1. **TB and apparent LAB (a) and apparent HAB (b) of OBZ in S or R egg supernatants at 4° or 37°C.** TB of the R strain was less than that of the S strain but the apparent LAB of both strains were similar at each temperature (LAB of R not shown). Similar results were obtained using MBZ. Each point is a mean ± SE of [illegible] experiments done in duplicate as outlined in Materials and Methods.

EGG

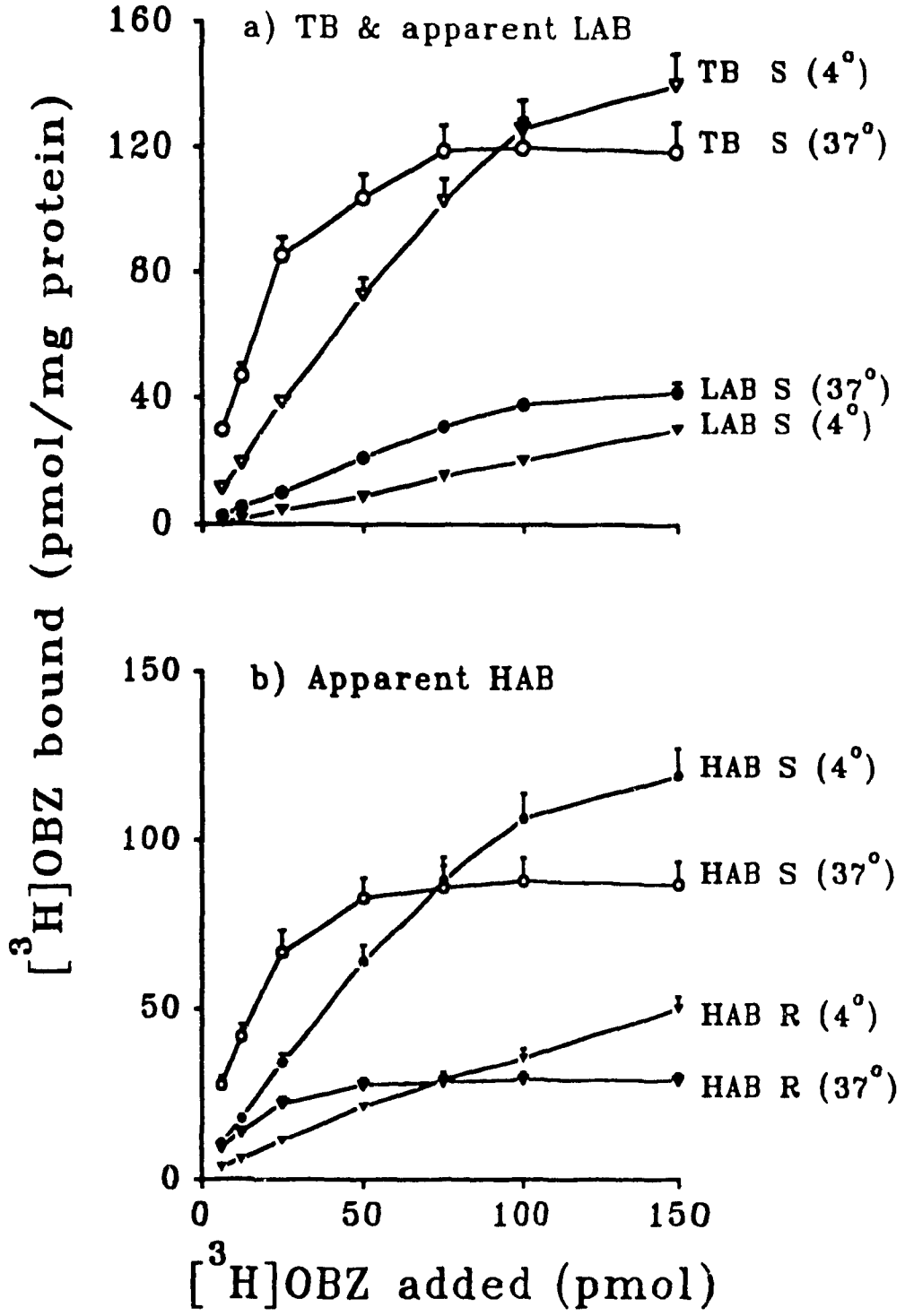

a) TB & apparent LAB
TB S (4°)
TB S (37°)
LAB S (37°)
LAB S (4°)
160
120
80
40
0
b) Apparent HAB
HAB S (4°)
HAB S (37°)
HAB R (4°)
HAB R (37°)
150
100
50
0
0
50
100
150
[3H]OBZ bound (pmol/mg protein)
[3H]OBZ added (pmol)

Fig. 9.2. TB and apparent LAB (a) and apparent HAB (b) of ABZ in S or R egg supernatants at 4° or 37°C. TB of the R strain was less than that of the S strain but the apparent LAB of both strains were similar at each temperature (LAB of R not shown). Each point is a mean ± SE of 4 experiments done in duplicate as outlined in Materials and Methods.

