EFFECTS OF BENZIMIDAZOLE ANTHELMINTICS ON NEMATODE PARASITES OF SHEEP

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Doctor of Philosophy

by

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DECLARATION

The contents of this thesis are the work of the author. The thesis has not been submitted previously for the award of a degree to any university. The following publications are based on the work contained in this thesis:

Scott, E.W., McKellar, Q.A., Armour, J., Bairden, K. and Bogan, J.A., 1988, Production of strains of the sheep parasite *Ostertagia circumcincta* by implantation of adult parasites into the abomasum of lambs, *Res. Vet. Sci.* **45**:120-121.

Scott, E.W., Mitchell, E.S., Armour, J., Bairden, K., Soutar, A. and Bogan, J.A., (in press), Level of benzimidazole resistance in a strain of *Ostertagia circumcincta* studied over several infections in lambs, *Vet. Parasitol.* (in press).

Scott, E.W., Bairden, K., Armour, J. and Bogan, J.A., The effect of treatment with increasing doses of fenbendazole on benzimidazole resistance in *Ostertagia circumcincta*, *Vet. Parasitol.* (submitted)

ABBREVIATIONS

kg	kilogram
mg	milligram
ug	microgram
ml	millilitre
M	molar
pmole	picomoles
r.p.m.	revolutions per minute
dpm	disintegrations per minute
cpm	radioactive counts per minute
e.p.g.	eggs per gram
ED50	effective dose 50% -dose which produces 50% of the desired effect on the population
LD50	lethal dose 50% - dose requires to kill 50% of the population
HPLC	high-performance liquid chromatography
ABZ	albendazole
ABSX	albendazole sulphoxide
ABSO	albendazole sulphone
CBZ	cambendazole
FBZ	fenbendazole
FBZSO	fenbendazole sulphone
FLZ	flubendazole
MBZ	mebendazole
OFZ	oxfendazole
OBZ	oxibendazole
PBZ	parbendazole
TBZ	thiabendazole
TCZ	triclabendazole
^{3}HABZ	tritiated albendazole
^{3}HFBZ	tritiated fenbendazole
^{3}HMBZ	tritiated mebendazole

SUMMARY

A benzimidazole resistant strain (HFRO strain) and a benzimidazole susceptible strain (Bearsden strain) of the sheep abomasal parasite *Ostertagia circumcincta* were obtained and passaged through parasite naive lambs. Using egg hatch assays to detect resistance day to day changes in the susceptibility of the eggs from adult *O.circumcincta* of these strains to the effects of the benzimidazoles, thiabendazole and albendazole were noted. Treatment of the lambs which were parasitised with the HFRO strain with fenbendazole at the normal dose rate caused a temporary increase in the resistance level measured by egg hatch assays. However, 12 treatments with fenbendazole over a 14 generation period with the HFRO strain produced a negligible rise in benzimidazole resistance for this strain.

A dose titration trial to determine the effectiveness of fenbendazole against adult parasites of the HFRO strain of *O.circumcincta* confirmed that the parasites were highly resistant to the effects of the benzimidazole anthelmintics. Treatment with increasing doses of fenbendazole used in the dose titration trial failed to increase the level of resistance in the eggs produced by the adult parasites remaining after treatment.

The establishment of 'clones' of the HFRO strain of *O.circumcincta* by implantation of pairs of adult parasites into the abomasa of lambs was investigated. The offspring of these parents showed a reduced level of resistance to the benzimidazoles measured by egg hatch assays but after treatment with fenbendazole selection for resistance occurred rapidly.

The DNA from the two strains of *O.circumcincta* was isolated and compared after sectioning with restriction endonucleases. The banding obtained after gel electrophoresis was similar for the two strains.

Studies were carried out on the motility of infective larvae of the HFRO and Bearsden strains of *O.circumcincta* in the presence of benzimidazole anthelmintics, their metabolites and physostigmine. Prolonged incubation in the presence of benzimidazoles

was required to reduce motility. Differences in the motility in the presence of physostigmine between benzimidazole resistant and benzimidazole susceptible strains of parasites were found. The activity of the benzimidazole metabolites against larval stages and eggs of *O.circumcincta* was studied and discussed.

A possible mode of action of the benzimidazole group of anthelmintics is binding to nematode tubulin causing depletion of microtubular structures with disruption of cellular transport systems. Tubulin was extracted from the pig intestinal nematode *Ascaris suum* and the sheep nematodes *O. circumcincta* and *Haemonchus contortus*. The benzimidazoles albendazole and mebendazole were shown to inhibit the binding of colchicine to tubulin extracts from *A.suum*. Tritiated albendazole, fenbendazole and mebendazole were synthesised and were used to study the binding of these drugs to tubulin extracts from *A.suum*, *O.circumcincta* and *H.contortus*. Binding of these benzimidazoles was variable and was influenced by the protein content of the tubulin extract. High concentrations of tritiated benzimidazoles bound to extracts synthesised using low numbers (<100,000) of infective larvae of *O.circumcincta* and *H.contortus*.

The differential binding of tritiated benzimidazoles to tubulin extracts from nematodes as a test for resistance in these parasites to the effects of benzimidazole anthelmintics was investigated using tritiated fenbendazole, albendazole and mebendazole. Extracts from *H.contortus* larvae which were resistant to the benzimidazoles bound less mebendazole and albendazole than extracts from benzimidazole susceptible larvae. With tubulin extracts from different strains of *O.circumcincta* results were more variable and depended on the ligand used in the assays.

Displacement of albendazole binding to tubulin extracts from *H.contortus* by albendazole and its metabolites , albendazole sulphoxide and albendazole sulphone, was found with albendazole proving to be the most potent inhibitor. However, in assays using *O.circumcincta* tubulin extracts albendazole, albendazole sulphoxide, albendazole

sulphone and colchicine failed to inhibit, consistently the binding of tritiated albendazole.

An ELISA to measure binding of anti-alpha and anti-beta tubulin antibodies to extracts from *O.circumcincta* larvae found reduced binding of both antibodies to extracts from first stage and third stage larvae of the benzimidazole resistant strain compared with similar extracts from the benzimidazole susceptible strain. Treatment of the larvae with benzimidazoles prior to the ELISA for tubulin did not alter the antibody binding to extracts from both the resistant and susceptible strains of *O.circumcincta*

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INTRODUCTION

ANTHELMINTICS

Anthelmintics are a group of agents used in the therapy of internal parasitic infections in animals. These drugs should kill parasites effectively without toxicity to the host, have good activity against adult and larval stages, persist in tissues for sufficient time to kill the parasites but have a short withdrawal period to avoid tissue residues and be inexpensive to use.

The anthelmintics can be subdivided into groups according to their activity against different groups of helminths e.g. nematocides, anticestodal and antitrematodal compounds. They can be classified on their spectrum of activity against parasites - broad spectrum or narrow spectrum. Some of the narrow spectrum anthelmintics were among the first to be used as anthelmintics in veterinary therapy. These narrow spectrum anthelmintics have activity against specific groups of parasites and include brotianide, bunamidine, clorsulon, diamphenethide, diethylcarbamazine, piperazine , praziquantel and salicylanilides e.g.closantel, rafoxanide, nitroxynil, oxyclosanide and niclosamide. Many of these compounds remain in use for their activity against trematode and cestode parasites. In the agricultural industry in the UK the most important group of anthelmintics used in the control of nematode parasite infestations are the broad spectrum anthelmintics including the benzimidazoles, pro-benzimidazoles, avermectins, organophosphorous compounds, imidathiazoles e.g. levamisole and tetrahydropyrimidines e.g. morantel and pyrantel. A brief description of the main groups of anthelmintics which are of importance to the UK agricultural industry is given below.

Avermectins

This is a group of macrocyclic lactones produced by the soil microorganisms *Streptomyces avermitilis* which were discovered in 1973 and investigations were

carried out as to their suitability as anthelmintics (Burg *et al.*,1979). Of the natural compounds avermectin B_1 has the greatest anthelmintic activity and chemical modification of this molecule by selective hydrogenation produces 22,23,dihydroavermectin B_1, ivermectin, the compound which is marketed as an anthelmintic in most countries including the UK and USA. As an anthelmintic ivermectin is highly potent, being active against some cattle and sheep parasites at doses as low as 0.025 mg/kg and overall ivermectin is about 1000 times more potent than thiabendazole (Egerton *et al.*,1981). Activity of ivermectin against different species of parasites can be variable with efficacy against intestinal dwelling parasites lower than that against abomasal parasites (Fisher, 1985). The pharmacokinetics of ivermectin may explain these differences in part (Bogan *et al.*, 1988) but there seems to be species variation in susceptibility to the effects of ivermectin. Ivermectin shows activity against ectoparasites (Ostlind *et al.*, 1979 ; James *et al.*, 1980), but has no activity against cestode and trematode parasites.

The route of administration of ivermectin influences the pharmacokinetics of the drug with subcutaneous administration giving higher plasma levels and a longer retention time compared with orally administered drug. In cattle after subcutaneous administration efficacy against abomasal parasites persists for 14 days (Barth , 1983) and for lungworm activity may extend for 21 days (Armour *et al.*, 1985). In sheep, however, when ivermectin is administered by the subcutaneous or oral routes the persistent effect is much less (McKenna ,1986; McKellar and Marriner, 1987). Slow release intraruminal devices are being developed to prolong the activity of the drug.

Ivermectin is believed to act by potentiating the release and binding of the neurotransmitter gamma-aminobutyric acid (GABA) allowing the accumulation of GABA at the nerve synapse and thus blocking transmission of nerve impulses (Fritz *et al.*, 1979; Campbell, 1981). This effect causes paralysis of the parasite which can then be removed

from its predilection site. Ivermectin also has a suppressive effect on reproductive processes (McKellar *et al*, 1988) and possibly other effects on parasite metabolism at the biochemical level. The neurotoxic effects of ivermectin do not occur in the mammalian parasite host because GABA receptors are confined to the central nervous system and the high molecular weight and polarity of ivermectin prevents the molecule from passing through the blood-brain barrier. In some breeds of dogs there can be defective transport mechanisms in the blood-brain barrier and ivermectin has been shown to cause neurotoxicity (Pulliam *et al*, 1985)

Ivermectin ('Ivomec', Merk, Sharp and Dohme) has been available as a subcutaneous injection for cattle in the UK since 1981 and has achieved widespread use especially as an autumn treatment prior to housing and in a pasture parasite control system which involves administration at 3,8 and 13 weeks after turn out onto pasture in the spring (Armour *et al*, 1987). For sheep ivermectin ('Oramec' , Merk,Sharp and Dohme) has been available in the UK since 1983 but the oral preparation has poorer efficacy against ectoparasites and persistence is reduced and therefore the drug has not achieved widespread use as in the cattle industry. Avermectin has been introduced to the Australian market for sheep where its main indication will be on farms where parasites are resistant to the other broad spectrum anthelmintics, the benzimidazoles and levamisole/morantel. An oral paste formulation of ivermectin ('Eqvalan', Merk, Sharp and Dohme) is marketed for horses and is used frequently in the field.

As with the other anthelmintics problems of parasite resistance may develop (see later). The ecological effect of the use of ivermectin, particularly in slow release devices, involving the inhibition of the development of dung beetles and their larvae, the decreased rate of disintegration of faecal pats and the effect on pasture may be of significance over long periods of anthelmintic usage (Wall and Strong , 1987). However, the climatic

conditions influence the speed of disintegration of faecal pats and a field study on the build-up of faecal material in pastures grazed by ivermectin treated and untreated animals failed to show any differences in pasture contamination (McKeand *et al.*, 1988).

Benzimidazoles and Pro-benzimidazoles

The benzimidazoles are a group of related anthelmintics with similar chemical structures based on the benzimidazole nucleus (Fig. 1.1). Thiabendazole (TBZ) was the first of this group to be introduced in 1961 and was shown to have antifungal as well as anthelmintic properties (Davidse,1975). Biochemical substitutions on the benzimidazole nucleus were used in the development of a range of anthelmintics with differing pharmacological properties and efficacy against parasites. The important members of the group are given in Table 1.1 along with their biochemical structures and dates of introduction. The later benzimidazoles have a carbamate group at position R^2 which increases activity and the substituent on position R^1 influences the solubility of the compound and hence its pharmacokinetics. TBZ is the only member of the group with antifungal activity. The earlier compounds e.g. TBZ, cambendazole (CBZ), parbendazole (PBZ), oxibendazole (OBZ), mebendazole(MBZ) and flubendazole (FLZ) have good activity against gastrointestinal parasites but poorer efficacy against lungworms and no activity against hypobiotic fourth stage larvae of *Ostertagia spp.* The modern benzimidazoles, namely fenbendazole,(FBZ), oxfendazole,(OFZ) and albendazole,(ABZ) are less soluble than the other members of the group and have better efficacy against nematode parasites including hypobiotic larval forms. Triclabendazole (TCZ) has no activity against nematode parasites but has good efficacy against trematodes, notably *Fasciola hepatica*, (Boray *et al.*, 1983). ABZ is the other benzimidazole with antitrematodal action but high doses of the drug are required compared with those to kill nematodes and efficacy against trematodes is much poorer than with TCZ.

Figure 1.1

Chemical structure of benzimidazoles

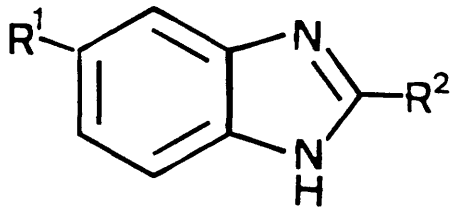

R1
N
R2
N
H

Table 1.1

Chemical structures of the benzimidazole anthelmintics

Name	R^1	R^2
Thiabendazole	H	4-thiazolyl
Cambendazole	$(CH_3)_2CH-O-CO-NH-$	4-thiazolyl
Albendazole	C_3H_7-S-	$-NH-CO_2CH_3$
Fenbendazole	C_6H_5-S-	"
Flubendazole	$F-C_6H_4-CO-$	"
Mebendazole	C_6H_5-CO-	"
Oncodazole	2-thienyl-CO-	"
Oxfendazole	C_6H_5-SO-	"
Oxibendazole	C_3H_7-O-	"
Parbendazole	C_4H_9-	"
Ricobendazole	C_3H_7-SO-	"

The pro-benzimidazoles are a group of compounds which are metabolised in the body to form benzimidazoles and owe their anthelmintic activity to the benzimidazole metabolite which is produced. Thiophanate is metabolised by organisms in the rumen microflora to produce a benzimidazole, carbendazim , with activity similar to the early benzimidazoles. Febantel is a phenylguanidine which is absorbed from the gastrointestinal tract and undergoes cyclisation in the liver to form FBZ and OFZ (Delatour *et al.*, 1982). Netobimin is a nitrophenylguanidine which undergoes metabolic reduction of a nitro group to an amino group prior to cyclising to form ABZ in the rumen or liver (McDougall *et al*, 1985).

The limited water solubility of the benzimidazoles makes them unsuitable for subcutaneous administration and all the benzimidazoles are administered orally as drenches, pastes, powders, intraruminal boluses or in feed blocks. OFZ is also available as an intraruminal injectable preparation.

The benzimidazoles have a similar mode of action (see later) and differences in efficacy against parasites are due to variations in solubility and resulting pharmacokinetics among the compounds. The relatively water insoluble modern benzimidazoles e.g. FBZ, OFZ and ABZ have greater *in vivo* anthelmintic activity than the more soluble compounds e.g. TBZ. Because of their mode of action the length of time that parasites are exposed to the benzimidazoles is important to get effective anthelmintic activity. TBZ given as a single oral dose to cattle failed to remove hypobiotic larvae of *O. ostertagi* (Armour, 1969) whereas administration of TBZ as an intraruminal infusion over 36 hours removed 90% of hypobiotic *O.ostertagi* (Prichard *et al*, 1978). In animals with a rumen this organ acts as a store for the benzimidazoles and allows steady release of the anthelmintic into the rest of the gastrointestinal tract prolonging its availability. In monogastric animals the small volume of gastrointestinal contents, the steady depletion of contents and the limited

solubility of the benzimidazoles reduces availability of the drugs. Dividing the dose of benzimidazole over several days in monogastric animals, especially carnivores, increases the availability of the drug and parasite exposure to the compound and therefore increases the efficacy (Burke and Roberson, 1978). There is evidence that dividing the dose of benzimidazoles increases the drug availability in ruminant animals (Bogan *et al.*, 1987). In addition malnutrition of the host or severe parasite infestations with diarrhoea, anaemia,pyrexia etc. can affect, detrimentally the pharmacokinetics of the benzimidazoles and uptake of the drug by the parasites (Prichard ,1980)

After oral administration of the benzimidazoles they are absorbed to some extent depending on their water solubility. Metabolism of the drugs occurs in the rumen, gastrointestinal tract and liver usually forming compounds with increased solubility or inactive products which are excreted. The main routes of metabolism for the benzimidazoles include reduction, oxidation (sulphoxidation, demethylation and hydroxylation) and conjugation prior to excretion (Van den Bossche *et al.*, 1982). The modern benzimidazoles , ABZ, FBZ and TCZ are metabolised by reversible sulphoxidation. Various enzymes e.g. liver microsomal oxidases, tissue oxidases and reductases are involved in the interconversions of sulphide to sulphoxide and the reverse. These reactions can occur in many tissues (Marriner and Bogan ,1981b) but there is limited interconversion of sulphides and sulphoxides in the rumen and gastrointestinal tract. There is a further slower, irreversible oxidation to the sulphone metabolite, ABZ, therefore is metabolised rapidly to ABZ sulphoxide (ABSX) with none of the parent ABZ identifiable in peripheral plasma. Further metabolism to the sulphone (ABSO) occurs along with formation of low levels of other metabolites (Gyurik *et al.*, 1981). Febantel, FBZ and OFZ are metabolically interconvertible with FBZ the sulphide, OFZ the corresponding sulphoxide and febantel the pro-benzimidazole which converts to FBZ

(Delatour *et al*, 1985; Marriner and Bogan, 1981b). The solubility of the compound which is administered has a significant effect on drug absorption and plasma levels of the drug. When administered orally small differences in solubility between the sulphide and sulphoxide forms, the sulphoxide being more water soluble, may have a significant impact on the drug availability and consequent efficacy against parasites (Marriner and Bogan, 1985).

Both the sulphide and sulphoxide forms of the modern benzimidazoles are thought to be anthelmintically active *in vivo* (Marriner and Bogan, 1980 ; Marriner and Bogan, 1981a) but the sulphone is inactive (Averkin *et al*, 1975). Considering that a possible mode of action of the benzimidazoles is as anti-tubulins in parasites both the sulphide and sulphoxide forms bind to tubulin although there is conflicting evidence as to the potency of these two forms of the drugs as anti-tubulins (Barrowman *et al*, 1984; Ireland *et al*, 1979).Because of the interconversion of the sulphides and sulphoxides in the tissues there may be little significance in identifying which form is most active biologically .

The pharmacokinetics of the benzimidazoles varies amongst species and with members of the group of compounds. Following are descriptions of the pharmacokinetics of some of the important benzimidazole compounds as applicable to sheep. (For a review of the anthelmintic activity of the benzimidazoles see Van den Bossche *et al*, 1982)

After oral administration TBZ is rapidly absorbed from the rumen and abomasum. In sheep after oral administration at the recommended dose rate of 44 mg/kg maximum concentrations in the plasma were reached at 2-4 h post-treatment and fell to undetectable levels by 36h post-treatment. The inactive metabolite of TBZ, 5-hydroxythiabendazole (Tocco *et al*, 1964), reached maximum plasma concentrations in 6h and was undetectable in plasma by 24h post-treatment (Weir, 1987). Thiabendazole and 5-hydroxythiabendazole are conjugated to glucuronide or sulphate in

the liver and excreted rapidly in the urine (Tocco *et al.*, 1964). Thiabendazole has good efficacy against adult and immature gastrointestinal nematodes but the rapid clearance from the body leads to poorer efficacy against lungworms and hypobiotic larvae of *Ostertagia spp.*

Albendazole has a lower water solubility than TBZ and therefore absorption is slower. In sheep after oral administration of ABZ none of the parent compound is detected in the plasma and it is only the metabolites ABSX and ABSO which are found. After oral administration of ABZ maximum concentrations of ABSX are found in the plasma at about 20 hours post-treatment and maximum concs. of ABSO are found 10-12 hours later (Marriner and Bogan, 1980) .Both ABSX and ABSO can be detected in plasma for about 72 hours post treatment. The increased persistance of ABSX in the tissues compared with TBZ produces better efficacy against immature gastrointestinal parasites including hypobiotic forms of *Ostertagia spp.* and lungworms. At increased dosage rates ABZ has activity against adult and some immature *Fasciola hepatica*. Ricobendazole,which is ABSX, is now available as a drench for sheep and has similar anthelmintic activity to ABZ. However, it does not require conversion from the sulphide form within the animal and therefore may have increased availability compared with the parent compound,ABZ.