EGG

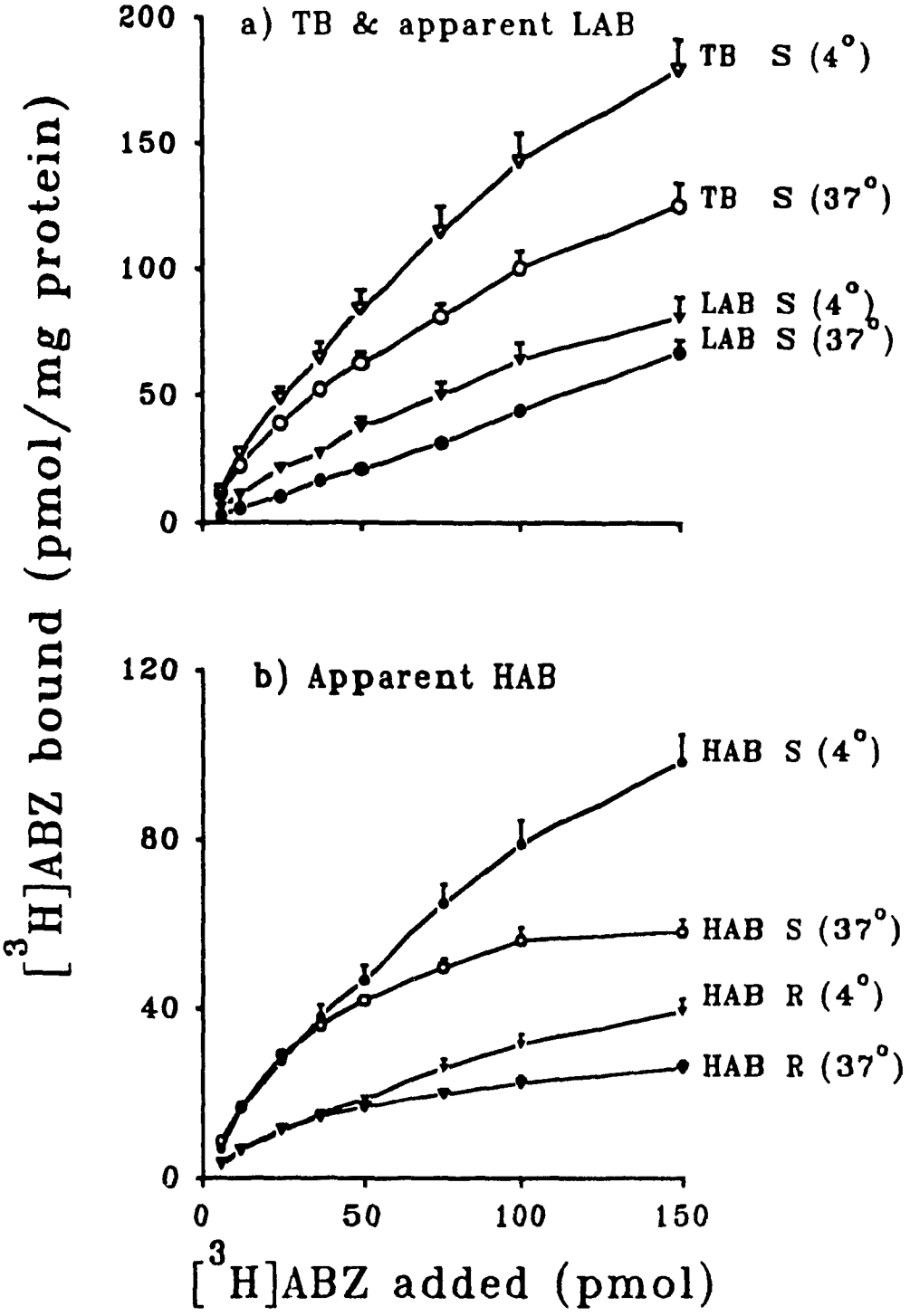

a) TB & apparent LAB
TB S (4°)
TB S (37°)
LAB S (4°)
LAB S (37°)
200
150
100
50
0
b) Apparent HAB
HAB S (4°)
HAB S (37°)
HAB R (4°)
HAB R (37°)
120
80
40
0
0
50
100
150
[3H]ABZ bound (pmol/mg protein)
[3H]ABZ added (pmol)

Fig. 9.3. **TB and apparent LAB (a) and apparent HAB (b) of OBZ in S or R adult worm superratants at 4° or 37°C.** TB of the R strain was less than that of the S strain but the apparent LAB of both strains were similar at each temperature (LAB of R not shown). Similar results were obtained using MBZ. Each point is a mean ± SE of 2 experiments done in duplicate as outlined in Materials and Methods.

ADULT WORM

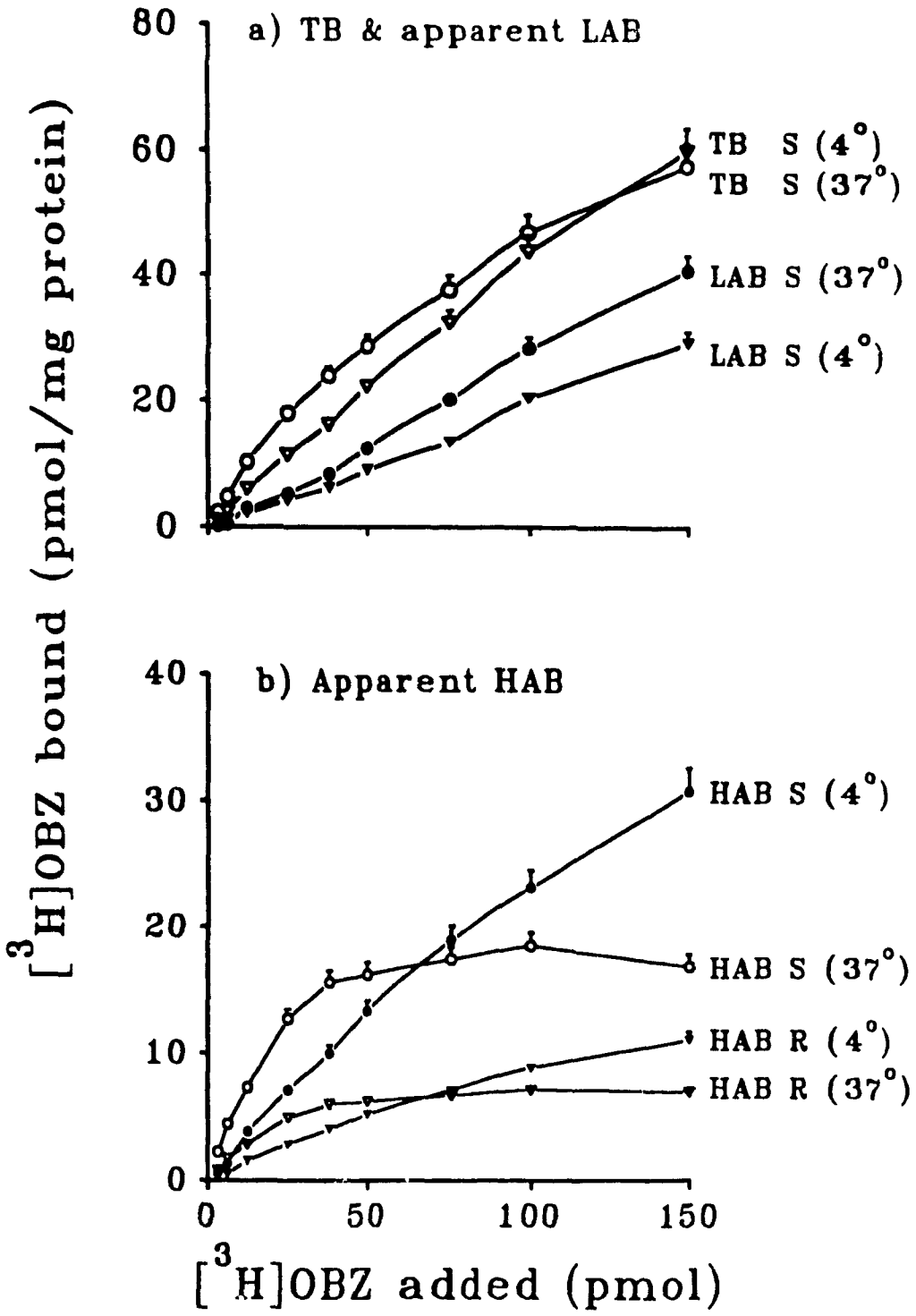
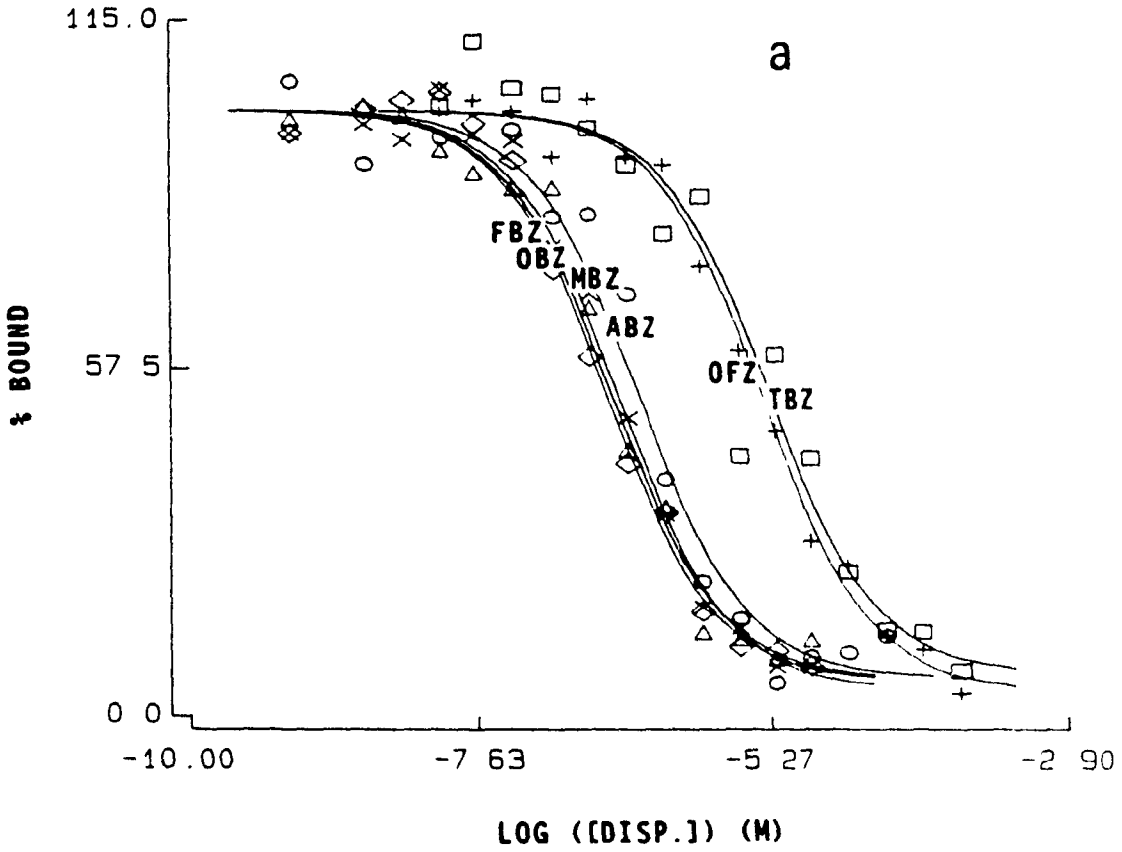