After oral administration of FBZ to sheep maximum plasma concentrations of FBZ are found at 24h. FBZ is converted to OFZ and peak concentrations of this metabolite are detected at 30h. Both FBZ and OFZ can be detected in plasma for up to 5 days. The sulphone metabolite of FBZ and OFZ, FBZSO, is detected in plasma for up to 7 days with maximum concentrations at 36h after oral administration of FBZ (Marriner and Bogan, 1981b). When OFZ is administered orally maximum plasma concentrations of the drug are detected at 30h and levels persist in the plasma for up to 7 days. The sulphone metabolite, FBZSO , reaches maximum plasma concentrations at 48h after oral administration and can be

detected in plasma for 8 days (Marriner and Bogan, 1981a). Both FBZ and OFZ have good activity against gastrointestinal nematodes, lungworms and hypobiotic forms of *Ostertagia spp.* These drugs have no activity against flukes.

The benzimidazoles are thought to have a similar mode of action and differences in efficacy of the drugs against groups of parasites probably reflect differences in the bioavailability of the drugs within the host animal. The more potent anthelmintics of the benzimidazole group of compounds have slower absorption and elimination rates (Prichard *et al*, 1978).

Studies to investigate the mode of action of the benzimidazole group of anthelmintics concentrated on the antimitotic properties of some members of the group. It was suggested that the antimitotic activity was due to disruption in the formation of the mitotic spindle (Seiler, 1975). Using oncodazole Hoebeke *et al*, (1976) studied the binding of this benzimidazole compound to rat brain tubulin and suggested that the antimitotic activity of the benzimidazoles was due to binding to tubulin. The attachment of benzimidazoles to tubulin molecules caused inhibition of the formation of microtubules resulting in disruption of cell division. A similar mechanism of tubulin binding and disruption of the microtubular framework within cells was suggested as a possible reason for the anthelmintic activity exhibited by the benzimidazoles (Davidse and Flach, 1978).

Borgers and De Nollin (1975) noted changes in the ultrastructure of the parasite *Asaris suum* after treatment of pigs with MBZ which had been infected experimentally with these parasites. The changes in ultrastructure of parasite intestinal cells noted by these authors included alteration in the distribution of cell organelles and loss of microvilli. These changes were identified at periods of 6-9h after oral treatment of the host with MBZ. The ultrastructural changes in intestinal cells of *A.suum* were obvious before alterations were noted in other cells of the parasites. Using radiolabelled MBZ

higher concentrations of the benzimidazole were detected in the parasite gut than in other parasite tissues. Further studies on the effects of MBZ on the ultrastructure of intestinal cells of *A.suum* and *Syngamus trachea* and the tegumental cells of *Taenia taeniaeformis* have been reported (Borgers *et al*, 1975a; Borgers *et al*, 1975c). After exposure to MBZ there was disappearance of cytoplasmic microtubules causing disruption in the migration of subcellular organelles with a failure of transport of secretory granules. This resulted in prolonged storage of secretory material within the cells with eventual lysis of the cell cytoplasm and disintegration of the cells. Cell autolysis occurred within 15-24h of treatment with MBZ. There was no effect on the ultrastructure of host cells with the cytoplasm and mitotic spindle appearing normal and microtubule distribution remaining unaltered. These authors suggested that the anthelmintic action of the benzimidazoles was due to differences in the sensitivity of host and parasite cells to the effects of the benzimidazoles.

Similar changes in parasite cell ultrastucture were noted in the cestodes, *Hymenolepsis nana*, after exposure to MBZ (Verheyen *et al*, 1976). Using cytochemical analysis of hydrolase activity in the parasites the degeneration of the parasites was found to be due to autolysis of the tegument. The authors suggested that the MBZ caused a block in the transport of secretory granules within the cell. This prevented adequate nourishment of the cell and the prolonged intracellular storage of the enzymes within the secretory granules resulted in cellular autolysis. Microtubules are thought to be essential for enzyme secretion by parasites and studies of *Nippostyrongylus brasiliensis in vitro* (Watts *et al*, 1982) indicated that secretion of acetylcholinesterase was inhibited by benzimidazoles. Reduction in secretion of acetylcholinesterase in the presence of benzimidazoles has also been shown in *Trichostrongylus colubriformis* and this was linked to disappearance of microtubules which were examined ultrastructurally (Sangster

et al., 1985)

The differences in the sensitivity of host and parasites to the effects of benzimidazoles may be due to differences in the structure of microtubules in their cells. Ultrastructural studies with *Ascaridia galli* and *T. colubriformis* revealed differences in the protofilament numbers in microtubules of cells of the parasites compared with mammalian cells (Davis and Gull, 1983).These authors found that mammalian cells had 13 protofilaments in microtubules whereas the parasites had cells with 11, 12 and 14 protofilaments in the microtubules.

In addition to studies on the effect of benzimidazoles on the ultrastructure of cells the binding of benzimidazoles to the protein tubulin has been studied. Experiments using extracts of tubulin from rat brain (Hoebeke *et al.*, 1976) identified the binding of oncodazole to the tubulin which prevented assembly of tubulin components into microtubules. Similar results were obtained using tubulin extracts from bovine brain (Friedman and Platzer, 1978). Extracts of tubulin from intestines of the pig nematode *A.suum* were used in studies of microtubule assembly in the presence of MBZ and colchicine (Kohler and Bachman,1980) These authors discovered that MBZ acted as a competitive inhibitor of colchicine binding to tubulin and this suggested that MBZ and colchicine bound to microtubules at a structurally close site. Differences in the affinity of benzimidazoles for tubulin extracted from *A.suum* intestines and pig brains were approximately two fold. This small difference in the affinity for the tubulin derived from the mammalian and parasitic sources may account partially for differences in the activity of the benzimidazoles against the host cells and parasitic cells. The uptake of MBZ into the parasites also may affect the activity of the drug with the worms accumulating higher concentrations of MBZ than in the surrounding medium. These results suggested that the pharmacokinetics of the benzimidazoles may be important in determining the differences

in toxicity of the drugs to the host and parasite cells.

Experiments on the binding of FBZ and MBZ to tubulin extracted from embryonic forms of *A.suum* indicated that inhibition of colchicine binding to these extracts was non-competitive and that tubulin extracts from embryonic forms of *A.suum* were very sensitive to the effects of the benzimidazoles (Friedman and Platzer, 1980a). At the embryonic stage the parasites are independent of external nutrients and the effect of anthelmintic on metabolic processes would be minimal. There was a 250-400 fold difference in the inhibition constants of colchicine binding in the presence of MBZ or FBZ to tubulin extracts from embryonic *A.suum* and tubulin extracted from bovine brain. This suggested that differences in binding of these benzimidazoles to tubulin from parasites and mammalian tissues may account for the differential toxicity of the benzimidazoles between host and parasitic cells. However Dawson *et al*, (1984) using tubulin from *Ascaridia galli* and mammalian tubulin found that MBZ, ABZ, FBZ, PBZ and OBZ had similar inhibitory activity against polymerisation of tubulin from nematodes and mammals. OFZ had greater activity against parasitic tubulin than mammalian tubulin. Gull *et al*, (1987) using purified tubulin extracts from *A.galli* found FBZ, PBZ and ABZ had similar potency as inhibitors of microtubule assembly against mammalian and nematode tubulin but OFZ and TBZ were more effective in preventing nematode microtubule assembly. Therefore, differential binding of benzimidazoles to tubulin from parasitic and mammalian sources did not account for differences in toxicity of some of the benzimidazoles to host and parasite cells.

Ireland *et al.*, (1979) using tubulin extracted from sheep brain found some correlation between antimicrotubular activity *in vitro* and anthelmintic activity with PBZ, FBZ, OBZ, ABZ, CBZ and TBZ but not for MBZ and OFZ. These authors suggested that the possible discrepancies between the *in vitro* and *in vivo* activity may be due to the

pharmacokinetics of the drugs in the host or differences in helminth and mammalian tubulin. Lacey *et al.*,(1987) compared the efficacy of some of the benzimidazoles measured as their ovicidal activity with the tubulin binding of the drug to mammalian tubulin, extracted from sheep brain. These authors found that the potency of the drugs in one assay reflected drug potency in the other assay. They suggested that these results indicated that inhibition of tubulin polymerisation was the mode of action of the benzimidazoles when acting as ovicidal drugs against parasite eggs.

The benzimidazoles are effective against nematode parasites but efficacy against the trematodes e.g. *Fasciola hepatica* is limited to ABZ and TCZ. Using tubulin extracted from *F. hepatica* colchicine binding to the extracts was inhibited by CBZ, PBZ, OBZ, MBZ and ABZ. TCZ had no effect on colchicine binding to *F.hepatica* (Fetterer, 1986). This suggested that the action of ABZ against *F.hepatica* may be due to binding to tubulin but TCZ may have other actions against flukes.

The disruption of metabolic processes within parasites may be involved in the activity of benzimidazoles. Parasites can differ from their hosts in a number of pathways of respiratory metabolism. There are differences in the enzymes of the respiratory pathways and energy synthesis in mitochondria between the host and nematode parasites. Nematode parasites produce incompletely oxidised organic acids as end products of metabolism (Behm and Bryant, 1979).

Mebendazole was found to inhibit the uptake of glucose by *A.suum* (Van den Bossche and De Nollin , 1973) and this inhibition was considered to be irreversible (Van den Bossche, 1972). A decrease in the glycogen content of the parasites' muscles along with decreased uptake of amino acids and fatty acids was reported for *A.suum* in the presence of MBZ (Van den Bossche and De Nollin, 1973). Inhibition of glucose uptake and depleted glycogen reserves were found in *S. trachea* (Van den Bossche, 1972) in the presence of

MBZ and similar effects were noted with PBZ and CBZ. This author noted that the reduction in glycogen reserves was related to the dose of benzimidazole to which the parasites were exposed. CBZ and MBZ were shown to inhibit glucose uptake, increase glycogen utilisation and decrease adenosine triphosphate (ATP) levels due to the activity of these benzimidazoles on metabolic enzymes in *Moniezia expansa*. Studies on intestinal material from *A.suum* revealed that uptake of sodium ions across the intestinal membrane was reduced after treatment with benzimidazoles (Beames *et al.*, 1976). This effect could be secondary to a reduction in glucose uptake by the parasites produced by the benzimidazoles. TBZ produces little effect on glycogen reserves in parasites. A substitution at position 5 or a carbamate group on the benzimidazole molecule may be essential to affect glycogen utilisation in nematode parasites (Van den Bossche, 1972).

Benzimidazoles can act as potent uncouplers of oxidative phosphorylation in mammalian mitochondria (Jones and Watson, 1967). The activity of various metabolic enzymes in nematode parasites is inhibited by benzimidazoles. Both cytoplasmic and mitochondrial malate dehydrogenase in *A.suum*, *F.hepatica* and *M.expansa* are inhibited by ABZ, PBZ, MBZ and TBZ (Tejada *et al*, 1987). CBZ and MBZ inhibited phosphoenolpyruvate and fumarate reductase in *M.expansa* (Rahman and Bryant, 1977). TBZ inhibited fumarate reductase in *H.contortus* (Prichard, 1970 and 1973). Fumarate reductase in *A.suum* was inhibited by MBZ, ABZ, FBZ, OFZ, ABSX and ABSO (Barrowman *et al.*, 1984). Also, succinate dehydrogenase and fumarate reductase in *Trichinella spiralis* were inhibited by MBZ (Boczon, 1976). However higher concentrations of the benzimidazoles are required to inhibit fumarate reductase in nematode parasites than concentrations which can inhibit tubulin polymerisation (Dawson *et al.*, 1984; Kohler and Bachmann, 1978).

The general structure of the benzimidazoles suggests that they are likely to interfere with reactions involving purine-like groups. When benzimidazoles interact with

metabolic enzymes in parasites the lethal effect of the drugs is due to a decline in the ATP available to the parasites and therefore, they are unable to maintain their situation within the host (Behm and Bryant, 1979).

Studies on the mode of action of benzimidazoles have revealed species differences in the biochemical effects on the parasites. Alteration in the microtubular transport systems within the parasites produced by the benzimidazoles may lead to changes in the metabolic reactions which occur within the parasites and the effect of the benzimidazoles on tubulin polymerisation and biochemical effects on respiratory enzymes may be linked (Behm and Bryant, 1985).

The aim of the studies detailed in this thesis was to investigate the effect of benzimidazoles on tubulin molecules derived from nematode parasites. The experiments were carried out using the modern benzimidazoles ABZ and FBZ. The efficacy of benzimidazole metabolites was investigated. Studies on the mechanism of action of the benzimidazoles were expanded to include studies on possible differences in the effects of benzimidazoles on anthelmintic resistant and susceptible strains of parasites.

Imidathiazoles

Tetramisole which is the racemic mixture of D and L forms, dexamisole and levamisole, was introduced into veterinary therapy in 1966. The L-isomer, levamisole, is more active and less toxic than the D-form and therefore levamisole is marketed separately as an anthelmintic. This drug is highly active against a wide range of nematodes but it is less active against immature gastrointestinal parasites than the benzimidazoles or ivermectin and has poor efficacy against hypobiotic fourth stage larvae of *Ostertagia spp.*

The mechanism of action of levamisole is due to ganglion stimulation producing reversible paralysis. At high concentrations levamisole causes inhibition of fumarate reductase in parasites inhibiting parasite metabolism. Levamisole is absorbed and

excreted rapidly (Bogan *et al*,1982). The drug can be administered by subcutaneous injection or orally and is now available as a topical preparation for cattle. Maximum blood concentrations occur at 30-60 min after subcutaneous administration and 2-3 hours after oral administration. Excretion is rapid occurring within 24 hours of drug administration.

A survey of anthelmintic usage in the UK in 1982 (Thomas *et al*., 1982) indicated that the percentage of the anthelmintic market held by levamisole was 30% for cattle, 15% for sheep and 30% for pigs. In cattle levamisole is used as a summer drench to remove adult and immature parasites when hypobiotic forms of *O. ostertagi* are not present. Several species of nematode parasites have become resistant to the anthelmintic effect of levamisole (see later). These resistant parasites tend to show side - resistance with morantel/pyrantel.

Levamisole can be used as an immunostimulant in the therapy of chronic infections and cancer (Renoux,1978). This ability to produce immunostimulation may be the reason for the synergistic effect observed when levamisole is used at low dose rates in combination with a benzimidazole anthelmintic (Bennet *et al*., 1980)

Tetrahydropyrimidines

This group of anthelmintics is similar in structure to the imidathiazoles and the members of veterinary importance are pyrantel and its methyl ester analogue, morantel. These broad spectrum anthelmintics have good activity against adult gastrointestinal nematodes but are less effective against immature, mucosal dwelling larval forms. They are thought to act as depolarising muscle relaxants in parasites (Coles *et al*, 1974) producing paralysis.

In the UK morantel is available only for cattle incorporated in an intraruminal continuous release bolus ('Paratect', Pfizer). However the drug is available as an oral

drench for use in sheep and cattle in other countries.Pyrantel is used in horses and small animals. Parasites have become resistant to the effects of these drugs and in most cases there is side - resistance with levamisole(see later).

ANTHELMINTIC RESISTANCE

Resistance is defined as a significant increase in the ability of individuals within a strain to tolerate doses of a compound which would prove lethal to the majority of individuals in a normal population of the same species. This resistance is genetically expressed and inherited (Kelly and Hall, 1979). Within a normal population there may be individual members with the characteristic necessary to develop resistance but these individuals do not dominate in the population unless there is selection pressure on the populations and these individuals have an advantage over other members of the population.

Drug resistance in populations of bacteria, protozoa, arthropods and some mammalian cells has developed rapidly and become widespread. However, drug resistance in helminths to the effects of anthelmintics has developed relatively slowly and is sporadic in its occurrence (Waller and Prichard, 1986) The timescale of development of resistance to anthelmintics has paralleled the introduction of the individual anthelmintics onto the agricultural market. Reports have been published of resistance in nematode parasites to all the major groups of modern anthelmintics including the benzimidazoles, the levamisole/morantel group and the avermectins. If there is resistance in a nematode population to one drug in the benzimidazole group there is usually resistance to all members of the benzimidazole group of compounds (Hall *et al*, 1978b; Martin *et al*, 1985) This is designated side-resistance and has been defined as resistance to a compound which is the result of selection by another compound with a similar mode of action (Prichard *et al*, 1980). Parasites which are resistant to the effects of levamisole show

side-resistance to morantel (Le Jambre and Martin, 1979; Sangster *et al.*, 1979). However the reverse is not necessarily the case and Waller *et al.*, (1986) reported resistance to morantel in a strain of *T.colubriformis* and the parasites remained fully susceptible to the effects of levamisole. Cross-resistance is the term given to resistance which has developed to a compound as the result of selection by another compound with a different mode of action (Prichard *et al.*, 1980). When parasites become resistant to two or more anthelmintic groups, either as a result of selection by each group independently, or as a result of cross-resistance then multiple-resistance has developed (Prichard *et al.*, 1980).

Resistance in *H.contortus* to the effects of the phenothiazine group of anthelmintics was reported in the USA by Drudge *et al.*, (1957). The introduction of the broad spectrum anthelmintics, e.g. the benzimidazoles led to a reduction and eventually cessation of use of the phenothiazines. However, resistance to these broad spectrum anthelmintics developed and was reported from the USA by Drudge *et al.*, (1964) in a strain of *H.contortus*. Further reports of resistance to benzimidazoles in *H.contortus* in Australia followed (Smeal *et al.*, 1968). Resistance to anthelmintics in other sheep nematodes developed and a strain of *T.colubriformis* resistant to benzimidazoles was reported by Hotson *et al.*,(1970) and *O.circumcincta* by Le Jambre (1977). Coles, (1986) suggested that parasites which were resistant to phenothiazines were able to develop resistance to benzimidazole anthelmintics rapidly (Tritschler *et al.*, 1987).

Resistance to anthelmintics in sheep nematodes is most prevalent in areas of Australia and several reviews decribing the importance of anthelmintic resistance have been written (Kelly and Hall, 1979; Prichard *et al.*, 1980; Donald, 1982; Waller and Prichard, 1986; Waller, 1987). A survey of resistance in sheep producing areas of the Northern Tablelands in Australia revealed that the incidence of benzimidazole resistant

H.contortus was widespread (Webb *et al*, 1979). The failure of the benzimidazoles to clear resistant *H.contortus* from sheep prompted a change to the use of levamisole or morantel on many properties and in the following period resistance to these anthelmintics was identified in *T.colubriformis* and *O.circumcincta* (Waller, 1986). However, resistance to levamisole or morantel in *H.contortus* was slower to develop and was first reported by Green *et al*, (1981). The use of benzimidazoles followed by levamisole and morantel led to multiple resistance to anthelmintics in *T.colubriformis* (Sangster *et al*, 1979), *H.contortus* (Green *et al*, 1981) and similar problems of resistance to anthelmintics in nematodes from goat farms (Hall *et al*, 1981). A survey of properties on the Northern Tablelands found a high incidence of anthelmintic resistance in *T.colubriformis* and *O.circumcincta* (Love, 1985). In other areas of Australia there is widespread resistance to anthelmintics in *T.colubriformis* and *O.circumcincta* (Waller, 1986).

In other countries anthelmintic resistance has been identified and has become a problem to sheep and goat producers. Surveys of anthelmintic resistance in New Zealand on sheep farms identified resistance in *T.colubriformis* and *H.contortus* (Kemp and Smith, 1982) and resistance has been identified in strains of *O.circumcincta* (Hughes and Seifert, 1983) and *Nematodirus spp* (Middleberg and McKenna, 1983). Resistance to anthelmintics on goat farms has been identified as a particular problem in New Zealand (Kettle *et al*, 1983).

The climate of Argentina, Brazil and Uruguay resembles that in areas of Australia and *H.contortus* infections can be a problem in some areas of all these countries. In Brazil *H.contortus* infections with parasites resistant to anthelmintics are widespread (Amaral, 1985) and strains of *T.colubriformis*, *O.circumcincta* and *Nematodirus spp* show resistance to anthelmintics (for review see Waller, 1986). Similar climatic conditions

and incidence of anthelmintic resistance in sheep nematodes are found in South Africa with strains of *H.contortus* resistant to benimidazoles (Berger, 1975) salicylanilides (Van Wyck *et al.*, 1987) and ivermectin (Van Wyk *et al.*, 1987).

Anthelmintic resistance was first reported in the USA but these cases occurred on research farms and there have been few reports of field cases of anthelmintic resistance in sheep nematodes (Coles, 1983).

In Europe resistance to anthelmintics was described in Switzerland by Jordi (1980). In the Netherlands there have been reports of resistance to anthelmintics in *H.contortus* (Boersema *et al.*, 1982: Eysker *et al.*, 1982: Eysker *et al.*, 1983) and a survey of anthelmintic resistance in the Netherlands was carried out and low levels of resistance were found in *H. contortus* on a large number of properties (Boersema *et al.*, 1987). Resistance in the U.K was reported first by Britt (1982) after a survey of sheep parasites from 33 flocks. A strain of *O.circumcincta* resistant to thiabendazole was identified on one of the farms. Further studies on this strain of *O.circumcincta* were described (Britt and Oakley, 1986). Other reports of benzimidazole resistance in strains of *O.circumcincta* were made (Cawthorne and Whitehead, 1983) and a survey of anthelmintic resistance in the south-east of England (Cawthorne and Cheong, 1984) found benzimidazole resistance in *H.contortus* on seven farms. During a survey of sheep farms in the north of England (Evans, 1988) parasites showing resistance to anthelminics were not identified. Levamisole resistance in sheep abomasal parasites was reported by Britt (1986). Currently a survey of anthelmintic resistance on sheep farms in the south of England is being conducted (Taylor, personal communication). In other European countries surveys for anthelmintic resistance have not been reported and the incidence of anthelmintic resistance is unknown

Resistance to anthelmintics in nematodes infecting species other than sheep has been

identified. The prevalence of resistance in cattle parasites to the effects of anthelmintics is low but there have been reports of benzimidazole resistance in *Trichostrongylus axei* from Australia (Eagleson and Bowie, 1986), of levamisole resistance in *Ostertagia ostertagi* in Belgium (Geerts *et al.*, 1987), morantel resistance in *Ostertagia ostertagi* in the Netherlands (Borgsteede, 1988) and benzimidazole resistance in *Cooperia oncophora* in New Zealand (Jackson *et al.*, 1987a). Resistance to benzimidazole in horse strongyles has been reported widely in Australia (Kelly *et al.*, 1981), the U.S.A. (Drudge *et al.*, 1979), in Canada (Slocombe and Cote, 1977), in Germany (Bauer *et al*, 1986) and in the U.K. (Round *et al.*, 1974; Britt,1984; Herd, 1986; Ryan *et al.*, 1987 and Lumsden and Ryan, 1987). Resistance in the pig parasite *Oesophagostomum spp* to pyrantel was reported in Denmark (Roepstorff *et al.*, 1987). A report of resistance to anthelmintics in dog parasites was made by Jackson *et al.*, (1987b). These authors identified a strain of *Ancylostoma caninum* resistant to pyrantel and oxantel discovered in New Zealand.

A number of methods, both *in vivo* and *in vitro,* are available to identify resistance to anthelmintics in nematode parasites. Initially resistance to anthelmintics may be noticed on the farm as a failure of the anthelmintic used clinically to cure parasitic gastroenteritis in sheep. Further studies can be instituted to identify an anthelmintic resistant strain of parasites and to isolate the strain. In some cases failure of anthelmintics to reduce parasite populations in sheep may be caused by factors other than anthelmintic resistance e.g. inadequate dosage of drug, poor management allowing immediate reinfection of animals and rapid drug metabolism. The latter can be a problem in goats especially with levamisole and cases of resistance identified by Kettle *et al.*,(1983) may have been due to inadequate dosing of goats with levamisole rather than levamisole resistance in the parasites (McKenna, 1984)

The faecal egg count reduction test (FECR test) is an *in vivo* test which can be used on

farms and commonly is used in the investigation of parasite resistance to anthelmintics. In Australia this test is used routinely to evaluate the success of worm control programmes and to try to investigate the reasons for failures in the control systems. This test compares the faecal egg counts in animals before and after treatment with an anthelmintic and the percentage reduction in faecal egg count is calculated. A group of untreated control animals is included in the test to monitor day to day fluctuations in the faecal egg counts. Faecal egg counts are taken 7-10 days after treatment with anthelmintics because levamisole and benzimidazoles have a suppressive effect on egg production (Martin *et al*, 1985) and faecal egg counts taken in the period immediately post treatment may give an overestimation for the depressive effect of the anthelmintics. Another problem with this test is that faecal egg counts may not reflect the adult worm burden carried by the animals for some species of parasites (Martin *et al*, 1985) and there can be between animal variation in worm burdens and egg output with large group sizes required for the test.

Reduction in egg count of <90% after treatment with anthelmintics is indicative of parasite resistance to anthelmintics (Webb *et al*, 1978; Kettle *et al*, 1981). To identify the species of parasites resistant to anthelmintics in mixed populations the faeces collected after treatment with anthelmintics can be cultured and the parasites identified at the infective larval stage (Presidente, 1985). This test has been used for the initial identification of parasite resistance to anthelmintics in sheep, goats and horses (Webb *et al*, 1979; Hotson *et al*, 1970; Kelly *et al*,1981) The test was used for the identification of resistance during surveys of the prevalence of anthelmintic resistance , reviewed by Presidente (1985).

The controlled anthelmintic efficiency test is the most reliable test to confirm the presence of resistance in nematode parasites but it is costly to perform. It is used to confirm resistance in isolates of parasites and to quantify the level of resistance in a

particular strain of parasite . All types of anthelmintic can be used in this assay. The test is a dose titration trial (Moskey and Harwood, 1941; Gibson, 1964; Powers *et al.*, 1982) comparing the worm counts of experimentally infected animals treated with increasing doses of anthelmintic with worm counts in untreated animals. Worm burdens should be assessed at necropsy approximately 7 days after treatment of the animals with the anthelmintic to ensure that all susceptible parasites are removed from the host (Prichard *et al.*, 1980). An LD50 (concentration of anthelmintic which kills 50% of the nematodes) can be calculated for the resistant parasites. This test has been used to confirm the presence of resistance in a range of parasite species in sheep and goats (for review see Presidente, 1985). A controlled anthelmintic efficiency test has been described for *T.colubriformis* using guinea pigs as an alternative animal model to sheep to reduce the cost of the test (Kelly *et al.*, 1981).

Several *in vitro* tests have been described to detect resistance in nematodes to the effects of anthelmintics. To detect benzimidazole resistance the *in vitro* egg hatch assay is used most commonly (Le Jambre, 1976; Coles and Simpkin, 1977). This assay involves the incubation of freshly collected parasite eggs in the presence of a range of dilutions of the benzimidazole, usually TBZ, and the assessment of the hatchability of the eggs. There are a variety of modifications to the method with incubation times varying from 24-72h and assessment of effectiveness of the anthelmintic given as either number of eggs failing to hatch to larvae or number of eggs failing to develop (Boersema, 1982). An ED50 (concentration of drug which prevented development of 50% of the eggs) can be calculated from a dose response curve. Some workers use a log-dose probit plot to calculate the ED50 (Le Jambre *et al.*, 1979) or Arc-sin transformations (Cawthorne and Whitehead, 1983). There can be day to day variation in the egg hatch assay results (Borgsteede and Couwenberg, 1987; Le Jambre, 1976). Le Jambre (1976) used comparison of ED50 for

a reference susceptible strain of parasites and a resistant strain to overcome the problem of day to day variability in the assay. Where a mixed infection of parasites is being tested the eggs can be cultured to the infective larval stage to identify the species which are resistant to the benzimidazoles (Whitlock *et al.*, 1980).

The *in vitro* egg hatch assay test can be modified to test resistance to other anthelmintics including levamisole, morantel and ivermectin. Eggs collected in the faeces are cultured in the presence of the drug and prevention of development of the free-living stages of the parasites to the infective larvae is measured (Waller and Lacey, 1986). This test is now being standardised as a larval development assay to detect resistance (Coles *et al.*, 1988; Lacey, personal communication; Taylor, personal communication). Dobson *et al.*, (1986) have described an egg hatch assay for use with levamisole.

An *in vitro* larval motility assay can be used to detect resistance to levamisole and morantel (Martin and Le Jambre, 1979). This assay assesses the paralysis of larvae in the presence of increasing concentrations of the anthelmintic. The motility of *H.contortus* in the presence of drugs has been assessed using a micromotility meter (Folz *et al.*, 1987). Differences have been found in the quantities of cholinesterase enzymes in benzimidazole resistant and susceptible *H.contortus*, *T.colubriformis* and *O.circumcincta* (Sutherland *et al*, 1988). This led to the development of a larval paralysis assay for benzimidazole resistance using incubation of infective larvae in the presence of physostigmine (Sutherland *et al.*, 1988).

A tubulin binding assay to measure the differential binding of tritiated benzimidazoles to tubulin extracts from benzimidazole resistant and susceptible parasites has been described (Lacey, 1985; Lacey and Prichard, 1986; Lacey and Snowdon, 1988). In this assay the extracts from benzimidazole resistant parasites bind less benzimidazole than extracts from benzimidazole susceptible parasites. A comparison between the ovicidal

activity of benzimidazoles and their ability to bind to tubulin extracts (Lacey *et al.*, 1987) showed good correlation for a range of benzimidazoles. However MBZ is the benzimidazole of choice for this assay (Lacey and Snowdon, 1988).

The mechanisms by which parasites have become resistant to the effects of anthelmintics are unknown. Changes in the carbohydrate metabolism in benzimidazole resistant parasites have been noted compared with metabolism in susceptible parasites (Bennet, 1981; Rew *et al.*, 1982). There was no reduction in the uptake of benzimidazoles by parasites resistant to these drugs (Prichard *et al*, 1978; Sangster and Prichard, 1984; Weston *et al.*, 1984). The benzimidazole anthelmintics are thought to affect microtubules in parasites. Differences were found in the binding of benzimidazoles to tubulin from benzimidazole resistant and susceptible strains of *Aspergillus nidulans* (Davidse and Flach, 1977). In nematodes Sangster *et al*, (1985) noted differences in the acetylcholinesterase secretion between benzimidazole resistant and susceptible strains of *T.colubriformis* incubated in the presence of benzimidazoles. This correlated with the presence of microtubules in intestinal cells of the benzimidazole resistant parasites compared with disappearance of microtubules in susceptible parasites examined ultrastructurally at intervals after incubation with the benzimidazoles. Binding of benzimidazoles to tubulin extracted from benzimidazole resistant strains of *H.contortus* was reduced compared with binding to tubulin from susceptible strains (Lacey and Prichard, 1986). Differential binding of benzimidazoles to tubulin extracted from benzimidazole resistant and susceptible strains of *O.circumcincta* and *T.colubriformis* also was shown(Lacey and Snowdon, 1988). Reduced binding of benzimidazoles to tubulin was suggested as the biochemical mechanism for development of resistance to benzimidazole anthelmintics (Lacey, 1985). Foster *et al.*, (1987) discovered a mutant form of the β tubulin molecule in a benzimidazole resistant strain of *Physarum*

polycephalum and suggested that this could be responsible for the benzimidazole resistance. The mechanism of resistance to levamisole and morantel has not been studied in sheep parasites. A levamisole and morantel resistant strain of *Caenorhabditis elegans* was found to have fewer acetylcholine binding sites than susceptible strains of this nematode (Lewis *et al.*, 1980).

The mechanisms by which resistance is genetically transferred are unknown. Studies on benzimidazole resistance in *H.contortus* have produced conflicting theories on the mode of inheritance of resistance in this species. Le Jambre *et al.*, (1979) found that thiabendazole resistance in *H.contortus* was not sex-linked, was inherited as a semi-dominant trait and probably involved more than one gene. Herlich *et al.*, (1981) also found that benzimidazole resistance in *H.contortus* was not sex-linked but these authors suggested that resistance was probaby inherited recessively and was multigenic. Studies on a levamisole resistant strain of *T.colubriformis* (Waller *et al.*, 1985) and the production of a mathematical model of the development of resistance in this strain (Dobson *et al.*, 1987) suggested that levamisole resistance in *T.colubriformis* was determined by a single dominant gene. Resistance to benzimidazoles in a strain of *T.colubriformis* was found to be controlled by more than one gene and resistance was inherited as a semi-dominant trait with some maternal influence (Martin *et al.*, in press). A similar mechanism for inheritance of benzimidazole resistance in *O.circumcincta* has been suggested (Martin, personal communication). Breeding studies using sheep nematodes to try to elucidate the genetic mechanisms controlling resistance are difficult because of the life-cycles of the parasites. However, work on the inheritance of anthelmintic resistance in the free-living parasite *C.elegans* may produce results which could be of relevance in the study of anthelmintic resistance in parasitic nematodes (Waller, 1986).

A wide range of factors may influence the development of resistance to anthelmintics in

the field. The prevalent species of parasites has an important influence on the speed of development of resistance with benzimidazole resistance predominating in areas where *H.contortus* infections are a major problem in sheep (Waller, 1986). The frequency of anthelmintic treatments has a direct relationship on the development of resistance (Barton, 1983; Martin *et al.*, 1982). If the interval between treatments is shortened to near the pre-patent period for the parasite then selection for resistance is increased because only parasites which can survive anthelmintic treatment are contributing eggs to the pasture which will develop into the next generation of parasites (Donald, 1982). Trials investigating the control of parasites by strategic dosing with anthelmintic at particular times during the grazing season and suppressive treatments throughout the season have shown that high levels of resistance are attained more rapidly with the suppressive treatments (Martin *et al*, 1982; Waller *et al.*, in press). Another important factor is the number of parasites contaminating the pasture at the time of anthelmintic treatment. Studies by Martin *et al*, (1981) indicated that resistance to benzimidazoles in a strain of *H.contortus* was slower to develop if a large number of parasites remained *in refugia* Coles, (1988) suggested that the ability of larvae of *Ostertagia spp.* to inhibit within the host and thus avoid the action of anthelmintics may reduce the speed of development of resistance.

Many parasite control schemes rely on a drench followed by moving the animals to clean pastures.This practice may encourage the development of resistance because the small proportion of parasites which can withstand anthelmintic treatment will be the source of eggs for development of the next generation of parasites. Increased levels of resistance with this type of management has been reported in *O.circumcincta* by Cawthorne and Whitehead, (1983) and in *H.contortus* by Eysker *et al.*,(1983). However, similar studies with strains of *H.contortus* and *T.colubriformis* failed to show increased

resistance in these parasites subjected to a dose and move regime compared with a set-stocked system (Waller *et al.*, in press).

Reversion to susceptibility in benzimidazole resistant strains of *H.contortus* and *T.colubriformis* was reported by Simpkin and Coles (1978). However, in other studies reversion to suseptibility in benzimidazole resistant parasites has not occurred (Hall *et al.*, 1982). The fitness of the resistant parasites to survive in the environment will influence the development of resistance. Comparison of the physiological characteristics of benzimidazole resistant and susceptible *H.contortus* suggested that the resistant parasites exhibited increased fitness (Kelly *et al.*, 1978). Increased fitness in anthelmintic resistant parasites also has been suggested by Martin (1987).

Treatment of a benzimidazole resistant strain of *O.circumcincta* with levamisole over a number of generations was found to cause reversion to benzimidazole susceptibility faster than in an untreated strain and this was termed counterselection (Donald *et al.*, 1980). Similar counterselection against benzimidazole resistance with levamisole was reported by Waller *et al.*, (1983) and Martin *et al.*, (1988). Counterselection against levamisole resistance by using a benzimidazole in *T.colubriformis* also has been reported (Waller *et al.*, in press).

Methods to control anthelmintic resistance are now being implemented in some countries. The development of new anthelmintics is unlikely because of the prohibitive costs involved and therefore control of nematode parasites must rely on the present anthelmintics.

Ivermectin has a persistent action and has activity against benzimidazole reistant and levamisole/morantel resistant strains of parasites (Waller, 1986). However resistance to ivermectin has been reported already in *H.contortus* in South Africa (Van Wyk *et al.*, 1987) and in Brazil (Echevarria and Trindade, in press) and in *T. colubriformis* in the

U.S.A. (Giordiano *et al.*, in press) and may develop in other areas. Studies with frequent dosing of ivermectin over a five year period failed to induce ivermectin resistance in strains of *H.contortus* and *T.colubriformis* (Waller *et al.*, in press). Closantel, a salicylanilide, is active against *H.contortus* as well as flukes and has a persistent action. This drug has proved useful in the control of anthelmintic resistant strains of *H.contortus* in Australia and recently has been introduced to the U.K. market. Resistance in flukes to the effects of salicylanilides may have developed as a result of intensive use of closantel to control *H.contortus* infections in sheep in Australia (Boray, personal communication.) The other drugs available to control nematodes are the benzimidazoles , levamisole and morantel and resistance to these drugs is prevalent in some areas. Combinations of drugs may have a greater effect against anthelmintic resistant parasites and low levels of levamisole, in combination with the benzimidazoles, can increase the efficacy of the benzimidazole anthelmintic (Bennet *et al.*, 1980). The use of parbendazole combined with a modern benzimidazole increases the duration of action of the benzimidazole and may increase the efficacy of the benzimidazole against resistant parasites (Hennessy *et al.*, 1985). Prolonging the exposure time to anthelmintics for benzimidazole resistant nematodes may increase the efficacy of the benzimidazole (Le Jambre, 1982). Studies to evaluate the use of controlled release devices and sustained release devices for the delivery of anthelmintics and their effect against anthelmintic resistant parasites are being carried out in Australia (Anderson, personal communication).

The dose of anthelmintic administered to the host animal can affect the efficacy of the drug and the development of anthelmintic resistance. A dose of anthelmintic which kills the entire population of parasites within the host will prevent survivors from contributing resistant offspring to the next generation (Dash *et al*, 1985). Recommendations for dosing animals include calculating the dose rate of anthelmintic using the heaviest sheep in

the flock rather than the average weight. If the average flock weight was used this would result in underdosing of some animals and there is evidence that this practise may accelerate the development of resistance (Waller and Prichard, 1986; Waller, 1987).

Rotation of anthelmintics, every approximately 12 months, in order to prevent the development of high levels of resistance has been recommended (Prichard *et al*, 1980; Donald and Waller, 1982; Dash *et al*, 1985). However, rapid alteration between anthelmintic groups can select for resistance more rapidly (Le Jambre *et al*, 1977).

In Australia parasite control schemes have been introduced to try to reduce the frequency of anthelmintic treatments given to sheep flocks and overcome some of the problems of anthelmintic resistance in nematodes at the same time as trying to prevent further development of resistance to anthelmintics. The 'Wormkill' programme was introduced for the Northern Tablelands of Australia (Dash *et al*, 1985) which involved strategic drenching of sheep flocks using a broad spectrum anthelmintic (benzimidazoles or levamisole/morantel) combined with closantel. The latter has a persistent effect and controls *H.contortus* infections. This scheme was publicised and has been successful during its initial phase of implementation. Other control schemes e.g. 'Drenchplan' used in the Central and Southern Tablelands and 'Wormbuster' used in South East Queensland have been developed. An advantage of these schemes, which are organised by the local department of agriculture in coordination with C.S.I.R.O., is that any problems involving parasite control which arise on farms are investigated and advice is available to farmers on the use of anthelmintics and pasture management.

Other methods to prevent gastrointestinal parasitism in sheep are being investigated and may be of use in the control of anthelmintic resistance. Advances in vaccine development and manufacture may lead eventually to the production of vaccines for roundworm infections in sheep. An alternative approach is the breeding of sheep which can

resist infection by parasites. In Australia a Merino ram which resisted infection by *T.colubriformis* and *H.contortus* was discovered and breeding programmes using offspring from this sheep may establish flocks which can resist infection by gastrointestinal parasites (Gray, 1987; Albers *et al*, 1987).