a) TB & apparent LAB
80
60
40
20
0
TB S (4°)
TB S (37°)
LAB S (37°)
LAB S (4°)
b) Apparent HAB
40
30
20
10
0
HAB S (4°)
HAB S (37°)
HAB R (4°)
HAB R (37°)
0
50
100
150
[3H]OBZ bound (pmol/mg protein)
[3H]OBZ added (pmol)

Fig. 9.4. LIGAND generated displacement curves for the inhibition of [^{3}H]OBZ (a) or [^{3}H]MBZ (b) binding by unlabelled BZs in S adult worm supernatants. The supernatants were preincubated with unlabelled BZs at 4°C for 30 min. Then the [^{3}H]BZ was added and the incubation continued for a further 30 min at 37°C. See Table 9.1 or Materials and Methods for details.


a
115.0
57 5
0 0
% BOUND
FBZ
OBZ
MBZ
ABZ
OFZ
TBZ
-10.00
-7 63
-5 27
-2 90
LOG ([DISP.]) (M)

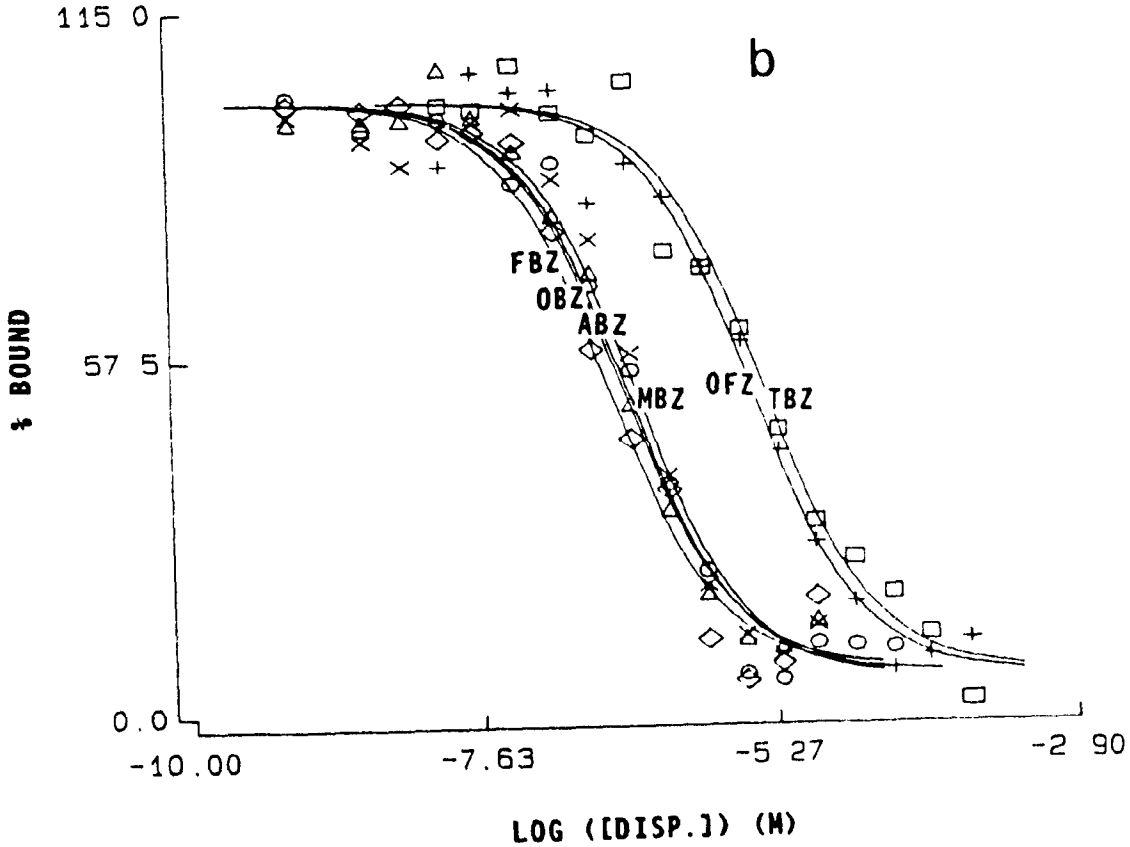

b
115 0
57 5
0.0
% BOUND
FBZ
OBZ
ABZ
MBZ
OFZ
TBZ
-10.00
-7.63
-5 27
-2 90
LOG ([DISP.]) (M)

Table 9.1 Inhibition of [^{3}H]BZ[a] binding to tubulin in adult worm supernatants[b] preincubated with unlabelled BZs[c] at 4° or 37° C.

[^{3}H]BZ	BZ[c]	4° C[d]			37° C[d]		
		IC_{50}[e]	K_a[f]	K_a[f]	IC_{50}[e]	K_a[f]	B_{max}[g]
[^{3}H]OBZ	FBZ	266	6.5	38	158	11	26
	OBZ	307	5.2	31	123	21	20
	MBZ	344	5.0	36	203	10	26
	ABZ	551	3.1	27	656	2.6	26
	OFZ	4886	0.36	31	1212	1.4	22
	TBZ	5666	0.3	29	3773	0.45	25
[^{3}H]MBZ	FBZ	275	6.7	29	221	13	28
	OBZ	332	5.5	26	167	16	24
	MBZ	440	4.2	26	164	16	29
	ABZ	422	4.4	29	872	3	26
	OFZ	3635	0.5	26	1518	1.7	24
	TBZ	7059	0.37	27	5016	0.42	24

a Final [(^{3}H)BZ] was 0.2 μM.

b Final [protein] was 100-200 μg/assay, derived from S strain.

c Final concentrations: 0-20 μM for FBZ, MBZ & OBZ; 0-50 μM for ABZ and 0-200 μM for OFZ & TBZ.

d Mean of 3 experiments each done in triplicate.

e IC_{50} = [BZ] inhibiting 50% of radioligand binding (nM).

f K_a = apparent association constant of the unlabelled BZ (x 10^6 M^{-1}).

g B_{max} = [High-affinity receptor] (pmol/mg protein).

9.4 DISCUSSION

The data in this Chapter show that BZ anthelmintics have the ability to bind tubulin of Haemonchus contortus over a wide range of temperature and that detection of BZ-resistance was not affected by temperature. TB of [^{3}H]OBZ or [^{3}H]MBZ at 37° is readily resolved into high-affinity binding (HAB) and low-affinity binding (LAB). However, saturation studies at 4° indicated that partition of [^{3}H]BZ binding of parasite supernatants into LAB and HAB may be influenced by temperature and the apparent HAB at 4° may include binding by tubulin and other proteins. The apparent HAB at 37° was saturable indicating that it may be specific binding and may involve tubulin only. Apparent HAB at 4° was not saturable within the [drug] and assay conditions used.