Studies desribed in this thesis were initiated to investigate a strain of *O.circumcincta* showing resistance to the benzimidazole anthelmintics. The effect of anthelmintics on the development of resistance in this strain of parasites was studied and factors contributing to increased anthelmintic resistance were discussed. Investigations with comparisons were carried out on various methods used to detect anthelmintic resistance. The biochemical action of benzimidazoles on nematode tubulin along with the examination of the role tubulin plays in the development of resistance to anthelmintics was investigated. Using the benzimidazole resistant strain of *O.circumcincta* studies were carried out on the genetic changes involved in the development of resistance and their mechanisms of inheritance.

RESISTANCE IN NEMATODES TO THE EFFECTS OF BENZIMIDAZOLES

GENERAL MATERIALS AND METHODS

Strains of Parasites

HFRO Strain - This strain of *Ostertagia circumcincta* was obtained from the Hill Farming Research Organisation and it was first isolated from premises at Hartwood Farm, Shotts when routine treatment of sheep with a benzimidazole anthelmintic failed to reduce faecal egg counts. Faecal material was collected from the sheep and taken to Glasgow University Veterinary School where larvae were cultured and the parasites used to infect lambs. Passage of this strain through lambs was carried out to produce sufficient larvae for investigation of the resistance status of the parasites and further studies on development of resistance. The passage history of this strain of *O.circumcincta* is shown in a flow chart (Fig. 2.1).

Moredun Strain - This strain of *O.circumcincta* was isolated from sheep at the Animal Diseases Research Association, Moredun Institute, Edinburgh.

Bearsden Strain - This strain of *O.circumcincta* has been maintained in the Parasitology Department at Glasgow University Veterinary School. The parasites have been passaged through lambs for approximately 15 years without treatment using anthelmintics.

V.R.S.G. Strain - This benzimidazole resistant strain of *Haemonchus contortus* was obtained from Dr. E. Lacey, C.S.I.R.O. Division of Animal Health, McMaster Laboratory, Sydney, Australia.

McMaster Strain - This benzimidazole susceptible strain of *H.contortus* was obtained from Dr. E. Lacey, C.S.I.R.O. Division of Animal Health, McMaster Laboratory, Sydney, Australia.

McMaster Strain - This benzimidazole susceptible stain of *O.circumcincta* was obtained from Dr. E. Lacey, C.S.I.R.O. Division of Animal Health, McMaster Laboratory, Sydney,

Australia.

KS79, KR79 and KS;KR Strains - These strains of *O.circumcincta* were obtained from Mr. P. Martin, C.S.I.R.O. Division of Animal Health, Parkville Laboratory, Melbourne, Australia. The KS79 strain was benzimidazole susceptible, the KR79 was benzimidazole resistant and the KS;KR strain was produced by infecting a lamb with a 40;60 mixture of larvae of the strains to produce a partially resistant strain of *O.circumcincta*

Passage of *O.circumcincta* HFRO, Moredun and Bearsden Strains through Lambs

Materials.

The animals used were parasite naive Dorset or Suffolk cross lambs and Scottish Blackface cross lambs.

Fenbendazole- Panacur 10% suspension, Hoescht, administered orally at the normal dose rate of 5mg/kg.

Method.

1. Lambs were infected by oral administration of 10,000 infective larvae of the relevant strain of *O.circumcincta*

2. At day 20-21 post-infection faecal samples were collected and a McMaster faecal egg count (see below) was carried out to confirm that patency had been reached.

3. At a variable interval after patency was attained some of the lambs were treated with FBZ at the normal dose rate of 5mg/kg administered orally.

4. At least 5 days were allowed to elapse after treatment before all faecal material was collected using a leather faecal bag with plastic liner changed daily. The faeces were used for a McMaster faecal egg count and the remainder was incubated at 27-28°C for at least 10 days in plastic pots. Infective larvae were collected using a Baermanisation technique (see below)

Collection of *O.circumcincta* Infective Larvae by Baermanisation

Method.

1. After incubation 20ml warm water was added to each pot and left to stand for 4 h.

2. The material was passed through a coarse wire mesh sieve and the fluid collected.

3. This was loaded onto two 20cm cloth milk filters (Maxa filters - McCaskie) under vacuum. The filters were placed on top of a Baerman funnel filled with warm water and left to sit overnight.

4. Larvae were collected from the base of the funnel and were stored at 4°C

McMaster Faecal Egg Count Method

(Method from Gordon and Whitlock, 1939)

Method.

1. Weighed out 3 g of faeces which were placed in a beaker and 42ml water added.

2. The faecal pellets were macerated using an electric homogeniser for approximately 3 min

3. The homogenate was filtered through a 250micron sieve. The filtrate was mixed and a 15ml aliquot was placed in a glass tube.

4. This was centrifuged at 1500g for 3-5mins and the supernatant removed.

5. Added 15ml saturated salt to the pellet and mixed well.

6. After thorough mixing an aliquot of the material was loaded into both chambers of a McMaster slide.

7. The eggs within the grid were counted by examining the slide under a binocular microscope. The number of eggs in each chamber was added and multiplied by a factor of 50 to give eggs per gram of faeces.

Calculation of Adult Worm Burden at Necropsy

(Method from Ritchie *et al*, 1966)

Method.

1. At necropsy the abomasum was removed from the lamb and opened along the greater curvature.

2. The abomasal contents were collected and the surface of the abomasum was washed with warm water. All washings and contents were collected and the volume made up to 2 litres.

3. After thorough mixing, two 200ml samples were collected and 20ml 40%v/v formalin was added to each. Equal portions of the samples (2-5ml) were stained with 45% iodine in potassium iodide and decolourised with 2.6% w/v sodium thiosulphate and examined under a binocular microscope. The number of adult parasites in each aliquot was counted.

4. The abomasum was divided at the pyloric - fundic juntion and the mucosa scraped off. This was finely chopped and digested with pepsin/HCl for 6h at 42°C.

5. Formalin was added to the digested material and the volume was adjusted to a suitable value. The material was mixed thoroughly and a 200ml sample was taken.

6. The number of parasites in the sample was counted by the method given above (3) and the total worm burden for the animal calculated by adding the number of parasites in the abomasal contents to the number in the digest.

Collection of Faeces from Parasitised Lambs

Method.

1. Leather harnesses were placed on each male lamb which had been infected experimentally with the relevant parasites.

2. The faeces were collected in a leather faecal bag with a plastic liner which was attached to the harness.

3. For collection of eggs for egg hatch assays the faecal bags were placed on the lambs for

2-4 h and the contents of the bag were used immediately or stored at 4°C.

Collection of *O.circumcincta* Eggs from Faeces of Lambs

Method I (Method modified from method described by Le Jambre, 1976)

Method.

1. Faecal material was collected by the method given above and this was used to obtain eggs for egg hatch assays and for tubulin extraction.

2. Approximately 3-4g of faeces were combined with 10ml saturated salt and homogenised for 1-2 min in an electric homogeniser. The faecal/salt mixture was passed through a 250 micron sieve and the filtrate was placed in a flat tray.

3. Several aliquots of faeces treated in this way were combined and salt solution was added to a depth of 5-7cm.

4. In saturated salt solution the parasite eggs float on the surface. A plastic sheet was placed on the surface of the faeces/salt mixture and after 1-2 min was removed carefully and the material adhering to the sheet was washed into a beaker using water. This step was repeated 4 times.

5. All washings were combined and passed through a 38micron sieve. The material which collected on the sieve was washed with water into a urine flask. The eggs of *O.circumcincta* cannot pass through a 38micron sieve and therefore collect on the mesh and can be washed from the surface of the mesh.

6. In water the eggs sink and the urine flask was allowed to stand for 1-2h at 4°C. The supernatant was poured from the urine flask and the concentrated egg suspension which had collected at the base of the flask was retained.

7 This egg suspension was examined under a binocular microscope to check for the presence of eggs and then the suspension was subjected to a further cleaning process.

8. The suspension was centrifuged at 1,400g for 5-10min and the supernatant removed.

Saturated salt was added in the ratio of 2 parts salt to 1 part egg suspension and the tube mixed.

9. This was centrifuged at 1,400g for 5 min. The supernatant was collected and passed through a 38micron sieve. The eggs collected on the mesh were washed into a collecting beaker with water.

10. To concentrate the egg suspension it was centrifuged at 2,000 g for 5 min and the supernatant removed.

11. Three aliquots of 10ul of the egg suspension were examined under the binocular microscope to estimate the number of eggs present.

Collection of *O.circumcincta* Eggs from Faeces of Lambs

Method II (Method modified from that described by Coles and Simpkin,(1977). This method was developed to try to reduce the time taken to collect eggs from faecal material.)

Method.

1. Homogenised 2 X 10-20g faeces in water using an electric homogeniser. The homogenate was placed in a 200ml centrifuge bottle and the volume made up to 200ml using water.

2. Centrifuged for 3 min at 2,000r.p.m using a Mistral refrigerated centrifuge at 4°C. Supernatant was removed.

3. The pellet was mixed with saturated salt and volume made up to 200ml using saturated salt. Centrifuged at 1,600 r.p.m. for 2.5 min.

4. The supernatant was decanted through a 125micron sieve to remove large particulate material. The filtrate was passed through a 38micron sieve and the eggs were washed from the mesh using water.

5. The egg suspension was examined under a binocular microscope and if necessary the cleaning and concentrating steps described for Method I (7-11) were carried out to

remove contaminating faecal material.

Egg Hatch Assay for the Detection of Benzimidazole Resistance in Nematode Parasites

(Method modified from Coles and Simpkin , 1977 and Le Jambre, 1976)

Reagents.

Suspensions of *O.circumcincta* eggs in water were isolated by the method given above.

Benzimidazole stock solutions - Stock solutions of ABZ and TBZ in methanol over a concentration range of 10ug/ml - 1mg/ml.

Sodium thiosulphate solution - 2.6% w/v of sodium thiosulphate.

Iodine solution - 45% iodine in potassium iodide.

Sterilin 16 well plastic plates.

Method.

1. Approximately 100 eggs per well were placed in the multiwell plates.

2. The volume in each well was adjusted to 990ul using tap water.

3. In duplicate 10ul of each concentration of the stock solutions of TBZ or ABZ were added to wells in the plate. In 2 wells (blanks)10ul water were added and in 2 wells 10ul methanol were added (methanol controls).

4. Plates were agitated to mix the contents of each well then incubated at 37°C for 48h.

5. Plates were removed from the incubator and the parasites were killed using one drop of iodine per well.

6. The iodine was decolourised using several drops of the sodium thiosulphate solution.

7. Plates were examined under a binocular microscope and the number of undeveloped eggs, larvated eggs and larvae present in each well were counted.

8. For each well the number of undeveloped eggs was expressed as a percentage of the total number of parasites in the well. This value was corrected for natural mortality by

subtracting the average value from the methanol control wells. The corrected values for each drug concentration were meaned and the value was expressed as a percentage of the total number of parasites which developed in the methanol control well.

9. This corrected percentage failing to hatch was plotted against the drug concentration. From the graph the effective dose 50% (ED50), the concentration of drug which prevents development of 50% of the eggs, was calculated.

10. In some studies the percentage failing to hatch was converted to probits and these values were plotted against the log of drug concentration. From this plot an ED50 was calculated.

Production of First Stage Larvae of *Ostertagia circumcincta*

Method.

1. Faeces were collected from lambs which had been infected experimentally with the relevant strains of *O.circumcincta* as described previously.

2. Faeces were incubated at 28°C for 48h in plastic pots.

3. Larvae were collected using the Method described previously (see Collection of infective larvae of *O.circumcincta* by Baermanisation).

Collection of Adult *Ostertagia circumcincta* from the Abomasum of Lambs at Necropsy

(Method modified from the modified Baermanisation technique described by Henriksen, 1965)

Method.

1. The abomasum was removed at necropsy and opened along its greater curvature

2. Aliquots of abomasal contents were placed on a double layer of gauze which was folded to form a bag.

3. This was suspended on rods in a beaker of warm water (30-37°C) and adult parasites migrated from the abomasal contents collecting in clumps at the base of the beaker.

4. Adult parasites were examined under a binocular microscope to determine their sex and then maintained at 37°C prior to use in transplant experiments.

Exsheathment of *Ostertagia circumcincta* Infective Larvae

Materials.

Double Strength Milton 2 Sterilising fluid (sodium hypochlorite 2%w/v, sodium chloride 16%w/v)

Larvae obtained after culture of faeces (see previously)

Method.

1. To approximately 15ml of larval suspension added 0.75ml Milton 2

2. Tube was mixed thoroughly and incubated at 37°C for up to 5 min. Larvae were examined at intervals during the period of incubation to check for exsheathment.

3. The mixture was divided into 2 aliquots which were placed in 10ml centrifuge tubes and the volume in each tube was made up to 10ml using water.

4. Tubes were centrifuged at 1,400g-2,000g for 3-5min and the supernatant was removed retaining a volume of 1ml.

5. The tubes were refilled with water and the above step repeated twice to ensure that all the hypochlorite solution was removed because it is toxic to larvae.

6. The final larval suspension was mixed well and aliquots were examined under a binocular microscope and an estimate was made of the number of larvae present.

RESULTS AND DISCUSSION

Routine Passages of *Ostertagia circumcincta* HFRO, Bearsden and Moredun Strains

Results

The passage history of the HFRO strain of *O.circumcincta* is shown in Fig. 2.1. Each treatment was with FBZ at the normal dose rate of 5mg/kg administered orally. The Bearsden susceptible strain of *O.circumcincta* was passaged in parallel with lambs infected on the same day and where possible eggs collected for egg hatch assays on the same day post-infection for the susceptible and resistant strains.

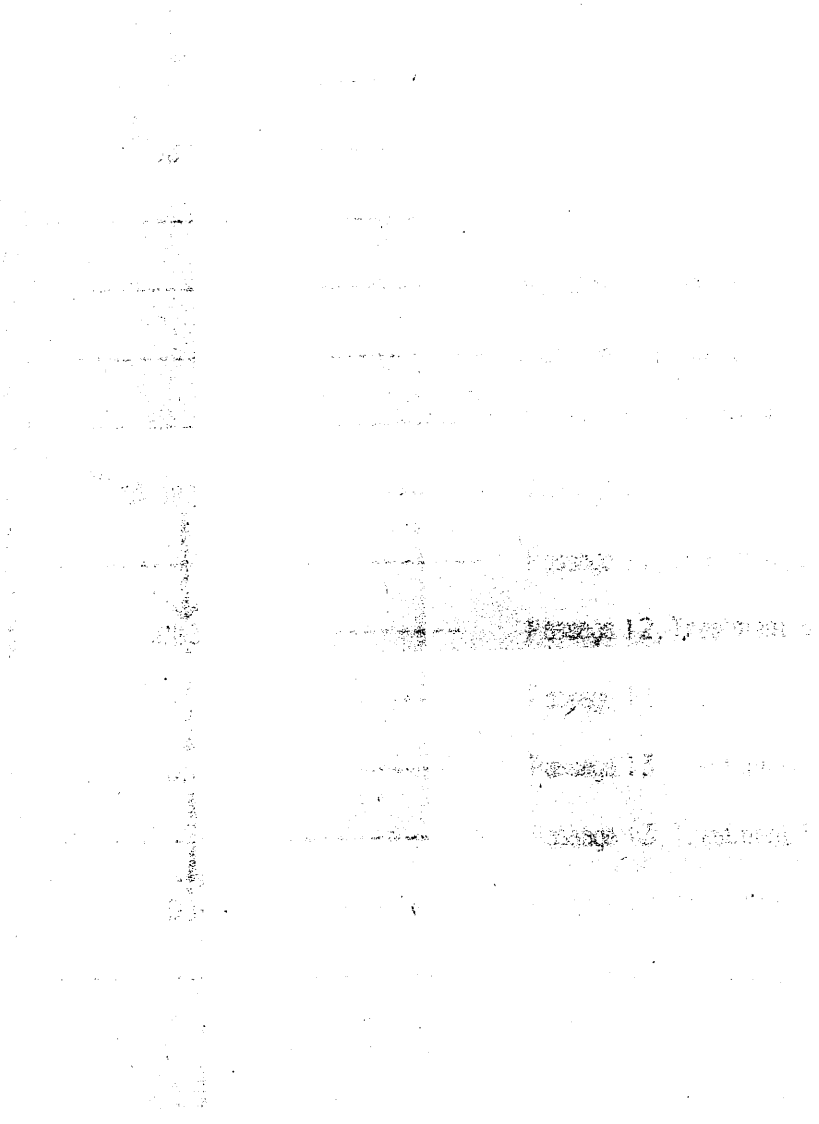
The results of the egg hatch assays for the HFRO strain of *O.circumcincta* are shown in Tables 2.1 and 2.2. The time in days after experimental infection of the lambs is stated along with the time after treatment of the lambs with FBZ where applicable. Table 2.3 shows results of egg hatch assays carried out on eggs from lamb DB4 which was infected experimentally with larvae of the HFRO strain which had been stored for 4 months at 4°C. Results of egg hatch assays on eggs collected from the faeces of lamb B2 which was infected experimentally with the Moredun strain of *O.circumcincta* are shown in Table 2.4 and Fig. 2.7. All the ED50s in Tables 2.1, 2.3 and 2.4 were calculated from graphs of probits against the log concentration of benzimidazole. Results in Table 2.2 were obtained from graphs of percentage failing to hatch against drug concentration. Figs. 2.2-2.7 show graphically the day to day changes in resistance as measured in egg hatch assays using TBZ or ABZ in the benzimidazole resistant strains of *O.circumcincta*

Discussion

The HFRO strain was identified when a group of ewes was treated with TBZ as a routine worming drench and this failed to reduce the faecal egg output measured in the ewes. There had been reports of benzimidazole resistance in *O.circumcincta* in the U.K. (Britt, 1982; Cawthorne and Whitehead, 1983) and the poor efficacy of TBZ observed in the case

Figure 2.1

Passage history of the HFRO benzimidazole resistant strain of *O.circumcincta*.



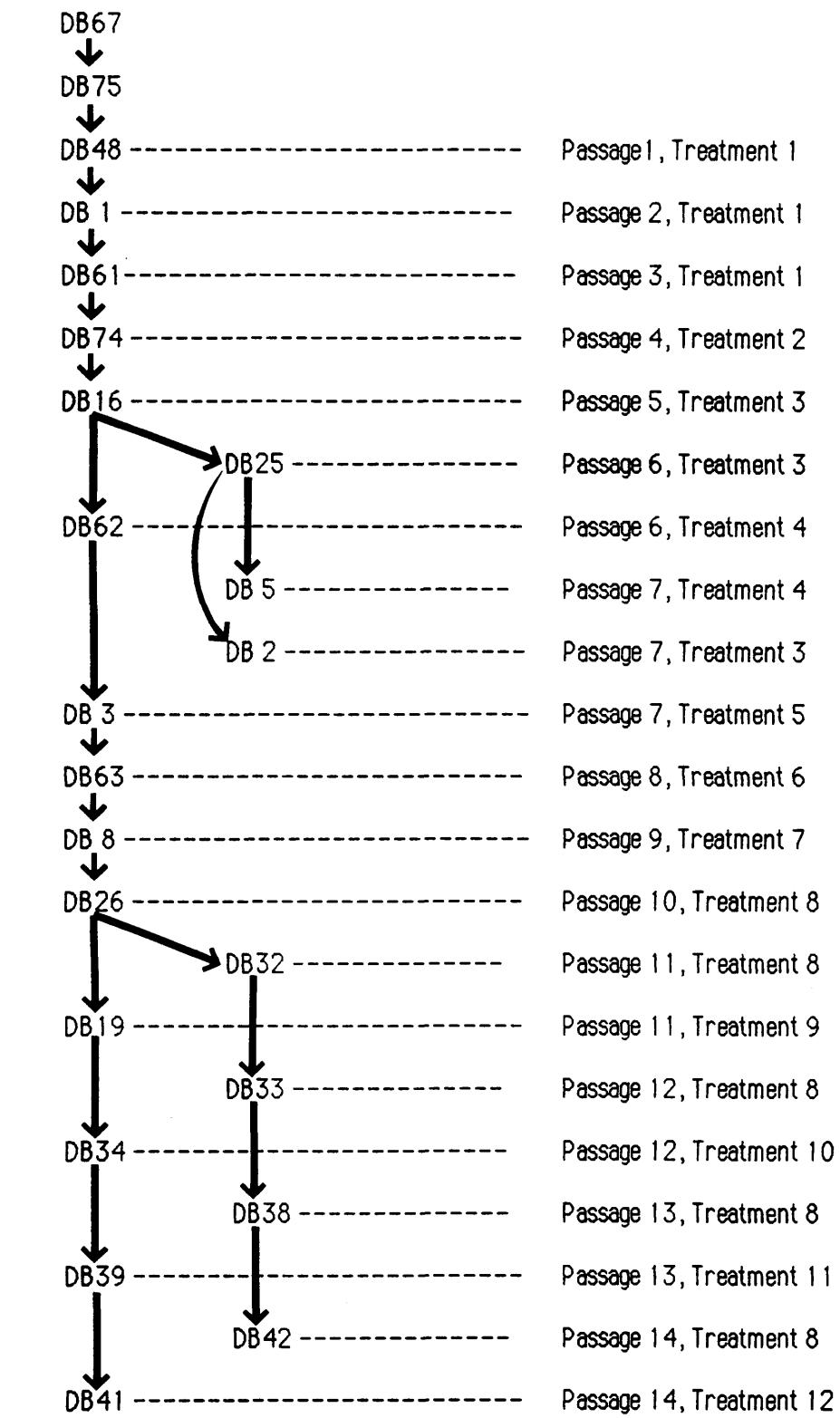

Lamb Number
Passage Number and Treatment Number using Fenbendazole at 5mg/kg
DB67
DB75
DB48 ---------- Passage1, Treatment 1
DB 1 ---------- Passage 2, Treatment 1
DB61 ---------- Passage 3, Treatment 1
DB74 ---------- Passage 4, Treatment 2
DB16 ---------- Passage 5, Treatment 3
DB25 ---------- Passage 6, Treatment 3
DB62 ---------- Passage 6, Treatment 4
DB 5 ---------- Passage 7, Treatment 4
DB 2 ---------- Passage 7, Treatment 3
DB 3 ---------- Passage 7, Treatment 5
DB63 ---------- Passage 8, Treatment 6
DB 8 ---------- Passage 9, Treatment 7
DB26 ---------- Passage 10, Treatment 8
DB32 ---------- Passage 11, Treatment 8
DB19 ---------- Passage 11, Treatment 9
DB33 ---------- Passage 12, Treatment 8
DB34 ---------- Passage 12, Treatment 10
DB38 ---------- Passage 13, Treatment 8
DB39 ---------- Passage 13, Treatment 11
DB42 ---------- Passage 14, Treatment 8
DB41 ---------- Passage 14, Treatment 12

Table 2.1

Results of egg hatch assays carried out during routine passages of the HFRO benzimidazole resistant strain of *O.circumcincta* using TBZ and ABZ

Lamb Number	Time (Days)		ED50 (ug/ml)	
	Post Infection	Post Treatment	TBZ	ABZ
DB74	23	-	1.53	2.37
DB16	25	-	3.36	3.83
	32	1	3.26	0.92
	33	2	2.01	5.34
	34	3	1.92	-
	39	8	0.98	0.92
DB62	27	-	2.81	4.53
	28	1	2.83	8.26
	29	2	3.06	2.56
	30	3	1.40	2.70
	34	7	1.68	2.82
	38	11	1.60	-
	42	15	-	3.50
	44	17	3.46	2.92
DB25	27	-	2.44	4.01
	28	-	1.31	2.02
	29	-	1.31	2.22
	30	-	1.91	4.43
	36	-	-	10.74
	38	-	-	3.66
	42	-	-	2.48
	44	-	1.30	-
	50	-	0.31	1.56
	51	-	0.19	0.34
	55	-	0.19	1.69
	56	-	-	3.47
	57	-	-	1.35
	59	-	-	2.39
	62	-	0.72	2.23
	64	-	-	2.29
	66	-	-	0.98
	69	-	-	2.87
	73	-	0.67	1.98

Table 2.1 (continued)

Lamb Number	Time (Days)		ED50 (ug/ml)	
	Post Infection	Post Treatment	TBZ	ABZ
DB 5	20	-	0.76	1.58
	22	-	1.00	1.97
	25	-	0.96	0.75
	27	-	1.96	2.19
	29	-	1.59	4.33
	32	-	0.80	2.25
	33	1	0.91	5.13
	34	2	0.71	5.90
	35	3	-	0.66
	39	7	1.13	2.19
	41	9	1.50	1.54
	46	14	1.59	0.92
	48	16	1.46	0.98
	55	23	1.70	3.74
	63	31	-	2.65
DB2	21	-	0.76	2.34
	26	-	0.81	0.68
	28	-	2.09	2.67
	30	-	1.85	4.66
DB3	33	6	2.47	6.37
	34	7	0.81	0.84
	38	11	1.34	2.63
DB63	30	-	1.48	2.02

Table 2.2

Results of egg hatch assays carried out during routine passages of the HFRO benzimidazole resistant strain of *O.circumcincta* using TBZ and ABZ

Lamb Number	Time (Days)		ED50 (ug/ml)	
	Post Infection	Post Treatment	TBZ	ABZ
DB 8	31	-	2.38	4.20
	39	7	1.78	2.55
DB26	23	-	1.50	2.81
	27	-	2.17	4.01
	34	-	1.60	1.83
	41	5	2.45	2.98
	47	11	1.65	0.81
	55	19	1.57	1.05
DB19	28	-	1.61	2.82
	35	-	1.42	1.99
	42	5	1.99	3.63
DB32	26	-	2.02	3.71
	40	-	1.32	2.48
DB34	35	-	1.60	1.83
	67	10	1.04	1.16
DB33	35	-	1.65	2.30
	67	-	1.12	1.07
DB41	28	-	1.92	2.00
	30	-	1.45	2.50
	42	11	1.36	1.49
	49	18	1.84	2.40
DB42	28	-	1.90	2.81
	30	-	1.80	2.23
	42	-	1.47	2.25
	49	-	1.78	2.41

Table 2.3

Results of egg hatch assays carried out on eggs passed in the faeces of a lamb inoculated with infective larvae of the HFRO benzimidazole resistant strain of *O.circumcincta* which were stored at 4°C for four months

Lamb Number	Time (Days)		ED50 (ug/ml)	
	Post Infection	Post Treatment	TBZ	ABZ
DB 4	22	-	1.42	2.24
	30	-	1.55	2.04

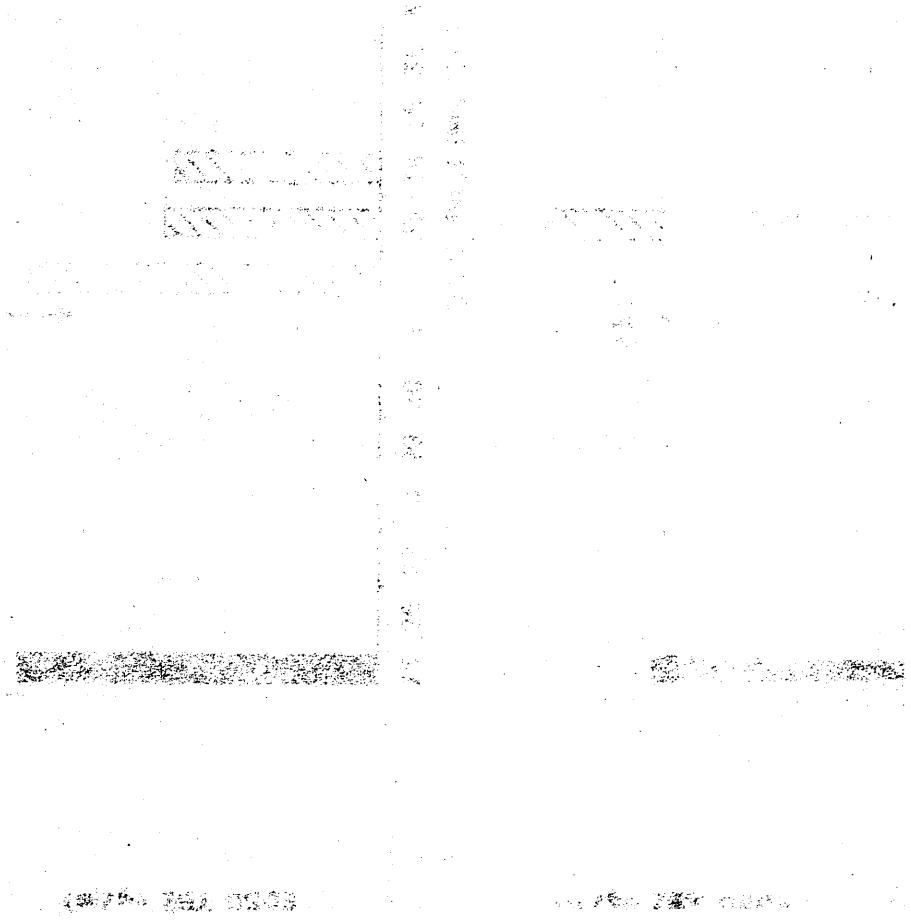
Table 2.4

Results of egg hatch assays carried out on eggs from the faeces of Lamb B2 infected with the Moredun benzimidazole resistant strain of *O.circumcincta*

Lamb Number	Time (Days)		ED50 (ug/ml)	
	Post Infection	Post Treatment	TBZ	ABZ
B 2	26	-	1.30	0.70
	28	-	1.48	0.59
	35	-	1.29	2.49
	40	-	1.01	1.83
	41	1	1.04	-
	42	2	0.68	1.12
	43	3	1.00	1.54
	47	7	1.49	1.18
	49	9	2.04	2.39
	54	14	2.04	4.22

Figure 2.2

Level of resistance as measured by the ED50 with TBZ and ABZ during Passage 5 of the HFRO strain of *O.circumcincta* in Lamb DB16. Treatment with FBZ (Treatment 3) was carried out on Day 31 post infection.



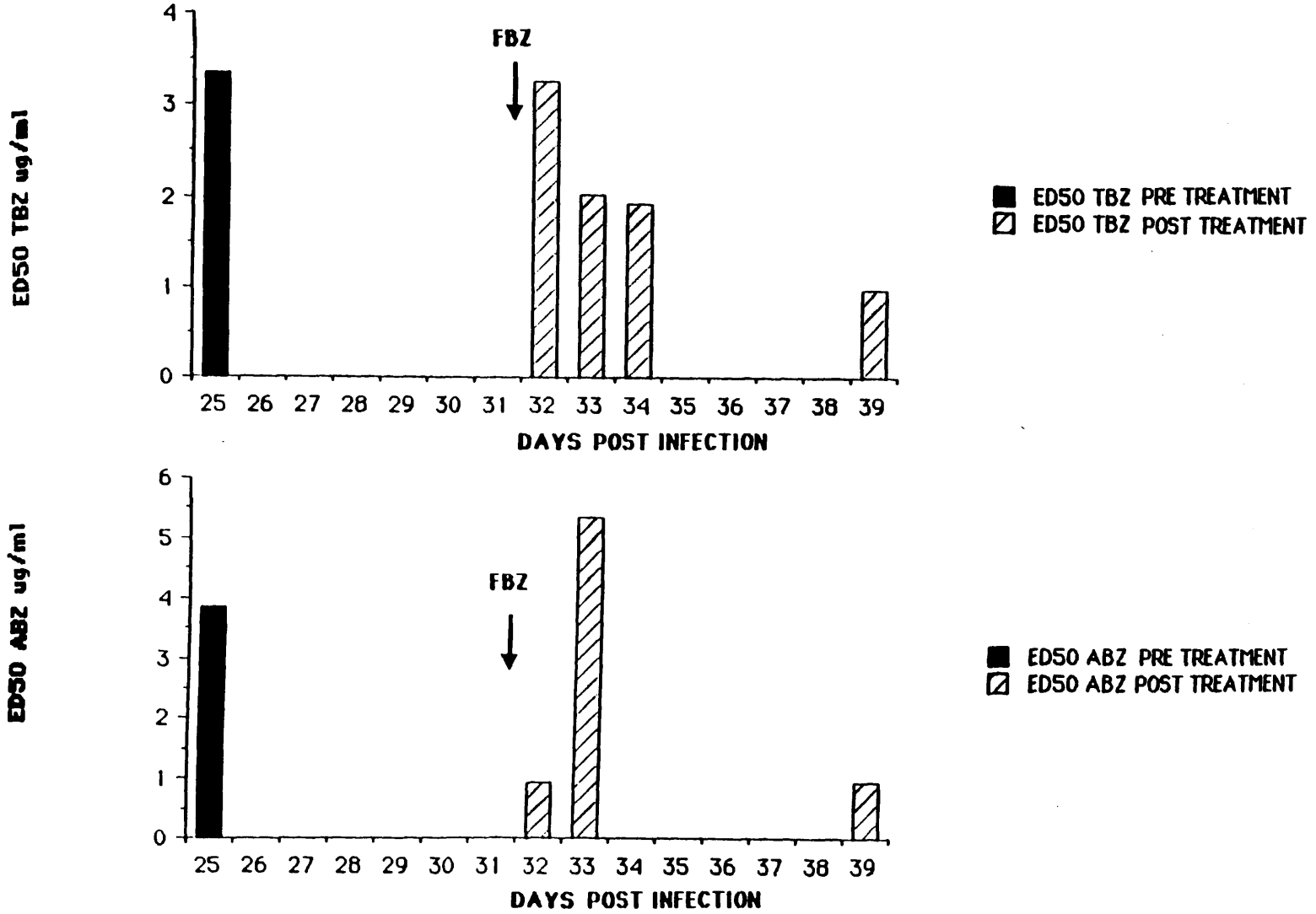
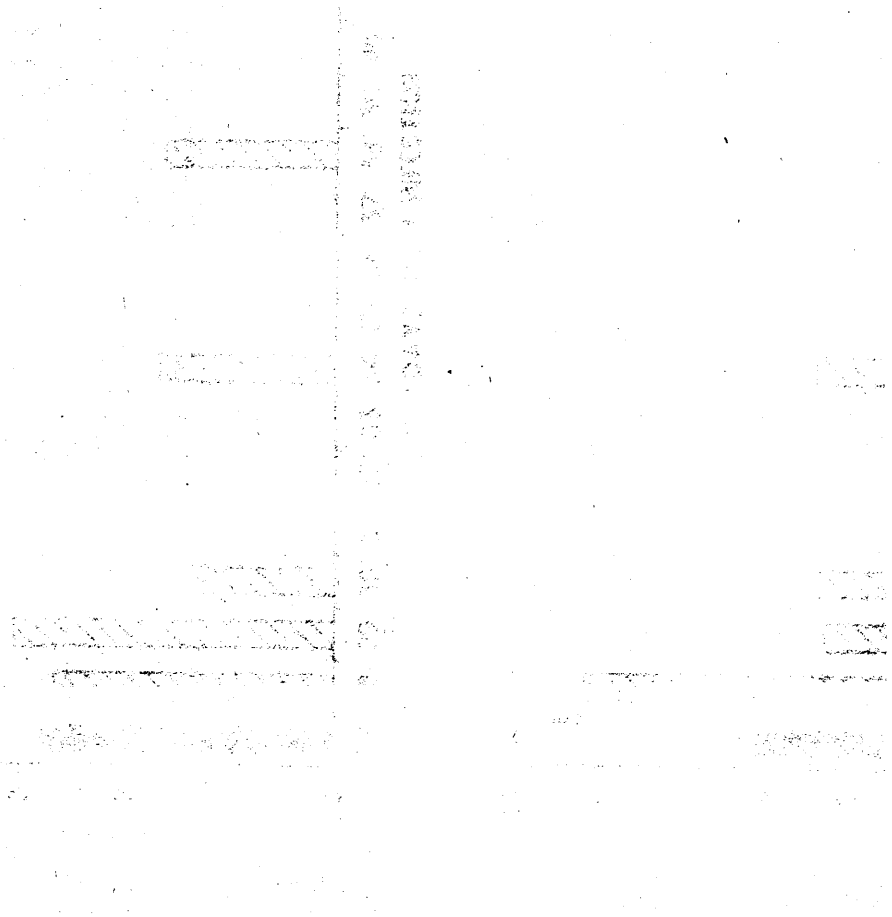

ED50 TBZ ug/ml
4
3
2
1
0
FBZ
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
DAYS POST INFECTION
ED50 TBZ PRE TREATMENT
ED50 TBZ POST TREATMENT
ED50 ABZ ug/ml
6
5
4
3
2
1
0
FBZ
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
DAYS POST INFECTION
ED50 ABZ PRE TREATMENT
ED50 ABZ POST TREATMENT

Figure 2.3

Level of resistance as measured by the ED50 with TBZ and ABZ during Passage 6 of the HFRO strain of *O.circumcincta* in Lamb DB62. Treatment with FBZ (Treatment 4) was carried out on Day 27 post infection.



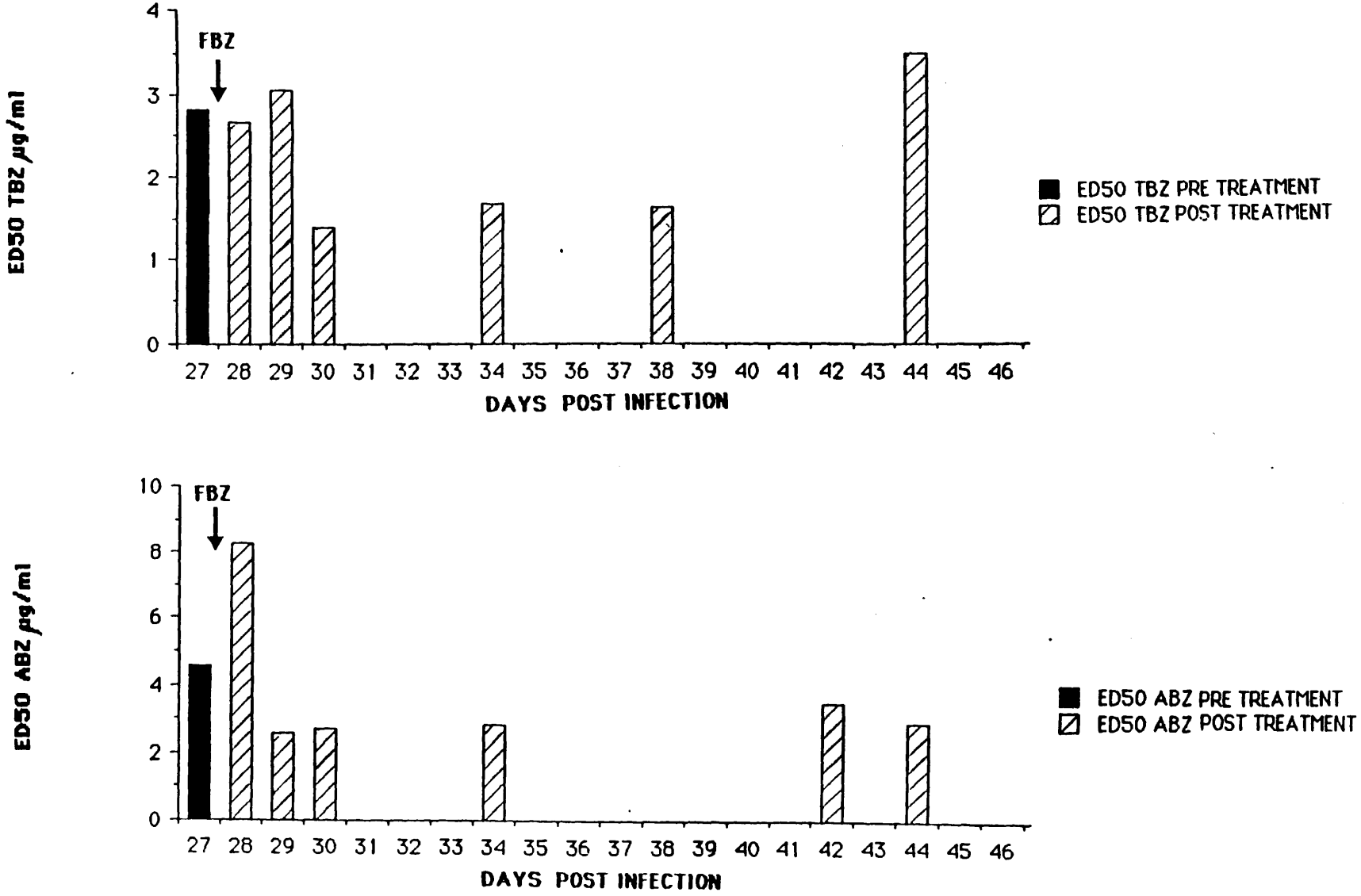
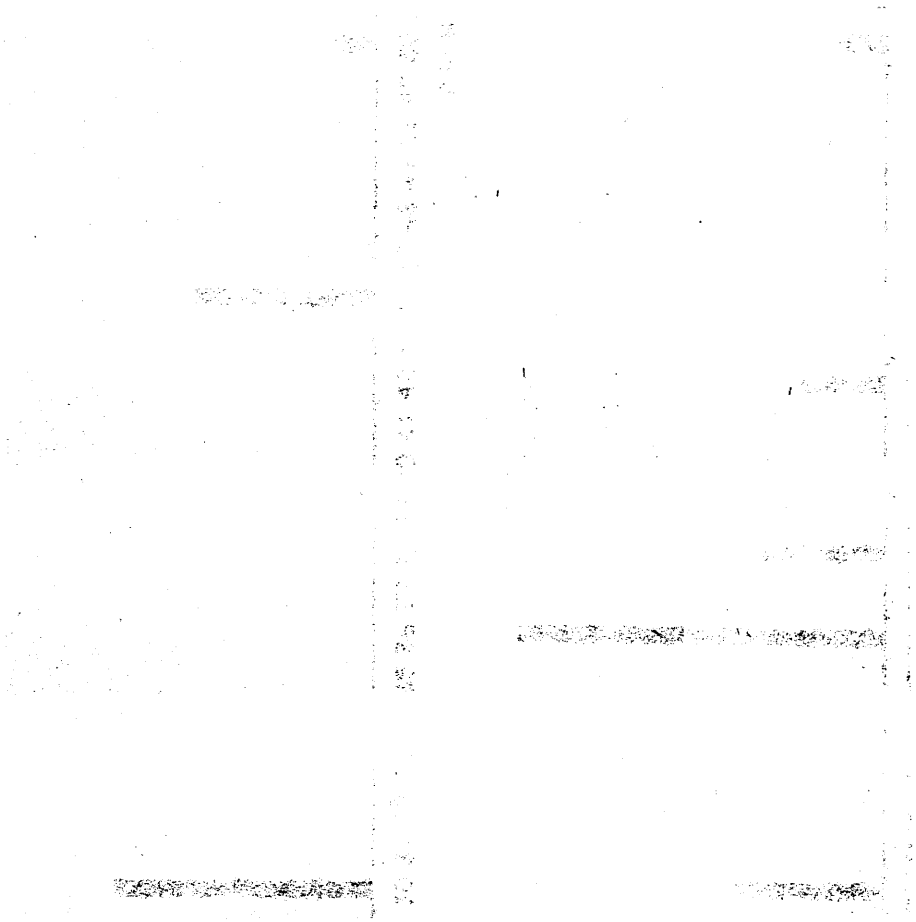