Inhibition studies in which the unlabelled drugs were preincubated with the supernatants at 4°, followed by incubation with the labelled drug at 37°, indicated a rank order of affinity broadly similar to that obtained by preincubating at 37°. Mammalian microtubule protein is polymerised at 37° and depolymerised into free tubulin dimers at low temperature (Dustin, 1984). This change in tubulin state may influence BZ binding of mammalian tubulin (Tang, 1988) suggesting that BZs may bind free tubulin rather than polymeric tubulin. The data presented in this Chapter suggest that H. contortus tubulin may not be affected this way probably because parasite tubulin under the in vitro conditions used may largely be free-tubulin at both 4° and 37°. Low tubulin content, which is typical of parasite helminths, would not favour polymerization (Dustin, 1984). However, inhibition studies in which the preincubation was done at 37° gave higher K_a values (lower IC_{50} values) indicating that tubulin of H. contortus had greater affinity for BZs at 37° than at 4°. However, the ability of ABZ to

inhibit other [^{3}H]BZs was not increased by a rise in temperature (Table 9.1). The reason for this anomally is not known. Nevertheless, the rank order of affinity of BZs was generally not affected by temperature. This suggests that all BZs can bind to the same sites or all of the sites of BZs respond similarly to temperature.

Chapter X

THE BINDING AFFINITY AND ANTHELMINTIC EFFICACY OF BZS

10.1 INTRODUCTION

The mode of action of benzimidazoles (BZs) as antifungal agents has been investigated (Davidse and Flach, 1977). Their antitumour activity and their ability to inhibit mammalian and fungal tubulin polymerisation has been known for some time (see Dustin, 1984 for review). Mebendazole, a BZ anthelmintic, was found to induce disintegration of the microtubular network in parasites (Borgers and De Nollin, 1975). Since then, more evidence has emerged, which suggests that BZs may exert their effects by binding tubulin and thereby inhibiting tubulin polymerisation to microtubules (see Lacey, 1988 for review).

Comparison of S and R strains of H. contortus, presented in the preceeding chapters suggests that BZ binding to tubulin is HAB and is the pharmacologically relevant component, since BZ-resistance is associated with a loss of HAB. LAB was not affected by BZ-resistance However, it is not clear whether some of the LAB occurs to some tubulin isoforms with low reactivity for BZs or whether the entire LAB is non-tubulin (non-specific) binding.

Although extensive evidence (see Lacey, 1988) suggests tubulin binding and subsequent inhibition of microtubule formation as the mode of anthelmintic action of BZs, attempts to correlate the *in vitro* activity of BZs against parasite tubulin to *in vivo* anthelmintic potency have met with limited success. Friedman and Platzer (1978) compared anthelmintic potency of some BZs, based on their interaction with bovine brain tubulin, and

Lacey et al. (1987a) compared the inhibition of polymerisation of mammalian tubulin and the inhibition of egg hatch by the BZs. Although some correlation was reported from these studies, the interaction of mammalian tubulin with BZs is different from that of parasite tubulin (Tang et al., 1987; Dawson et al., 1984) and selective toxicity of these BZs for parasites suggests a fundamental difference between mammalian and parasite tubulin-BZ interaction. Lacey (1988) compared anthelmintic potency to the ability of BZs to bind to parasite supernatants. Although useful information was reported, these data were based on total binding and adult worm or larval supernatants have been shown to contain LAB involving non-tubulin proteins (Chapter V). However, in egg supernatants, nonspecific binding is low and remains so at infinite ligand concentration. Therefore, uncorrected BZ binding to egg supernatants may correctly represent tubulin binding.

The egg hatch assay is the most used technique for assessment of BZ resistance (Boersema, 1984). However, the exact relationship between ovicidal activity and anthelmintic potency is not well understood. Although, BZs with strong inhibitory activity against mammalian tubulin were potent inhibitors of egg-hatch and non-inhibitors failed to prevent egg-hatch, some BZs such as oxfendazole (OFZ) and rycobendazole (ABZSO) with strong anthelmintic efficacy, in vivo, did not inhibit mammalian tubulin nor inhibit egg-hatch (Lacey et al., 1987a). These studies suggest that the effects of different BZs, in vitro, may not predict effects of some BZs or their metabolites in vivo.

In this study I have measured the ability of various BZs to bind tubulin (HAB) in egg supernatants of H. contortus and compared it to the known anthelmintic potency of these BZs.

10.2 MATERIALS AND METHODS

The ability of a series of unlabelled BZ compounds to bind to tubulin was assessed from their ability to competitively displace (inhibit) [^{3}H]OBZ or [^{3}H]MBZ binding to tubulin in egg supernatants of the S strain, at 37o C. The procedure was basically the same as that described elsewhere (Chapter V and IX) except that the unlabelled and the labelled drugs were added at the same time to allow direct competition for the receptors. All the BZs were routinely compared in the same run using materials from the same sample. The data were mathematically analysed as outlined in Chapter V.

10.3 RESULTS

Results for the competitive inhibition of [^{3}H]OBZ (Table 10.1 and Fig. 10.1) tubulin-binding, using S egg supernatants were compared with the known anthelmintic potency (recommended dose in mg/kg body weight) against sheep nematodes. Based on IC_{50} and K_a values, the performance of the BZs could be ranked as follows:

FBZ $\geq$ MBZ $\geq$ OBZ > ABZ >> OFZ > ABZSO > TBZ >> $ABZSO_2$.

A similar rank order was obtained when [^{3}H]MBZ or [^{3}H]OBZ was displaced. The data refer to HAB at 37° C which has been shown to involve tubulin only and to be the major component of TB at B_{max} for the egg supernatants (Chapter III).

The anthelmintic potency of these BZ compounds (adopted from Campbell, 1990) may be rated as follows,

FBZ = ABZ = OFZ = ABZSO > MBZ > OBZ >> TBZ >>> $ABZSO_2$.

$ABZSO_2$ is not used as an anthelmintic and is probably inactive <u>in vivo</u>. The B_{max} values were similar for all compounds.

Table 10.1 Competitive inhibition of [^{3}H]OBZ[a] binding to tubulin in egg supernatants[b] by unlabelled BZs[c].

BZ[c]	IC_{50}[de]	K_a[df]	B_{max}[dg]	Dose rate[h]
FBZ	112	16	92	5
OBZ	144	12	86	10
MBZ	142	12	82	15
ABZ	280	6.3	93	5
OFZ	1474	1.2	91	5
ABZSO	1734	1.0	98	5
TBZ	3854	0.8	83	44
$ABZSO_2$	8251	0.05	85	-

a Final [(^{3}H)OBZ] was 0.1 μM.

b Final [protein] was 100-200 μg/assay, derived from S strain.

c Final concentration: 0-20 μM for FBZ, MBZ & OBZ; 0-50 μM for ABZ and 0-200 μM for ABZSO, $ABZSO_2$, OFZ & TBZ.

d Each value is a mean of 3 experiments determined in triplicate.

e IC_{50} = [BZ] inhibiting 50% of radioligand binding (nM).

f K_a = apparent association constant of the unlabelled BZ (x 10^6 M^{-1}).

g B_{max} = [High-affinity receptor] (pmol/mg protein).

h Dose rate (mg/kg body weight); data adopted from Campbell (1990).

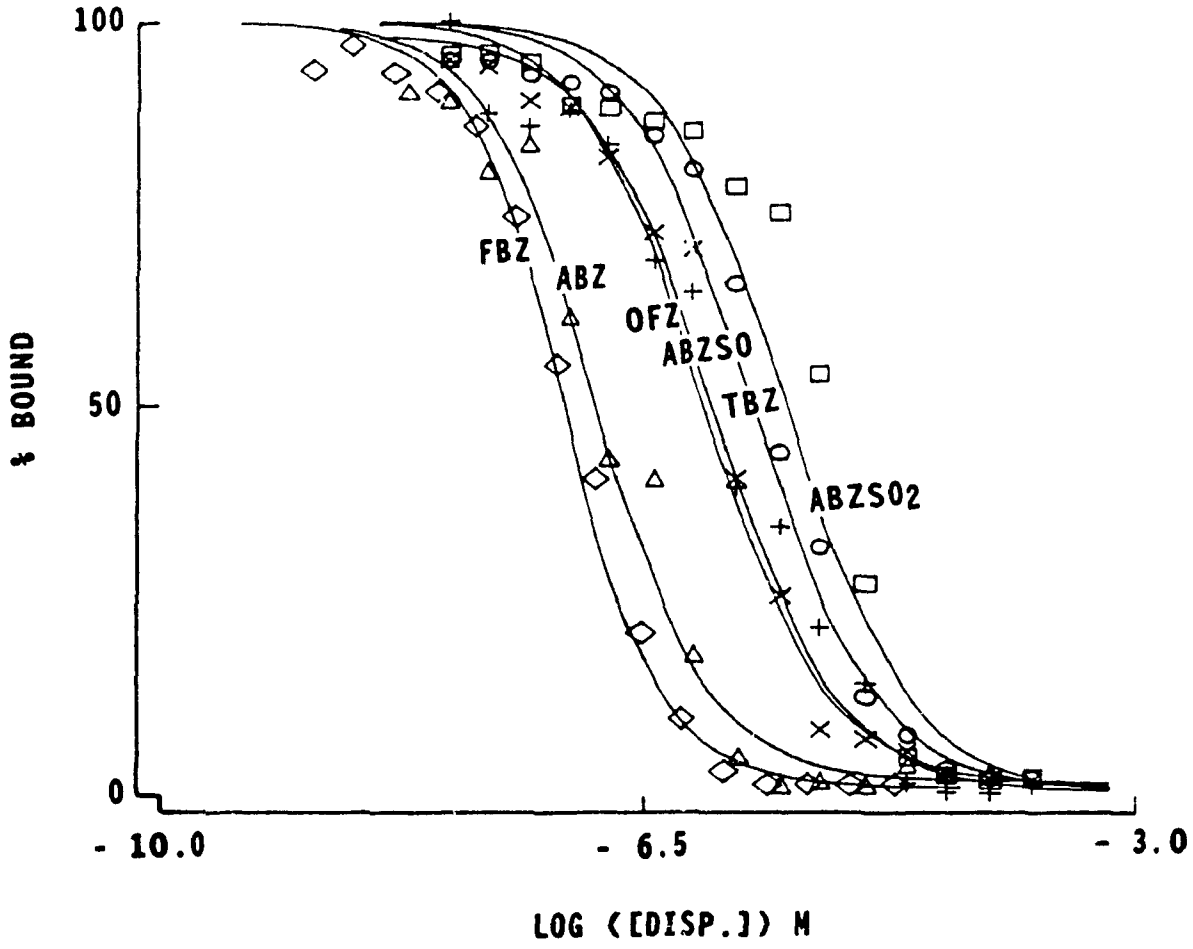


Fig. 10.1. Competetitive inhibition of [^{3}H]OBZ binding by unlabelled BZs in S egg supernatants. [^{3}H]OBZ, unlabelled BZ and egg supernatants were added together and incubated as described in Materials and Methods. See Table 10.1 for details. Displacement curves were generated using LIGAND.

10.4 DISCUSSION

Competitive inhibition studies, in which the labelled and unlabelled ligands were added at the same time to the egg supernatants, measured direct competition between two drugs (the labelled and the unlabelled) for the high-affinity receptors. Also, adding the ligands at the same time shortened the assay procedure. When the binding affinity (IC_{50} or K_a values) of the BZs studied was compared to their anthemintic potency (recommended therapeutic doses) some BZs gave unexpected results (Table 10.1). The affinity of FBZ, MBZ, OBZ, ABZ, TBZ and $ABZSO_2$ for tubulin correlated approximately with their known anthelmintic efficacy against sheep nematodes. OFZ and ABZSO which are very potent anthelmintics in terms of recommended therapeutic doses, demonstrated a low affinity for tubulin relative to other drugs with similar anthelmintic potency. It has been suggested as a general guide that a potent drug should be able to inhibit 50% of the radioligand at concentrations known to cause a pharmacological response in vivo (Creese, 1985). Based on this criterion OFZ and ABZSO may not be potent drugs on their own. Furthermore, [^{3}H]OFZ apparently bound to tubulin in a highly reversible manner and may have bound to tubulin and non-tubulin proteins with low-affinity (Chapter IV). This may have made it difficult to separate LAB and HAB for [^{3}H]OFZ (Fig. 4.1). The anthelmintic activity of OFZ and ABZSO may be potentiated by other factors in vivo. First, the sustained plasma levels of OFZ (Marriner and Bogan, 1981b) and ABZSO (Marriner and Bogan, 1980) may enhance the anthelmintic activity of these drugs against blood ingesting and tissue dwelling parasites. Secondly, OFZ (Marriner and Bogan, 1981b) and ABZSO (Lanusse and Prichard, unpublished observations) are reversibly converted by ruminal microflora into FBZ and ABZ respectively. OFZ and ABZSO may

have high anthelmintic potency against gastrointestinal parasites because they are converted to metabolites (Fig. 10.2) which bind tubulin strongly, with the parent compound acting as a metabolic reservoir effectively maintaining a high concentration of active metabolite in worms in vivo. ABZ was absent in plasma following treatment of sheep with ABZ (Hennessy et al., 1989) or was briefly present in very low levels following treatment of sheep with the prodrug of ABZ, netobimin (Lanusse and Prichard, 1990). However, high levels of ABZ were detected in gut 72 h postreatment of cattle with netobimin (Lanusse and Prichard, manuscript in preparation). Despite some differences between sheep and cattle, high sustained plasma levels of ABZSO were detected in all of these cases.

FBZ, OBZ, MBZ and ABZ may owe their high anthelmintic performance primarily to their high affinity for tubulin. The concentration of high-affinity BZs need not be high to effect maximum occupation of the receptors on tubulin. MBZ was absorbed rapidly from the gut into blood in sheep and it was quickly metabolised (Behm et al., 1983). Its absolute plasma concentration was low compared to other BZs. OBZ might also be rapidly metabolised. The rapid absorption from the gut removes the drug from the gut dwelling parasites while the rapid elimination from plasma removes the drug from tissue dwelling parasites and reduces the opportunity for the active drug to be recycled to the gut. This may explain why the recomended dose rate of MBZ is elevated compared to other high-affinity drugs. OBZ and MBZ whose metabolites have no affinity for tubulin may have to depend on their high affinity for tubulin and a little elevated dose rate to compensate for fast metabolic losses. TBZ is a low affinity drug (Table 10.1) which is also quickly absorbed from the gut and quickly eliminated from the body (Prichard et al., 1981; see Gottschall et