ED50 TBZ µg/ml
FBZ
0
1
2
3
4
27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46
DAYS POST INFECTION
ED50 TBZ PRE TREATMENT
ED50 TBZ POST TREATMENT
ED50 ABZ µg/ml
FBZ
0
2
4
6
8
10
27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46
DAYS POST INFECTION
ED50 ABZ PRE TREATMENT
ED50 ABZ POST TREATMENT

Figure 2.4

Level of resistance as measured by the ED50 with TBZ and ABZ during Passage 6 of the HFRO strain of *O.circumcincta* in Lamb DB25. The lamb was not treated with FBZ during this passage.



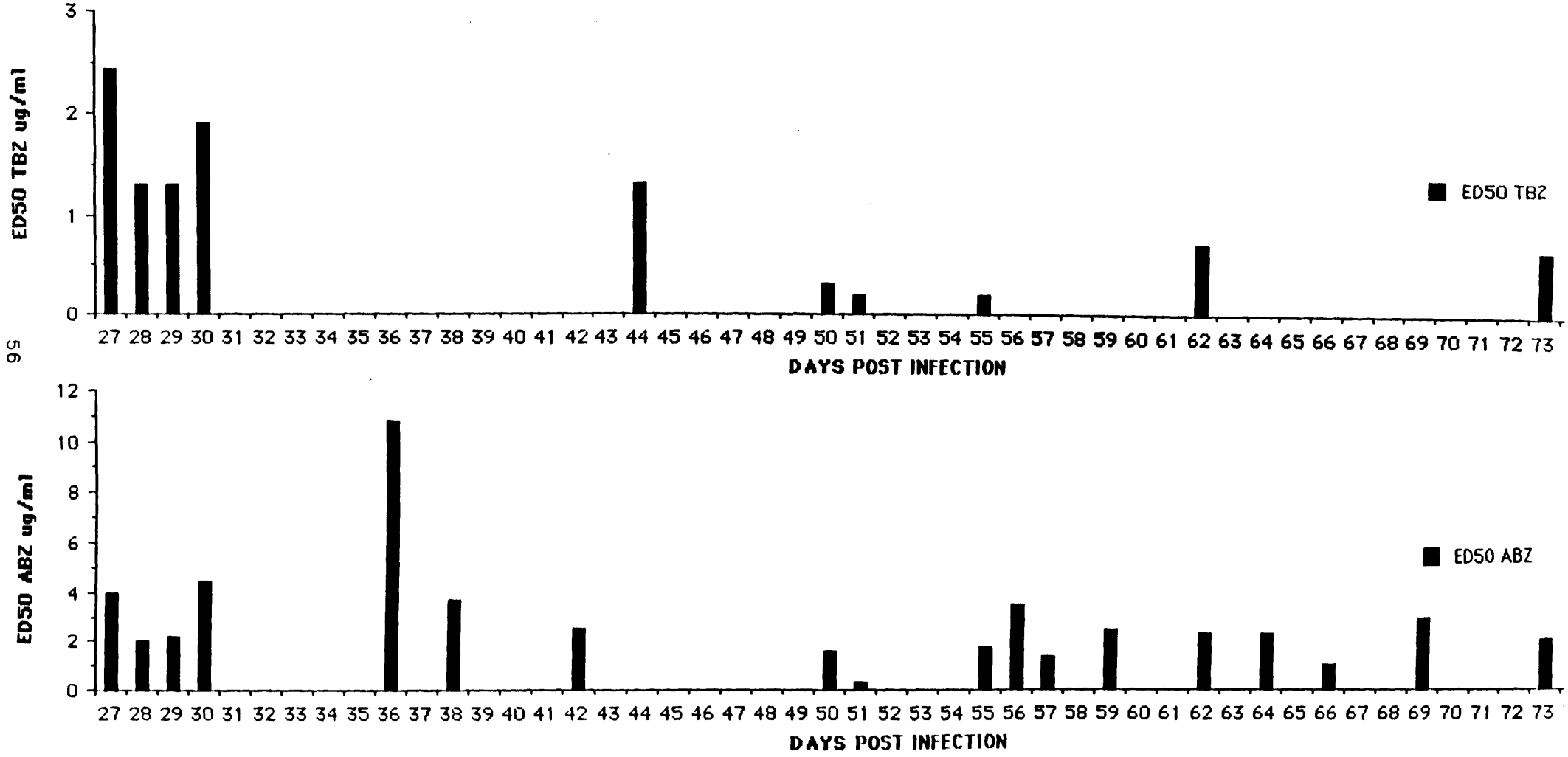
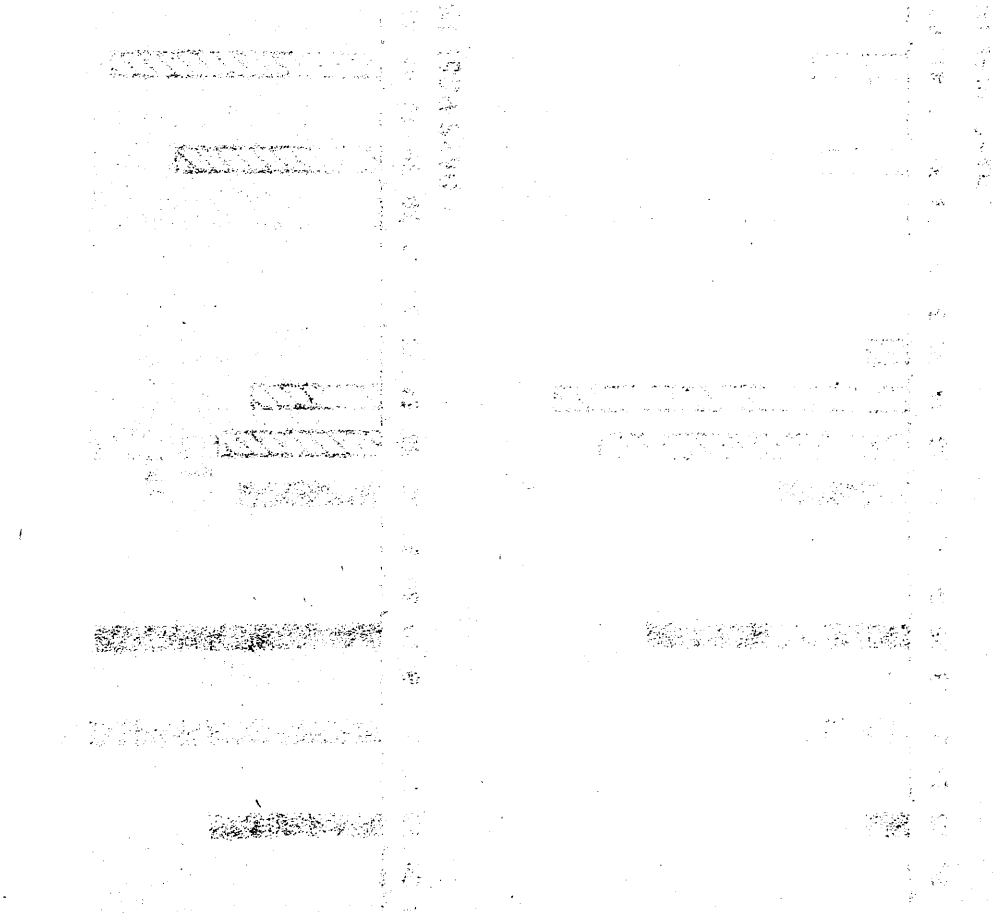

ED50 TBZ ug/ml
3
2
1
0
ED50 TBZ
27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73
DAYS POST INFECTION
ED50 ABZ ug/ml
12
10
8
6
4
2
0
ED50 ABZ
27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73
DAYS POST INFECTION

Figure 2.5

Level of resistance as measured by the ED50 with TBZ and ABZ during Passage 7 of the HFRO strain of *O.circumcincta* in Lamb DB5. Treatment with FBZ (Treatment 4) was carried out on Day 32 post infection.



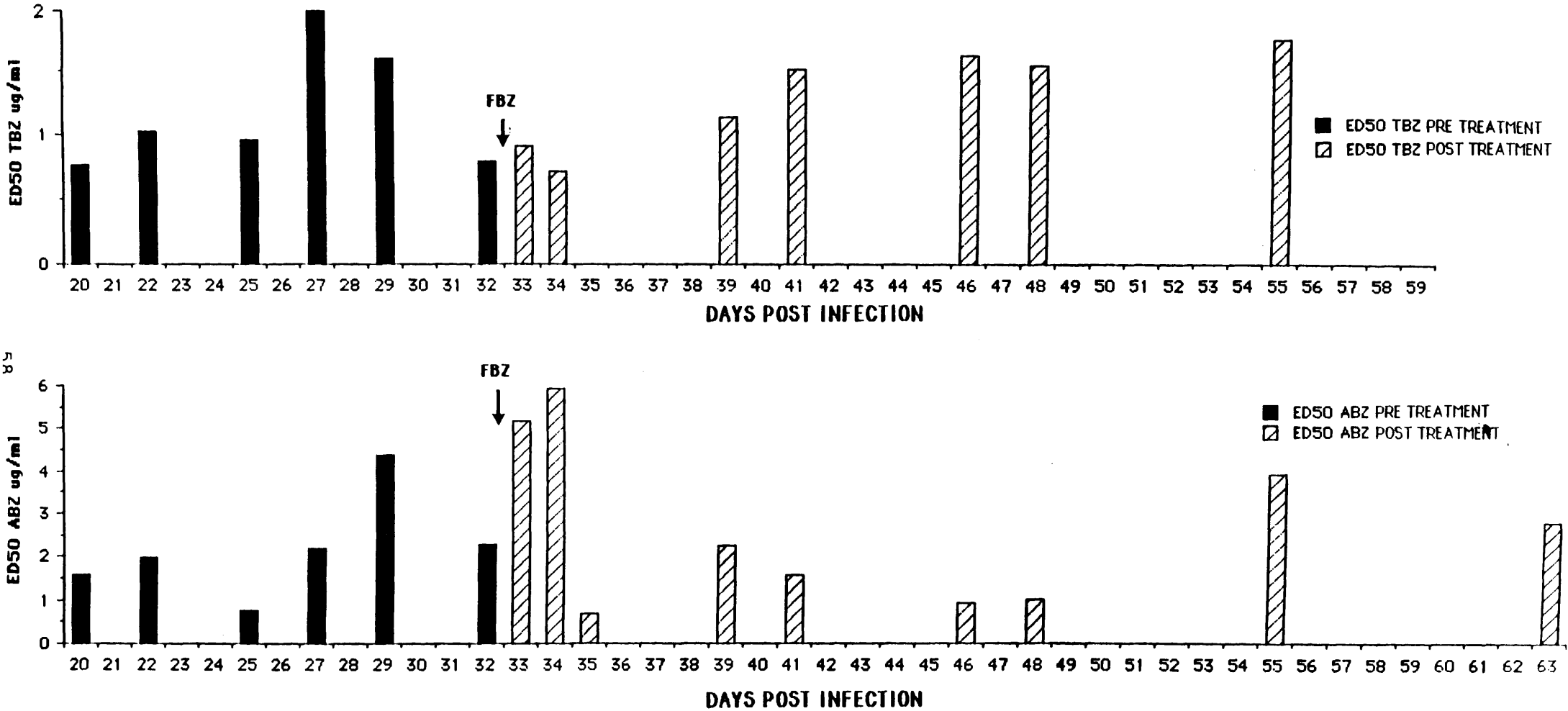
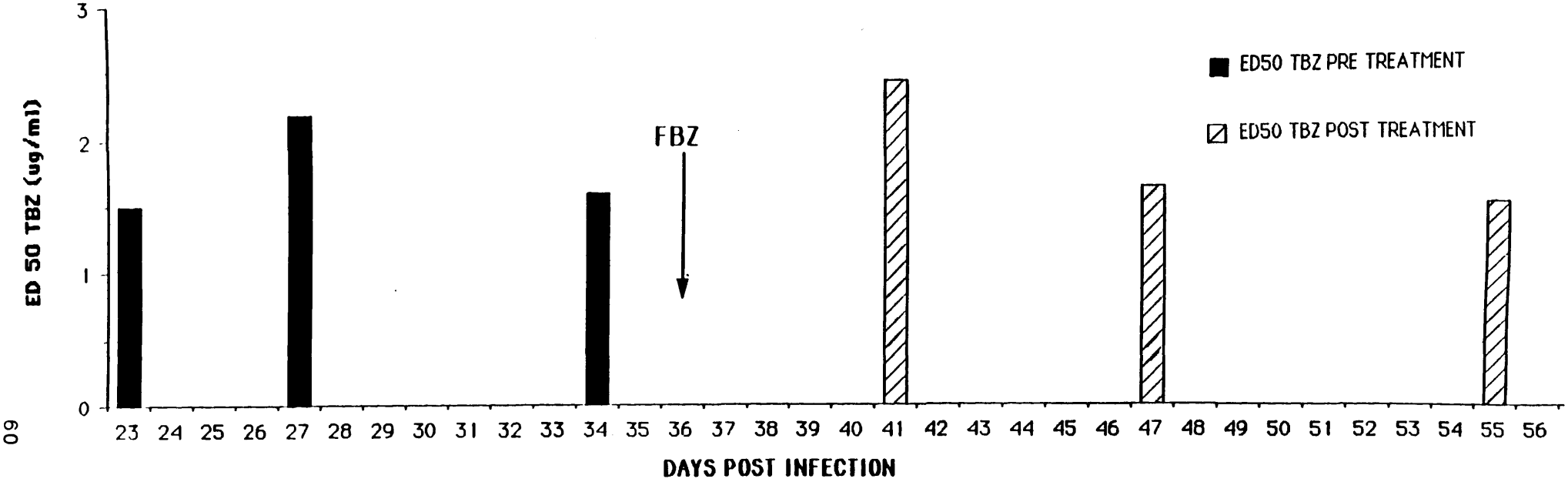

ED50 TBZ ug/ml
2
1
0
FBZ
ED50 TBZ PRE TREATMENT
ED50 TBZ POST TREATMENT
20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59
DAYS POST INFECTION
ED50 ABZ ug/ml
6
5
4
3
2
1
0
FBZ
ED50 ABZ PRE TREATMENT
ED50 ABZ POST TREATMENT
20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63
DAYS POST INFECTION

Figure 2.6

Level of resistance as measured by the ED50 with TBZ and ABZ during Passage 10 of the HFRO strain of *O.circumcincta* in Lamb DB26. Treatment with FBZ (Treatment 8) was carried out on Day 36 post infection.


ED 50 TBZ (ug/ml)
3
2
1
0
FBZ
ED50 TBZ PRE TREATMENT
ED50 TBZ POST TREATMENT
23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
DAYS POST INFECTION