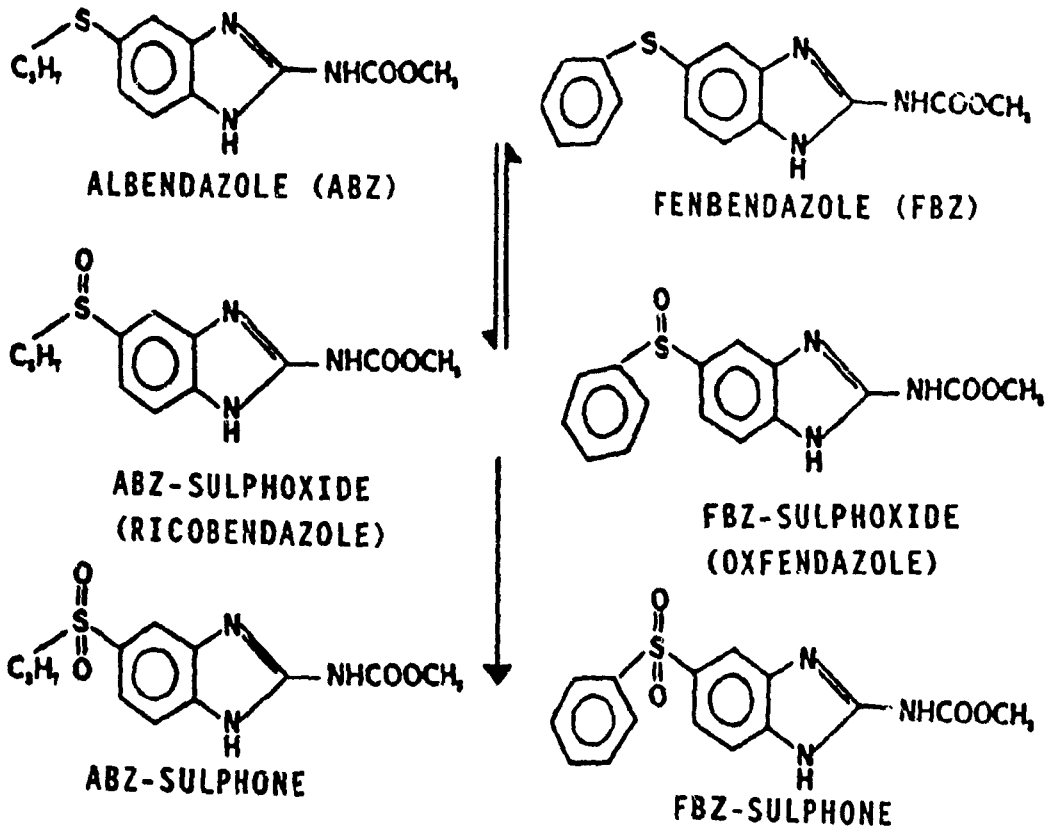


Fig. 10.2 Metabolism of albendazole (ABZ) and fenbendazole (FBZ).

al., 1990 for review). TBZ has, therefore, to be administered in large amounts to be effective. Very high concentrations of $ABZSO_2$ were required to displace [^{3}H]OBZ. The K_a and IC_{50} values suggest that $ABZSO_2$ may not be potent as an anthelmintic. The conversion of ABZSO to $ABZSO_2$ is irreversible (Gottschall et al., 1990). OFZ and ABZSO are low affinity drugs and although administered in low doses (Table 10.1), they are slowly eliminated from the body and some of their metabolites (FBZ and ABZ, respectively) bind strongly to parasite tubulin. Binding to tubulin and inhibition of tubulin polymerisation may not in itself be lethal immediately after exposure to BZs. The efficacy of BZs in vivo may depend on the impairment of the parasite's ability to maintain itself in its predilection site in the face of the physiological pressures from the host. The rate of elimination of the parasite needs to be faster than the rate of elimination of the impairing dr for the latter to be effective as an anthelmintic. The tubulin binding affinity and availability of an adequate BZ concentration in the host and consequently in the parasite to achieve and sustain maximum binding for long enough may be major factors in determining anthelmintic potency against gastrointestinal and tissue dwelling parasites.

CHAPTER XI

GENERAL DISCUSSION

The mechanism of BZ-resistance in H. contortus was investigated We need to know the biochemical mechanism of BZ-anthelmintic resistance because knowledge of the mechanism will help define and monitor resistant strains and substantiate the mode of action of BZs. The knowledge can assist in making decisions on which drugs to use in the field in order to delay the development of resistance and whether new BZ drugs will be effective against resistant strains. Biochemical characteristics may also be helpful markers for genetic research. If the mechanism of resistance is understood other BZ-like drugs or synergists may be designed to improve worm control based on tubulin binding.

In this thesis the specific (high-affinity) binding of various BZs in supernatants prepared from unembryonated eggs, L_3 and adult worms of BZ-susceptible and resistant strains of H. contortus has been examined and compared. The tubulin content and tubulin isoforms of the susceptible and resistant strains were also compared. The binding affinities of some commonly used BZs were compared. The binding data were analyzed using computer programs based on the fundamental principles of receptor-ligand interactions. The main findings will now be discussed.

It had been reported for some parasitic nematodes including H. contortus that homogenates of BZ-resistant strains bind less BZ drug than those of BZ-susceptible strains (see Lacey, 1990 for review). However in those studies only total binding was considered. The data were not analyzed for possible multiple classes of binding sites nor corrected for

non-specific binding. It could not be decided whether all types of bindings in parasite homogenates were reduced or whether resistance affected only a specific component of the total binding. In terms of biochemical kinetics, it was not clear whether resistance affected the binding affinity (K_a) and/or the number of receptors (B_{max}). It had been suggested in literature that BZs act through binding to tubulin (Lacey, 1988). It was, therefore, proposed that tubulin binding of the BZ-resistant strain was altered. The nature of this alteration was unknown and the tubulin of the susceptible and resistant strains had never been compared. In this thesis the total and the low- and high-affinity binding of [^{3}H]BZ anthelmintics- MBZ, OBZ, ABZ and OFZ -in whole supernatants from unembryonated eggs, third stage larvae or adult worms of BZ-susceptible or -resistant strains have been examined and compared. The total binding of all the [^{3}H]BZ used was reduced for the resistant strain compared with the susceptible one. Similar observations were reported previously (Lacey and Prichard, 1986; Lacey, 1988). The total binding of OBZ and MBZ was readily separated into low-affinity and high-affinity binding. However, OFZ bound with low-affinity and the total binding of this ligand could not be clearly separated into low-affinity and high-affinity binding. The total binding of ABZ was also separable into low- and high-affinity binding although limited work was done using this ligand due to an inadequate supply.

The binding affinity, K_a, and maximum binding, B_{max}, for the high affinity binding of OBZ and MBZ were calculated using the computer programs, EBDA and LIGAND. 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. The K_a was not

affected by resistance. There was a 3-5 fold loss of B_{max} by the resistant strain. Low-affinity binding was similar between the resistant and susceptible strains at each stage of development. BZ binding was also performed using tubulin-enriched supernatants. Low-affinity binding to parasite preparations devoid of tubulin was observed but high-affinity binding occurred only to preparations containing tubulin. Tubulin was responsible for the high-affinity binding. BZs bound to other proteins with low-affinity.

BZ-resistance affects all stages (eggs, larvae and adults) (Lacey, 1988). This is not suprising because tubulin is ubiquitous (Dustin, 1984) and all stages would be similarly affected unless there were stage specific tubulin isoforms. However, studies in vitro suggest that eggs or immature worms may be more susceptible to BZs than adult worms (Kirsch and Schleich, 1982; Rapson et al., 1985; Rew et al , 1986). The supernatants from eggs showed greater high-affinity binding per mg protein than those of larvae which in turn showed greater high-affinity binding than those of adult worms. It was shown by SDS-PAGE, Western blot and ELISA analysis that the tubulin content per mg protein decreased from egg, through larva to adult worm. Egg supernatants bound more drug, perhaps because egg cells contain more tubulin than the cells of larvae and adult worms. Tubulin is the principle protein of the mitotic spindle and may be abundant in rapidly dividing cells, such as eggs. Mitotic activity is expected to decrease from egg through larva to adult stage. High-affinity binding may depend on tubulin content which in turn may depend on the mitotic requirements of the organism. While cells which require greater tubulin utilisation might be more sensitive to BZs, it is also probable that it is the timing of critical physiological events (such as cell division) which

is important for susceptibility to BZs in all developmental stages. Thus eggs and immature worms may die quickly upon exposure to BZs but adult worms with little mitotic activity may die more slowly.