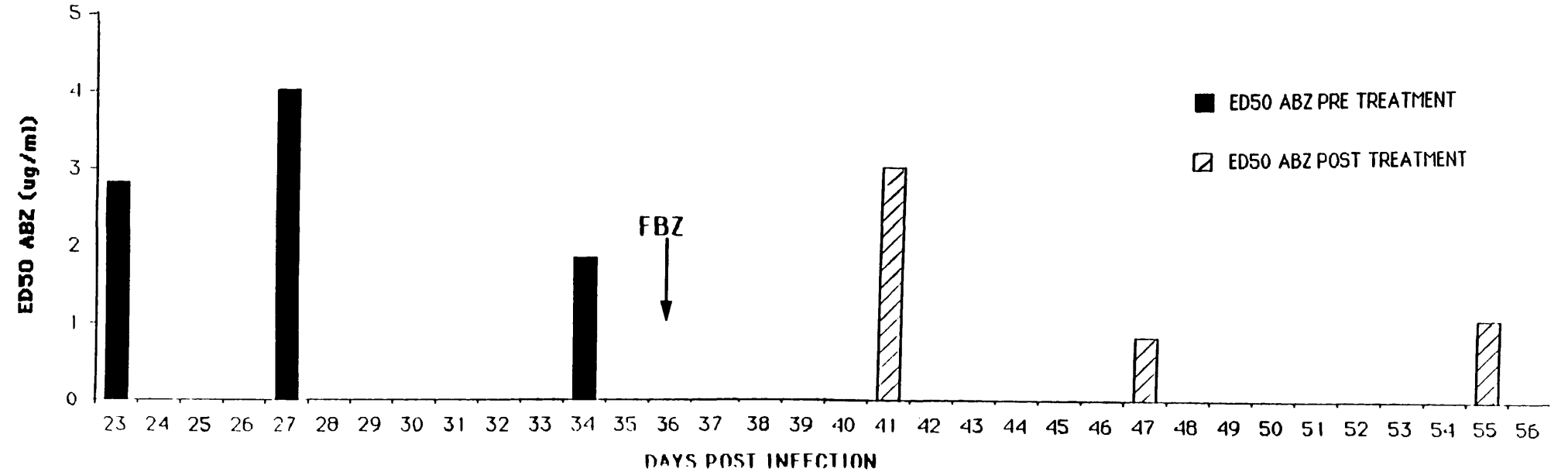
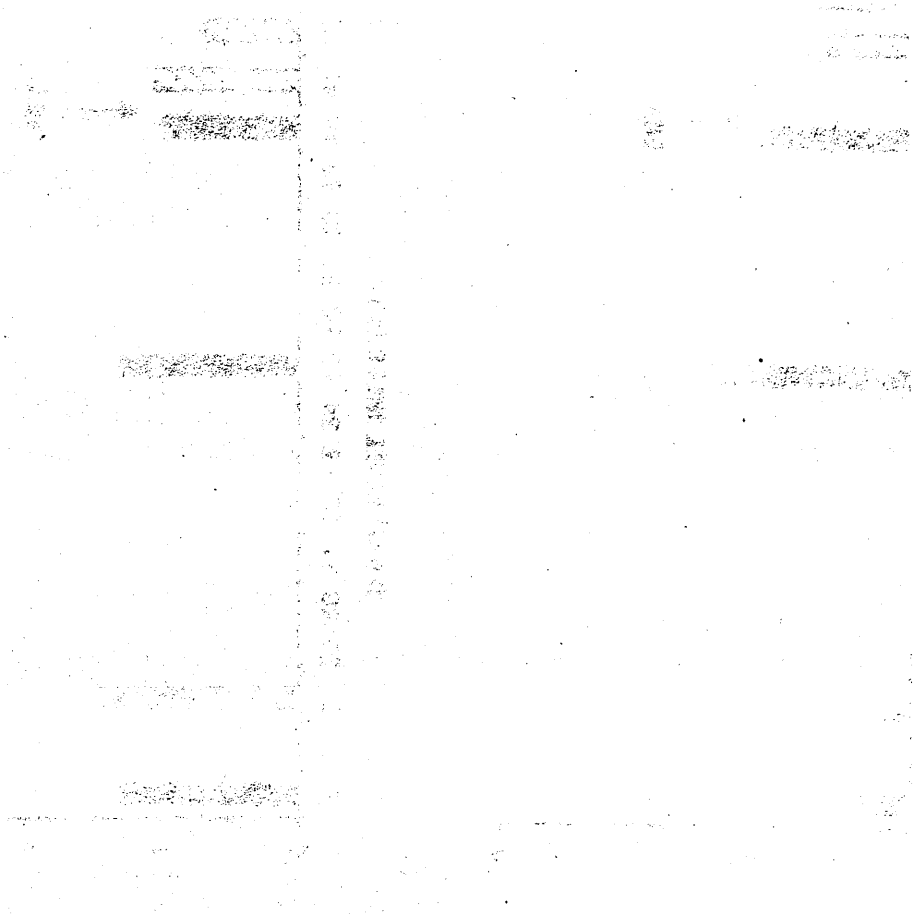
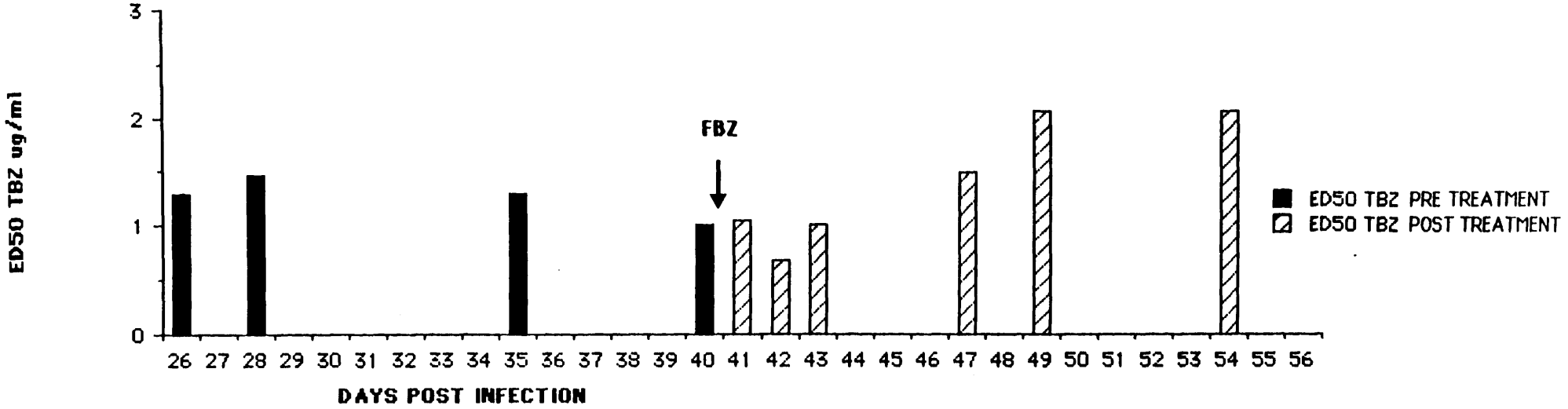
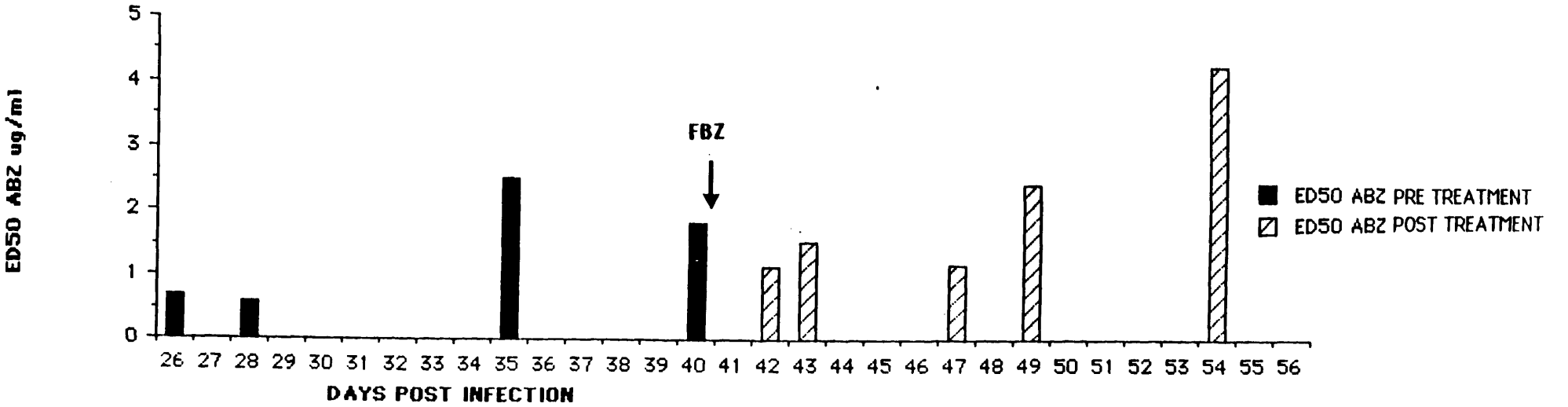

ED50 ABZ (ug/ml)
5
4
3
2
1
0
FBZ
ED50 ABZ PRE TREATMENT
ED50 ABZ POST TREATMENT
23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
DAYS POST INFECTION

Figure 2.7

Level of resistance as measured by the ED50 with TBZ and ABZ of the Moredun strain of *O.circumcincta* in Lamb B2. Treatment with FBZ (Treatment 1) was carried out on Day 40 post infection.




ED50 TBZ ug/ml
3
2
1
0
FBZ
26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
DAYS POST INFECTION
ED50 TBZ PRE TREATMENT
ED50 TBZ POST TREATMENT


ED50 ABZ ug/ml
5
4
3
2
1
0
FBZ
26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
DAYS POST INFECTION
ED50 ABZ PRE TREATMENT
ED50 ABZ POST TREATMENT

described above suggested that anthelmintic resistance may have developed on this farm. TBZ had been used on the farm as the anthelmintic for routine treatment of ewes and lambs for many years.

Faeces were collected from the ewes on the farm and were cultured to provide infective larvae which were used to infect lambs experimentally (DB67 and DB75). These passages were used to complete isolation of the possibly benzimidazole resistant strain of *O.circumcincta* and to increase the number of infective larvae which could be used in future studies on anthelmintic resistance in the parasite strain.

Lamb DB48 was infected experimentally with the strain and this was classified as Passage 1 for convenience because studies on the strain were to be initiated from this point. The previous passages used to isolate the strain and increase the number of available larvae have not been taken into account when passages were counted.

During Passage 1 the lamb (DB48) was treated with FBZ at a dose rate of 5mg/kg administered orally. The faecal egg output was monitored and after treatment the faeces were cultured to provide infective larvae for further passages. The faecal egg output from DB48 dropped after treatment but sufficient larvae were cultured to continue passages of the strain. Two further passages through lambs DB1 and DB61 were carried out without treatment and larvae cultured from the faeces of DB61 were stored at 4°C for 4 months prior to infecting DB74 the following spring.

The egg hatch assay was used to test for resistance in the eggs produced in the faeces of DB74 on day 23 post infection. An egg hatch assay using eggs of the Bearsden benzimidazole susceptible strain on Day 25 post inoculation gave an ED50 of 0.16ug/ml for TBZ. The resistance ratio calculated for the HFRO strain and Bearsden strain using TBZ in the egg hatch assays was 9.56 indicating that the HFRO strain was highly resistant to benzimidazoles.

When lamb DB 74 was treated with FBZ at a dose rate of 5mg/kg administered orally

the faecal egg count fell to approximately 50 e.p.g. and at this low level it was impossible to collect sufficient eggs to carry out the egg hatch assays. However, faeces were cultured and sufficient larvae were obtained to infect a lamb for the next passage (DB16). During all the passages 5-14 egg hatch assays were carried out to measure changes in resistance.

Generally in the *in vitro* egg hatch assay TBZ is the benzimidazole used to test for resistance. The HFRO strain was being selected with FBZ and it was therefore decided to carry out the egg hatch assays using one of the broad spectrum benzimidazoles, ABZ, in addition to TBZ. FBZ cannot be used in egg hatch assays because of its low ovicidal effect and poor solubility (Boersema, 1982). From Figs. 2.2-2.6 changes in resistance were paralleled fairly well with these drugs although fluctuations in resistance appeared more marked when ABZ was used in the assays. The resistant strain was selected using FBZ and although there is side resistance with all benzimidazoles the use of one of the more modern compounds with a structure similar to FBZ, ABZ in this case, in the egg hatch assay may improve the sensitivity of the test. However, the resistance ratio calculated by comparing the HFRO and Bearsden strains is similar for TBZ and ABZ e.g. for passage 8 (DB63) the resistance ratio with TBZ was 9.02 and for ABZ was 10.00.

In all passages there was day to day variation in the ED50 calculated from the egg hatch assays using both ABZ and TBZ. The ED50 for the period around Day 27 post-infection was relatively high. It may be that the more resistant members of the population are fitter and more prolific laying eggs at this point. Alternatively responsiveness of the eggs to the effect of benzimidazoles may alter with the stage of infection. Perhaps some within-host differences at particular stages of the infection may influence the level of resistance in the parasite eggs which are produced (see later).

After noting changes in the level of resistance of the HFRO strain during Passage 5 (DB16) (Fig. 2.3) it was decided to run an untreated infection (DB 25) along with the pressurised infection (DB62) during the next passage. Results of the egg hatch assays are

shown in Table 2.1 and Figs. 2.3 and 2.4. The ED50 was similar for each infection at Day 27 post infection. After treatment of DB62 with FBZ the ED50s measured in eggs from this treated animal were higher than ED50s measured in eggs from the untreated lamb (DB25). At Day 28 post infection (24h post treatment) the ED50s were 1.31 ug/ml TBZ and 2.02 ug/ml ABZ for DB 25 (untreated) and 2.83 ug/ml TBZ and 8.26 ug/ml ABZ in DB62 (treated). This apparently higher ED50 immediately post treatment was unexpected. Benzimidazoles are considered to act by binding to tubulin causing disintegration of microtubules in parasite cells (Borgers and De Nollin, 1975). After treatment of the lamb with FBZ it was thought that the FBZ would bind to tubulin in the resistant eggs. During the egg hatch assay less TBZ or ABZ would then be required to prevent hatching because of the presence of bound FBZ. This should produce a reduced ED50. However, the results from DB62 and DB25 are at variance with this theory. In other passages shown in Figs. 2.2, 2.5 and 2.6 there is an increase in the ED50 after treatment with FBZ although for these passages there is no untreated passage to allow a direct comparison betweeen treated and untreated and changes in ED50 could reflect day to day variation in the ED50 which was found in all passages. The changes in ED50 after treatment appeared more pronounced when ABZ was used in the egg hatch assays.

After treatment there may be a change in the egg laying population with only some females producing eggs immediately post treatment with FBZ. During some passages (4-8) there was a fall in faecal egg count after treatment with FBZ which rose again after 5-7 days . One week post treatment in all passages the ED50 had returned to around the pre-treatment level and this may correlate with the return to normal egg laying capacity observed from faecal egg counts.

During the untreated passage (DB25) results of egg hatch assays using both TBZ and ABZ show day to day variability. Unfortunately the first egg hatch assay was carried out on Day 27 post infection rather than immediatly after patency of the infection (Day 21 post

infection). For DB25 the level of resistance measured using ABZ in the egg hatch assay showed a high level for Days 27-30 reaching a peak at Day 36 then falling to a low level at Day 50-51 before rising again later in the infection although never attaining the high level encountered during the early part of the infection. A similar pattern was found using TBZ in the egg hatch assays but fewer egg hatch assays were carried out with this drug. During Passage 7 (DB5) egg hatch assays were performed at patency (Day 20 post infection) and the results (Fig. 2.5) indicated a low ED50 at Day 20 rising to a higher level by Day 27-29 post infection. The pattern of day to day variation in results of the egg hatch assay with the HFRO strain of *O. circumcincta* seems to indicate a low level of resistance at patency which rises rapidly to reach a higher level by Day 27 , remains high up to day 40-45 then falls to a low level around day 50-55 before rising again later in the infection. Changes in the level of resistance during an infection have also been noted by Borgsteede and Couwenberg (1987). These authors measured the level of resistance in experimental infections with a benzimidazole resistant and benzimidazole susceptible strain of *H.contortus* using an egg hatch assay from Day 22-77 post infection. They found a low level of ED50 at Day 22 which rose steadily with highest values for the ED50 Days 40-60 post infection then a slight decrease in the ED50 towards the end of the infection. The pattern was similar in both the resistant and susceptible strains. Throughout the infection the variation in the ED50 was by a factor of 2. Le Jambre (1976) noted day to day variation in the egg hatch assay results and considered that changes in the ED50 may reflect differences in the stage of development of the eggs at the time the egg hatch assay was started. To overcome this problem it was recommended that a susceptible strain was tested at the same time and under the same conditions as the resistant strain and the resistance ratio calculated. This ratio appears to be an accurate measure of resistance and shows little day to day variation. From Borgsteede and Couwenberg (1987) it would appear that changes in the ED50 found during an infection are similar for the resistant

and susceptible strain therefore the resistance ratio will remain constant. Throughout passages of the HFRO strain a susceptible strain of *O.circumcincta*, the Bearsden strain, was run in parallel and an ED50 for this strain measured at the same time as the resistant strain in order to ensure that the resistance level was measured accurately.

The reasons for the changes in the ED50 noted above are unknown. In a review of methods used to detect anthelmintic resistance Boersema (1982) described the variations in methods used in the egg hatch assays by various authors and described some of the results for ED50s found for benzimidazole susceptible strains of *H.contortus* achieved using these methods. For the Bearsden strain of *O.circumcincta* the ED50 was approximately 0.16ug/ml TBZ and 0.20 ug/ml ABZ which is high compared with results for susceptible strains assayed by Cawthorne and Whitehead (1983) who gave ED50s for two susceptible strains of *O.circumcincta* as 0.038ug/ml TBZ and 0.036ug/ml TBZ. These discrepancies between resistance levels are probably due to differences in the technique used in the egg hatch assay or differences in the strains of *O.circumcincta* The Bearsden strain has been maintained at Glasgow Veterinary School for approximately 15 years without pressurisation and should be susceptible to the effects of benzimidazole anthelmintics. Results of tubulin binding assays with MBZ (see later) comparing different strains of *O.circumcincta* showed the Bearsden strain to be highly susceptible to benzimidazoles. The higher ED50s calculated for the Bearsden strain compared with ED50s for susceptible strains measured by Cawthorne and Whitehead (1983) are not likely to be due to possible resistance in the Bearsden strain. However, differences in the actual ED50 values found in assays carried out in different laboratories are probably not as important as the comparison of ED50s between resistant and susceptible strains studied in individual laboratories.

The time of incubation used during the egg hatch assay also may influence the results of the assay. Le Jambre (1976) used incubation for 72h, Coles and Simpkin (1977) and

Boersema (1982) used 48h, Cawthorne and Whitehead (1983) used 24h and most of the egg hatch assay methods used in Australia involve 24h incubation. During passage 7 (DB 2) the egg hatch assay was carried out comparing 24h and 48h incubation on Day 21 post infection and the ED50s calculated were identical. When calculating ED50s in the above assays the hatched larvae and eggs showing development (embryonated eggs) were counted as alive and those eggs which failed to develop were counted as dead and the percentage failing to develop was used when calculating the ED50. Workers in Australia have noted higher resistance factors because they count only hatched larvae as alive and consider all eggs, developed and undeveloped, as dead. Smith-Buijs and Borgsteede (1986) found high levels of embryonated eggs in low drug concentrations of benzimidazoles with their susceptible strain of *H.contortus* in egg hatch assays, whereas the majority of eggs of the resistant strain hatched to larvae at low concentrations of benzimidazoles. These authors suggested that in the susceptible strains the benzimidazoles prevent hatching of the eggs whereas in the resistant strains the benzimidazoles have little effect on hatching. Similar results were found during the routine passages of the Bearsden and HFRO strains of *O.circumcincta* and the percentage of embryonated eggs was high in egg hatch assays immediately post treatment of the HFRO strain with FBZ and in assays during the later period of an infection (Day 60 onwards). In order to maintain consistency of results embryonated eggs were counted as developed in all assays. However, contrary to results found with the HFRO and Bearsden strains of *O.circumcincta*, Hall *et al* (1978a) found more consistent results in egg hatch assays with *H.contortus* and *T.colubriformis* when embryonated eggs were counted as dead rather than alive.

Another problem with the egg hatch assay is the stage of development of the eggs at the start of the period of incubation with drug in the egg hatch assays. Coles and Simpkin (1977) noted that embryonation of nematode eggs occurs rapidly and these authors stated that eggs for the egg hatch technique should be collected from fresh faeces and tested within

4h of expulsion from the host animal. Le Jambre (1976) also noted that variation in ED50s from day to day may reflect changes in the stage of development of the eggs when tested, brought about by variation in the ambient temperature during the time of egg collection. Faecal egg counts during infections with *H.contortus* are considerably higher than with *O.circumcincta* therefore sufficient eggs can be collected over a short period of time. During the early passages of the HFRO and Bearsden strains faecal material was collected from the lambs overnight and material from the top of the faecal bags was used for collection of eggs for the egg hatch assay to try to ensure that the eggs were as freshly passed as possible. The variation in the stage of development of the eggs collected may have had some effect on the egg hatch assay results increasing variability of results.

From Passage 7 onwards a different collection schedule was followed with faeces collected over a 3-4h period. With this shorter period of collection and therefore more predictable age of eggs the results of the egg hatch assays still showed day to day variation. Smith- Buijs and Borgsteede (1986) studied the effect of storage of eggs on the results of the egg hatch assays. These authors found that storage of eggs for 0, 24 and 72h at 4°C prior to use in the egg hatch assay had no effect on the resultant ED50. However the hatchability of the eggs was reduced to between 40-50% when eggs were stored for 72h and storage for 1week at 4°C totally prevented eggs from hatching. It is possible that prolonged storage of larvae is more detrimental to hatchability in *H.contortus* rather than to *O.circumcincta* and *T.colubriformis* (Smith-Buijs and Borgsteede, 1986)

Le Jambre (1976) found that higher concentrations of benzimidazoles were required to prevent hatching of embryonated eggs compared with unembryonated eggs. The reasons for this reduced susceptibility of embryonated eggs to benzimidazoles may be due to changes in the metabolism of eggs after embryonation (Weston *et al*, 1984).The uptake of TBZ was reduced in embryonated eggs compared with unembryonated eggs possibly because of the lower level of lipid in the embryonated eggs. Uptake of benzimidazoles is thought to be by

passive diffusion into the eggs and therefore the lipid content of the embryonated egg, which can be approximately 60% less than the lipid content of the undeveloped egg, can have an effect on the uptake of lipid soluble benzimidazoles. As the eggs develop oxygen uptake increases and there is evidence of an increasing level of aerobic metabolism in embryonated eggs compared with undeveloped eggs. If a possible mechanism of action of the benzimidazole is inhibition of fumarate reductase which is associated with anaerobic metabolism a shift to aerobic metabolism will reduce the effect of the drug and therefore embryonated eggs may be less susceptible to the effect of the benzimidazoles.