Compared with the eggs, more of the binding in larvae and adults was low-affinity binding. Most of the low-affinity binding of the latter stages was probably non-specific binding involving proteins other than tubulin. There was little low-affinity binding of the eggs and some or all of it may have occurred to tubulin because it appeared to be partly saturable. This raises a possibility of a low-affinity site on some tubulin isoforms in addition to the high-affinity site. Any low-affinity site on tubulin of larvae and adults may have been masked by the non-specific binding of non-tubulin proteins. It remains to be seen, however, if a low-affinity site on tubulin does in fact exist. Since low-affinity binding is not affected by resistance, it would be interesting to know if resistant worms can be controlled through this site by raising the drug dose rate or altering the ligand chemistry.

The results presented in this thesis suggest that, notwithstanding the differences among the stages, BZ-resistance can be diagnosed using either eggs, larvae or adult worms. The specific BZ-binding assay corroborated the egg hatch assay. The results are not inconsistent with tubulin, an ubiquitous protein, being the target of BZ-anthelmintic action. It has been reported, however, that various tissues, developmental stages and species of organisms may contain different tubulin isoforms (Dustin, 1984). Nevertheless, the results in this thesis suggest that the tubulin isoforms or the amino acid sequences responsible for BZ-binding are present in all developmental stages.

It has been suggested in literature that all BZs might act on the

same receptor(s) (Lacey, 1988, 1990). This is supported by reports that resistance arising from selection with one particular BZ affects other BZs as well (Berger, 1976; Colglazier et al., 1975; Prichard et al., 1980). Whereas BZs appear to be equally potent against susceptible gastrointestinal nematodes (when administered at their respective recommended dose rates), there are several claims of superior performance by some BZs against worms resistant to other BZs (Simpkin and Coles, 1978; Donald, 1983; Slocombe et al., 1989). It is not clear, therefore, whether the mechanism of resistance is similar for all BZs or for all strains of all species. Several BZs were used in this study to see if the binding was altered for all or some of them.

The ability of various BZs to bind tubulin was assessed by displacement (inhibition) assay to determine their IC_{50} and K_a values. The ability of unlabelled BZs- FBZ, MBZ, OBZ, ABZ, ABZSO, $ABZSO_2$, OFZ and TBZ - to bind tubulin was determined from their ability to inhibit the binding of [^{3}H]MBZ or [^{3}H]OBZ to tubulin in parasite supernatants. Cross-displacement studies suggested that the different BZ drugs interacted with the same receptor (tubulin) in both S and R strains. The IC_{50} values of most of these BZs demonstrate high-affinity binding since they fall far below the drug concentrations expected in the gastrointestinal tract. Several observers have reported maximum concentrations (C_{max}) of 5-10 μM in plasma of sheep or cattle (Prichard et al., 1978b, 1981; Marriner and Bogan, 1980, 1981a, b). Lanusse and Prichard (in preparation) have observed C_{max} values of 5-20 μM in the ileum and abomasum of cattle after administration of recomended doses BZ. The uptake of TBZ by T. colubriformis in vitro was passive but the worms were able to concentrate the drug 5-6 times compared to the surrounded medium (Sangster and

Prichard, 1984). TBZ concentration inside the worms after treatment of sheep with the recommended dose was the equivalent of 20 μM (Sangster and Prichard, 1986).

It had been suggested that tubulin binding and subsequent inhibition of microtubule formation is the mode of anthelmintic action of BZs (Lacey, 1990). However, the correlation between the *in vitro* activity of BZs against parasite tubulin and the *in vivo* anthelmintic potency of BZs had not been attempted using a **specific** BZ-binding assay. Some studies had attempted to do so using purified mammalian tubulin or whole homogenates using a **total** BZ-binding assay (see Lacey, 1990 for review). As pointed out above, total BZ-binding binding could be misleading because low-affinity binding is not accounted for. In assays for diagnosing BZ-resistance or for anthelmintic screening, a specific BZ-binding assay would be of advantage since one does not need to purify the tubulin in order to perform such tests. Moreover, many purification procedures seem to denature tubulin and alter binding kinetics (Tang, 1988). In this thesis, the binding affinities (see IC_{50} and K_a values) of several BZs were measured using a specific BZ-binding assay and the results were compared to the known anthemintic potency (recommended therapeutic doses) of these BZs. The IC_{50} and K_a values correlated with the known anthelmintic potency of the BZ compounds except for OFZ and ABZSO whose K_a values were lower than could be expected from anthelmintic potency. It is possible that OFZ and ABZSO depend on conversion to FBZ and ABZ, respectively, for anthelmintic activity. The anthelmintic potency of a BZ may depend on the binding affinity for tubulin and/or metabolic conversion to more potent forms and other pharmacokinetic features.

BZs have the ability to bind tubulin of *H. contortus* over a wide

range of temperature. Although the affinity (K_a) was reduced at 4°C compared with 37°C, the detection of BZ-resistance was not affected by temperature. However, the expression and extent of BZ-resistance in C. elegans is temperature dependent (Driscoll et al., 1989; Woods et al., 1989). In H. contortus, the significance of the tubulin's ability to bind BZs at low temperature is not clear. It suggests, however, that the BZ binding-site is not markedly altered by a switch in temperature although the reaction may proceed more slowly at low temperature. This is in contrast to mammalian tubulin whose binding characteristics are sharply affected by a switch in temperature (Tang, 1988).

Studies in fungi (including yeast) and the free-living nematode, C. elegans suggest alteration in β-tubulin gene as the mechanism of BZ-resistance in these organisms (Foster et al., 1987; Sheir-Neiss et al., 1978; Orbach et al., 1986; Thomas et al., 1985; Driscoll et al., 1989). At the beginning of this study, there had been no report to suggest that BZ-resistance in parasitic nematodes is caused in a similar manner. Western blots of tubulin separated by 2-D PAGE showed that the ß-tubulin isoform pattern of the susceptible strain was different from that of the resistant strain but that the α-tubulin isoform patterns of the 2 strains were similar. BZ-resistance was associated with a decrease in high affinity BZ binding to tubulin and an alteration in ß-tubulin isoform pattern. The linkage between the binding and the specific isoform type remains undetermined. BZ-resistance did not affect high-affinity K_a values. The similarity in K_a values suggests that the receptor(s) in the S and R strains are similar or closely related although diminished in amount in the R strain. The resistant population may be heterogeneous consisting of mainly the resistant worms (tubulin isoforms) but with a proportion of the

susceptible worms (tubulin isoforms). This would explain why the K_a of the S and R strains are similar while the B_{max} of the R strain is less than that of the S strain. If this hypothesis was true one would expect the B_{max} of the R strain to diminish further upon subjection to more selection pressure. This reduction was observed upon selection of the R4 strain although reversion occurred after a few generations. Reversion (instability) of BZ-resistance was observed previously (Simpkin and Coles, 1978). The B_{max} of the CBZ-resistant strain was reduced compared with the S strain from which it was selected.

Whereas it can be concluded that alteration in β-tubulin caused reistance in the R strain, it cannot be said whether BZs bind β-tubulin only. BZs are known to inhibit colchicine binding and yet the binding site of colchicine may be located on α-tubulin (Schmitt and Atlas, 1976; Williams et al., 1985 and Serrano et al., 1984). It remains to be clarified how the binding domains of BZs and colchicine overlap or how the binding of one ligand interferes with the binding of another. It may well be that BZs bind at the interface between α- and β- tubulin in which case mutations in α-tubulin may be too rare to be observed in BZ-resistance.

The following may be summarised or concluded from this study:

(1) High-affinity binding, which is recommended as most important criteria for relevant binding, was demonstrated in parasite homogenates.

(2) BZs specifically bound tubulin with high-affinity.

(3) BZs bound tubulin over a wide range of temperature (4° and 37°C).

(4) Anthelmintic potency of BZs may depend on the K_a (which in turn may depend on the chemical structure) and on metabolic conversion to

more or less potent forms.

(5) All BZ anthelmintics were affected by resistance.

(6) However, some BZ structures might perform better than others against resistant worms e.g MBZ might perform better than OBZ against resistant H. contortus but this point needs to be verified in vivo. The situation with other worm species could also be different.

(7) The susceptible and resistant strains contained similar total amounts of tubulin per mg protein.

(8) The tubulin content per mg protein decreased from egg through larval to adult stage.

(9) The decrease in tubulin content per mg protein coincided with a decrease in high-affinity binding per mg protein from egg through larval to adult stage.

(10) Resistance was associated with a loss of high-affinity binding.

(11) Resistance was also associated with an alteration of ß-tubulin isoform pattern.

(12) The above points put together have established tubulin as the pharmacologically relevant receptor of BZs.

Important questions remain and form the basis for further research prospects:

(i) It cannot be said as yet whether the binding site of BZs is located on ß-tubulin or at the interface of α and ß tubulins.

(ii) Whether the BZs can bind relatively simple amino acid sequences or whether the receptor(s) comprise complex tertiary structures needs to be determined.

(iii) The basic sequences of amino acids and the structure that

comprises the binding site needs to be known in order to answer the above and other questions relating to BZ-resistance.

(iv) It remains to be seen whether alteration in ß-tubulin is a common and/or the only mechanism of BZ-resistance in parasitic nematodes. BZ-resistance in a fungus was associated with an alteration of a ß-tubulin isoform compared to a wild type (Foster et al., 1987). In sharp contrast, BZ-resistance in a free-living nematode was ascribed to a complete deletion of a ß-tubulin gene (Driscoll et al., 1989).

(v) The strains used in this study were laboratory strains which have lived free from some of the environmental selection pressures found in the field, for some time It would be of value to repeat this work using a freshly isolated fully BZ-susceptible strain. The new susceptible isolate could be compared with a resistant strain selected from it.

(vi) The answers to the above questions could lead to the development of drugs aimed at slowing or overcoming resistance. The identification, cloning and expression of the genes responsible for BZ-susceptibility and/or resistance would be a good starting point. The gene products (tubulin) would then be characterised and sequenced. Relevant sequences could then be manipulated and artificially synthesized and screened using a specific BZ-binding assay. A variety of BZ-like structures could be tested for affinity for such tubulin sequences and superior structures selected. Identified genes can also be used as probes in the diagnosis of BZ-resistance or as markers for genetic research. The processes which control the selection and inheritance of BZ-resistance could be better understood using such markers.

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APPENDIX I

ABBREVIATIONS USED

A	=	adult worm
ABZ	=	albendazole
ABZSO	=	albendazole sulphoxide = rycobendazole (ricobendazole)
$ABZSO_2$	=	albendazole sulphone
B_{max}	=	maximum BZ binding at infinite ligand concentration
	=	the maximum receptor concentration
BSA	=	bovine serum albumin
BZ	=	benzimidazole
BZs	=	benzimidazoles
CBZ	=	cambendazole
CLC	=	colchicine
2-D PAGE	=	two dimensional polyacrylamide gel electrophoresis
DMSO	=	dimethyl sulphoxide
dpm	=	disintegrations per minute
E	=	unembryonated egg
EC_{50}	=	effective concentration of BZ causing 50% egg hatch inhibition
ED_{50}	=	effective dose of anthelmintic causing 50% reduction of the worm burden
ED_{90}	=	effective dose of anthelmintic causing 90% reduction of the worm burden
EGTA	=	ethylene glycol bis-(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid
ELISA	=	enzyme-linked immunosorbent assay
FBZ	=	fenbendazole
GTP	=	guanosine-5'-triphosphate
HAB	=	high-affinity binding
IC_{50}	=	concentration of BZ inhibiting 50% radioligand specific (high-affinity) binding
IEF	=	isoelectric focusing
K_a	=	apparent association constant at equilibrium

L	=	larval
L_3	=	third stage (infective) larvae
LAB	=	low-affinity binding
MBC	=	methyl-benzimidazole carbamate = carbenazin
MBZ	=	mebendazole
MES	=	2[N-morpholino]ethanesulphonic acid
NDZ	=	nocadozole
NSB	=	non-specific binding
OBZ	=	oxibendazole
OFZ	=	oxfendazole
PDT	=	podophyllotoxin
PLL	=	poly-L-lysine
pmol	=	picamole(s)
R	=	thiabendazole-resistant
S	=	thiabendazole-susceptible
SB	=	specific binding
SDS-PAGE	=	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SF	=	susceptibility factor = the ratio of B_{max} of S to that of R
TB	=	total binding
TBZ	=	thiabendazole
TFS	=	tubulin-free supernatants

APPENDIX II

PUBLICATIONS

Some of the work described in this thesis has beer published or will be published as follows:

1. Lubega, G.W and Prichard, R.K. (1990). Specific interaction of benzimidazole anthelmintics with tubulin: high affinity binding and benzimidazole resistance in Haemonchus contortus. *Molecular and Biochemical Parasitology*, 38: 221-232.

2. Lubega, G.W. and Prichard, R.K. (1991). Specific interaction of benzimidazole anthelmintics with tubulin: Comparison of developing stages of thiabendazole-susceptible and thiabendazole-resistant strains of Haemonchus contortus. *Biochemical Pharmacology*, 41, 93-101.

3. Lubega, G.W. and Prichard, R.K. (1991). Beta-tubulin and benzimidazole resistance in the sheep nematode, Haemonchus contortus. *Molecular and Biochemical Parasitology*, 47, 128-139.

4. Lubega, G.W. and Prichard, R.K. (1991). Interaction of benzimidazole anthelmintics with Haemonchus contortus tubulin: Binding affinity and anthelmintic efficacy. *Experimental Parasitology*, In press.

5. Lubega, G.W. and Prichard, R.K. (1991). Effect of benzimidazole (BZ) anthelmintic treatment of Haemonchus contortus on the specific interaction of BZs with tubulin. Manuscript in preparation.

Some of the work was presented at conference proceedings:

1. Lubega, G.W. and Prichard, R.K. Pharmacology of benzimidazole resistance in Haemonchus contortus. American Association of Veterinary Parasitologists, Orlando, Florida, 16-18 July 1989.

2. Lubega, G.W. and Prichard, R.K. Molecular basis of benzimidazole resistance in Haemonchus contortus. Canadian Society of Zoologists, North York, Ontario, 17-19 May 1989.

3. Lubega, G.W. and Prichard, R.K. Mechanism of benzimidazole resistance in the sheep nematode, Haemonchus contortus. American Society of Parasitologists, 65th Annual Meeting, Michigan State University, East Lansing, Michigan, 26-30 June 1990.

4. Prichard, R.K., Lubega, G.W., Bédard, M. and Matlashewski, G. Benzimidazole resistance in Haemonchus contortus: Molecular basis of resistance. 14th symposium of the Scandinavian Society for Parasitology, Elsinore, Denmark, 2-4 August 1989.

5. Prichard, R.K., Lubega, G.W., Tang, L., Guénette, S., Bédard, M., Maingi, N., Scott, M.E. and Matlashewski, G. Anthelmintic resistance in nematodes: Extent, recent understanding and future directions for control and research. 25th Annual Meeting of the Australian Society for Parasitology (Silver Jubilee), Magnetic Island, Townsville, Australia, 26-29 September 1989.

6. Prichard, R.K., Tang, L. and Lubega, G.W. L'attachement des drogues antiparasitaires benzimidazoles à la tubuline de nématode. 57e Congrés de l'ACFAS, Montréal, Québec, 15-19 mai 1989.

7. Lubega, G.W. and Prichard, R.K. Interaction of benzimidazole anthelmintics with Haemonchus contortus tubulin: Binding affinity and anthelmintic efficacy. American Association of Veterinary Parasitologists. Seattle WA, 28-30 July 1991.