The uptake of the benzimidazoles into the eggs is affected by the nature of the benzimidazole and possibly the solvent used in the egg hatch assay. Because of the low solubility of FBZ this benzimidazole is not suitable for use in the egg hatch assay. Kirsch (1978) in studies on the ovicidal effect of FBZ found that after oral administration of FBZ to the host parasite eggs passed in the faeces showed atypical development. However, increasing drug concentrations of FBZ in egg hatch assays with *T.colubriformis* did not increase the ovicidal effect. This was thought to be due to poor solubility of the drug limiting its uptake into the eggs. FBZ is metabolised to OFZ and testing this metabolite in egg hatch assays failed to show any ovicidal effect at the highest concentrations where the drug could be solubilised (Boersema *et al*, 1982). Failure of this drug to prevent egg hatching could be due to lack of absorption of the drug through the shell of the eggs (See later for discussion on the ovicidal activity of ABSX and ABSO). Bone and Coles (1987) have decribed the use of a toxin from *Bacillus thuringiensis israelensis* in combination with benzimidazoles in egg hatch assays using *T.colubriformis*. The bacterial toxin caused a decrease in the ED50s possibly by increasing the uptake of drug into the eggs by an alteration in egg permeability (Bone and Coles, 1987). This work suggests that uptake of benzimidazoles into eggs occurs by passive diffusion and any alteration in permeability of the egg shell will affect drug uptake.

Differences in the solvent for the benzimidazole used in the egg hatch assays may be partly responsible for differences in the ED50s found between different workers. Le Jambre (1976) used TBZ in 0.1%sodium chloride, Coles and Simpkin (1977) used DMSO, Hall *et al.*, (1978a) used commercial drug preparations diluted in distilled water and Cawthorne and Whitehead (1983) used methanol. High concentrations of solvents are lethal to nematode eggs and therefore for the assays a concentration of < 5% methanol was used for each concentration of the drug. DMSO acts as an agent which can increase drug penetration into tissues and this may account for some of the differences in the ED50 when this drug was used as the solvent for benzimidazoles in egg hatch assays carried out in other laboratories. However, a control well with the equivalent concentration of solvent was used throughout the egg hatch assays to ensure that any measured effect on hatchability of the eggs was due to the benzimidazole anthelmintic. The percentage of the eggs failing to hatch in control wells varied from day to day therefore controls were necessary for each assay. Day to day variation in hatchability was noted also by Coles and Simpkin (1977).

After isolation of the HFRO strain of *O.circumcincta* the level of resistance of the parasites as measured by the egg hatch assay was studied over the 14 passages with or without pressurisation using FBZ. The ED50s for both TBZ and ABZ were very similar at Passage 4 (DB74), Passage 8 (DB63) and Passage 14 (DB41) indicating that constant pressurisation with FBZ did not increase the level of resistance in the strain. Martin *et al.*, (1984) found that after producing a highly resistant strain of *O.circumcincta* in a field experiment by treating with TBZ 17 times per year for two years further treatments over the next three years failed to increase the level of resistance. The level of resistance to TBZ estimated using a single *in vitro* egg hatch assay each year remained at 14 times higher than an untreated benzimidazole susceptible field strain. However, with a less resistant strain of *O.circumcincta* which had a resistance level 3 times the

susceptible level they found that treatment with TBZ 5 times yearly in the field increased the resistance level to 6 times that of the susceptible over two years. The HFRO strain may be highly resistant and therefore further selection cannot increase the overall resistance level which is approximately 10 times that of the Bearsden susceptible strain. However, from the results in Fig. 2.4 it is possible to encounter a higher level of resistance in egg hatch assays which were carried out at a particular time during the course of an infection.

Removal of pressurisation also appeared to have little effect on the level of resistance in the HFRO strain of *O.circumcincta* From Passage 11 onwards two lines, a FBZ pressurised and an unpressurised line, were maintained and studied in parallel. There was no significant difference in the ED50s measured for the differentially treated lines. Rapid reversion to benzimidazole susceptibility was not observed. This is contrary to the results obtained by Simpkin and Coles (1978) using *T.colubriformis* and *H.contortus*. These authors found that the level of resistance measured by the egg hatch assay fell if parasites were passaged through lambs without pressurisation and pressurisation with TBZ selected for worms producing eggs which were more resistant to TBZ, CBZ and PBZ but not MBZ in egg hatch assays. This differential resistance exhibited by individual benzimidazoles was thought to be due to the polygenic nature of the inheritance of resistance (see later). The reversion to susceptibility on removal of selection pressure with benzimidazoles noted by Simpkin and Coles (1978) was not found by other authors in separate trials. Hall *et al.* (1982) using benzimidazole resistant strains of *T.colubriformis* and *H. contortus* passaged the parasites over 12 generations in the laboratory without TBZ and found no significant change in the level of resistance measured by egg hatch assays. These authors noted that compared with the field situation there was no chance of dilution of the strain with susceptible parasites which may have remained on pasture. However, they implied a similarity with a self contained flock stating that it was unlikely that reversion to

susceptibility would occur if a population of parasites had been selected heavily for resistance without the reintroduction of large numbers of susceptible parasites. Maintenance of a high level of benzimidazole resistance was also noted by Le Jambre *et al.* (1982) in a strain of *H.contortus* when selection pressure was removed over 4 generations and these authors suggested this implied that the resistant parasites were as fit as the non-resistant parasites and therefore were not likely to be removed from the population even when selection pressure with benzimidazoles was removed.

Donald *et al.*, (1980) used a benzimidazole resistant strain of *O.circumcincta* in trials comparing the level of resistance measured by egg hatch assays and anthelmintic efficiency trials in untreated field infections with infections treated with levamisole every 4 weeks. They found that levamisole selected positively against benzimidazole resistance. Similar results were found by Waller *et al.* ,(1983) and Martin *et al.* , (1988) using *Ostertagia spp.* where counter selection with levamisole over several generations reduced the level of benzimidazole resistance compared with an untreated strain where reversion to susceptibility did not occur. The reason for this counter selection using an anthelmintic with a different mode of action in the field is unknown but may involve changes in the population dynamics of the parasites and therefore an alteration in the ability of the benzimidazole resistant parasites to survive. For reversion to susceptibility to occur the susceptible phenotype must have a fitness advantage over the resistant phenotype. However, experience with insecticidal resistance suggested that after intense selection the genome of the insects becomes reorganised in order that the resistant phenotype becomes as fit as the susceptible even in the absence of the selecting agent (Georghiou and Taylor, 1976 ; McKenzie *et al.*, 1982; McKenzie and Purvis, 1984). In this situation where the resistant parasites are not disadvantaged in the absence of the selecting agent the resistance level in the population will remain stable until introduction of different genetic material or a change in the factors affecting the fitness of the

parasites.

In the field situation the rate of development of resistance depends on the percentage of the population exposed to the selecting agent (Martin *et al*,1981). The percentage of the population of the parasites whichescapes selection is known as the population *in refugia*. For nematode parasites the free living larval stages on the pasture can escape selection with anthelmintics and act as a source of susceptible parasites maintaining the gene pool of susceptible characteristics. Where a small proportion of the larvae are *in refugia*, resistance to the selecting agent develops rapidly and where an increased proportion of the larvae are *in refugia* , resistance is slower to develop. The increase in the level of resistance was inversely proportional to the percentage of the population *in refugia*. All experiments with the HFRO strain of *O.circumcincta* were carried out in the laboratory with experimental infection of lambs using larvae obtained from previous passages and therefore none of the parasites were *in refugia* This could account for the high level of resistance found at the initial egg hatch assay (Passage 4, DB74) because resistant parasites would be selected intensively after the initial treatment (Passage 1, Treatment1) without dilution by unselected parasites and also the failure to revert to susceptibility on removal of selection pressure (Passages 11-14).

After treatment of lamb DB74 with FBZ at the normal dose rate the faecal egg count of the lamb fell to approximately 50 eggs per gram making collection of eggs for egg hatch assays impossible. Prior to infection of the lamb the larvae had been stored at 4°C for 4 months throughout the winter period when suitable hosts which could be used to continue passaging the parasites were not available. Although from results of a single egg hatch assay prior to treatment with FBZ the parasite population was resistant the fall in faecal egg count after treatment suggested that the resistance level may have fallen. The effect of storage of larvae over a four month period was then studied using lamb DB4. Larvae collected from the faeces of DB74 prior to treatment with FBZ were stored at 4°C for 4

months then used to infect DB4. This lamb was infected at the same time as lamb DB63 during the routine passages and the results of egg hatch assays on eggs passed in the faeces of the lambs are shown in Tables 2.1 and 2.3. Storage of larvae over the period had no effect on the level of resistance and the results for DB4 infected with stored larvae from DB74 (Passage4, Treatment 1) were similar to results for DB63 (Passage 8, Treatment 5). The reason for the fall in faecal egg count after treatment of DB74 with FBZ cannot be explained by a fall in resistance over the period of storage of the larvae. However, during the initial passages of the HFRO strain there was a tendency for faecal egg counts to fall immmediately post-treatment with anthelmintics and rise again after approximately 5-7 days. This fall in egg count immediately post treatment has been noted by other workers (Martin, personal communication) and it could be due to reduced reproductive function in the resistant parasites over the period of treatment.

A strain of benzimidazole resistant *O.circumcincta* was obtained from the Moredun Institute and the resistance level as measured by egg hatch assays was compared with the HFRO strain. Results of egg hatch assays on eggs passed in the faeces of lamb B2 which was infected with the Moredun strain are shown in Table 2.4 and Fig. 2.7. Egg hatch assays during the initial period post-infection (Days 26 and 28) showed a low ED50 for ABZ compared with the ED50 for TBZ. In the majority of egg hatch assays on the HFRO strain of *O.circumcincta* the ED50 for ABZ was higher than the ED50 using TBZ. Side resistance to the other benzimidazoles may have been less with the Moredun strain than the HFRO strain. Immediately post treatment with FBZ there was no effect on the ED50 but in the later part of the infection (Days 49 and 54) the level of resistance was higher. This is the opposite effect to the HFRO strain where during the later stages of infection the ED50 had a tendency to fall (see previously). It is possible that the population dynamics within the host differ for these two strains of *O.circumcincta* with individual parasites within the population laying different numbers of eggs at different periods during the infection.

However, calculation of the mean ED50 for the Moredun strain for both TBZ and ABZ suggested that this strain was less resistant than the HFRO strain and further studies on this strain were abandoned in favour of the more resistant HFRO strain of *O.circumcincta*

Dose Titration Trial

The dose titration trial was set up to examine the effect of treatment with increasing doses of FBZ on the level of benzimidazole resistance in the HFRO strain of *O.circumcincta* measured by an anthelmintic efficiency test and *in vitro* egg hatch assays.

Experimental Design.

Experiment 1. A dose titration trial (Moskey and Harwood, 1941; Gibson, 1964 ; Prichard *et al*, 1980; Powers *et al*, 1982) was carried out using 3 groups of 5 male lambs. The lambs were infected on Day 0 with 10,000 *O.circumcincta* infective larvae cultured from the faeces of lamb DB25 (Passage 6, treated on 3 occasions). Patency was shown by positive faecal egg counts on Day 21, and on Day 23, 4 lambs of each group were treated with FBZ administered orally at dose rates of 5,10, 20 and 40 mg/kg respectively with the remaining lamb in each group used as an untreated control. Lambs were slaughtered one week post treatment and worm burdens were measured (see General Materials and Methods).

Experiment 2. Five male lambs were infected experimentally on Day 0 with 10,000 *O.circumcincta* infective larvae cultured from the faeces of lamb DB25 . On Days 31, 35 and 38 parasite eggs were collected from the lambs' faeces to be used for *in vitro* egg hatch assays. On Day 44, 4 lambs were treated with FBZ administered orally at dose rates of 5, 10, 20 and 40 mg/kg and one lamb was an untreated control. On Day 49 and Day 51 parasite eggs were collected from the lambs' faeces and used in egg hatch assays. On Day 58 the lambs were slaughtered and parasite burdens calculated.

Experiment 3. Five female and five male lambs were inoculated on Day 0 with 10,000

infective larvae of *O.circumcincta* cultured from the faeces of DB25. On Day 21, 4 of the female lambs were treated with FBZ administered orally at dose rates of 5, 10, 20 and 40 mg/kg and the remaining female was left untreated. On Day 29 all 5 female lambs were killed and their worm burdens calculated. At necropsy, faeces were collected from the terminal colon and rectum and used to obtain parasite eggs for egg hatch assays. On Days 23 and 24, parasite eggs were collected from the faeces of the 5 male lambs. On Day 29, 4 of the male lambs were treated with FBZ administered orally at dose rates of 5, 10, 20 and 40 mg/kg and one lamb remained as an untreated control. On Days 35 and 36 parasite eggs were collected from the lambs' faeces and used in egg hatch assays. On Day 42, the 5 male lambs were slaughtered and worm burdens calculated.

Results

The results of the dose titration trials in Experiments 1, 2 and 3 are given in Table 2.5. The parasite burdens found at necropsy are listed together with the percentage reduction in parasite numbers compared with those of the control animals. Dose titration curves for each of the experiments are shown in Fig. 2.8 and a mean dose titration curve using combined data from all three experiments is shown in Fig. 2.9. The means were calculated by adjusting the values for Experiment 2 by a factor of 1.44 to give the equivalent worm burden in the control animal as was found in the other experiments. The mean values for percentage reduction in worm burden were 20%, 28%, 50% and 67% for lambs treated orally with 5mg/kg, 10mg/kg, 20mg/kg and 40mg/kg of FBZ respectively.

The ED50s calculated from egg hatch assays using TBZ or ABZ in Experiment 2 are given in Tables 2.6 and 2.7 respectively. Results from the assays conducted prior to and after treatment of the lambs with the stated doses of FBZ are shown. The results are represented graphically in Figs. 2.10 and 2.11. Tables 2.8 and 2.9 show the ED50s calculated from egg hatch assays carried out during Experiment 3 using TBZ and ABZ respectively. For the 5 male lambs results were obtained before and after treatment of the lambs with the stated

doses of FBZ. Included in the table are the egg hatch assay results obtained for eggs collected at necropsy, 7 days after treatment with FBZ of the 5 female lambs in Experiment 3. In all cases egg hatch assays were carried out at least 5 days after treatment of the lambs with FBZ to avoid possible fluctuations in results (see previously). Graphs of the results of egg hatch assays in Experiment 3 are shown in Figs. 2.12 and 2.13.

In Experiment 2, a mean value for percentage eggs failing to develop at each concentration of TBZ or ABZ used in the egg hatch assay was calculated using the values obtained from all 5 male lambs on the three occasions prior to treatment with FBZ. These values were used to plot a dose response curve of mean percentage failing to develop against drug concentration for TBZ and ABZ shown in Fig. 2.14. From these dose response curves the mean ED50 for the HFRO strain of *O.circumcincta* was calculated at 1.61 ug/ml TBZ and 2.61 ug/ml ABZ. The data obtained from egg hatch assays prior to treatment of the lambs with FBZ in Experiment 3 were treated in a similar way. Mean values for percentage of eggs failing to develop were used to plot the dose response curves shown in Fig. 2.15 The mean ED50s for the parasites prior to treatment with FBZ were 1.65 ug/ml TBZ and 2.87 ug/ml ABZ.

Discussion

The HFRO strain of *O.circumcincta* was shown to be highly resistant to the effects of the benzimidazole anthelmintic in the dose titration trials. The normal therapeutic dose rate for FBZ in sheep is 5mg/kg and in all of these experiments this dose had poor efficacy with percentage reductions in parasite burden of 0%, 67% and 56% in Experiments 1, 2 and 3 respectively. Data from the egg hatch assays also indicates a high level of resistance (Tables 2.6-2.9 and Figs. 2.10-2.13).

The variability in the effect of FBZ on adult worms was marked. In Experiment 1, three groups of lambs were used and therefore the results are a mean of three animals treated at

Table 2.5

Results of dose titration Experiments 1, 2 and 3 on lambs infected with *O.circumcincta* and treated with FBZ administered orally at dose rates of 0, 5, 10, 20 and 40mg/kg, showing adult worm counts at necropsy, mean worm counts for each experiment and percentage reduction in worm counts compared with control animals.

Dose FBZ	Experiment 1					Experiment 2		Experiment 3			
(mg/kg)	Gp 1	Gp 2	Gp 3	Mean	%*	M	%*	M	F	Mean	%*
control	4750	4100	4350	4400		3050		3600	5250	4425	
5	7650	3900	4500	5350	0	1000	67	1750	2100	1925	56
10	3050	4300	3400	3583	19	2150	30	1300	4250	2775	37
20	3200	3400	1500	2700	39	2150	30	600	1300	950	78
40	1900	2500	950	1783	60	100	97	3200	50	1025	63

*Percentage reduction in mean worm counts compared with the control animals

M-Male lambs F- Female lambs

Figure 2.8

Dose titration curves plotting percentage reduction in worm burden against dosage of FBZ (mg/kg) used orally in lambs in dose titration Experiments 1-3.

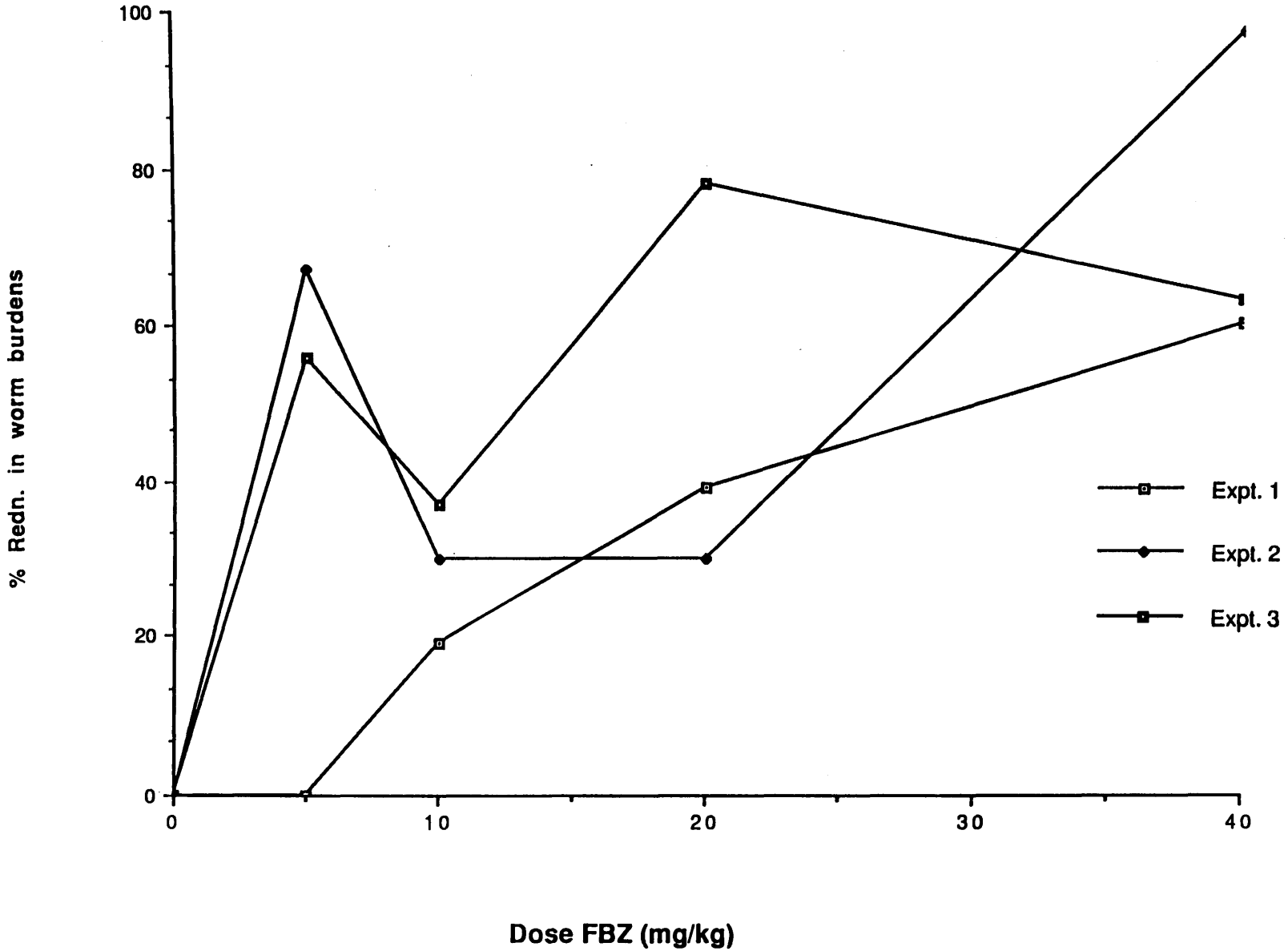
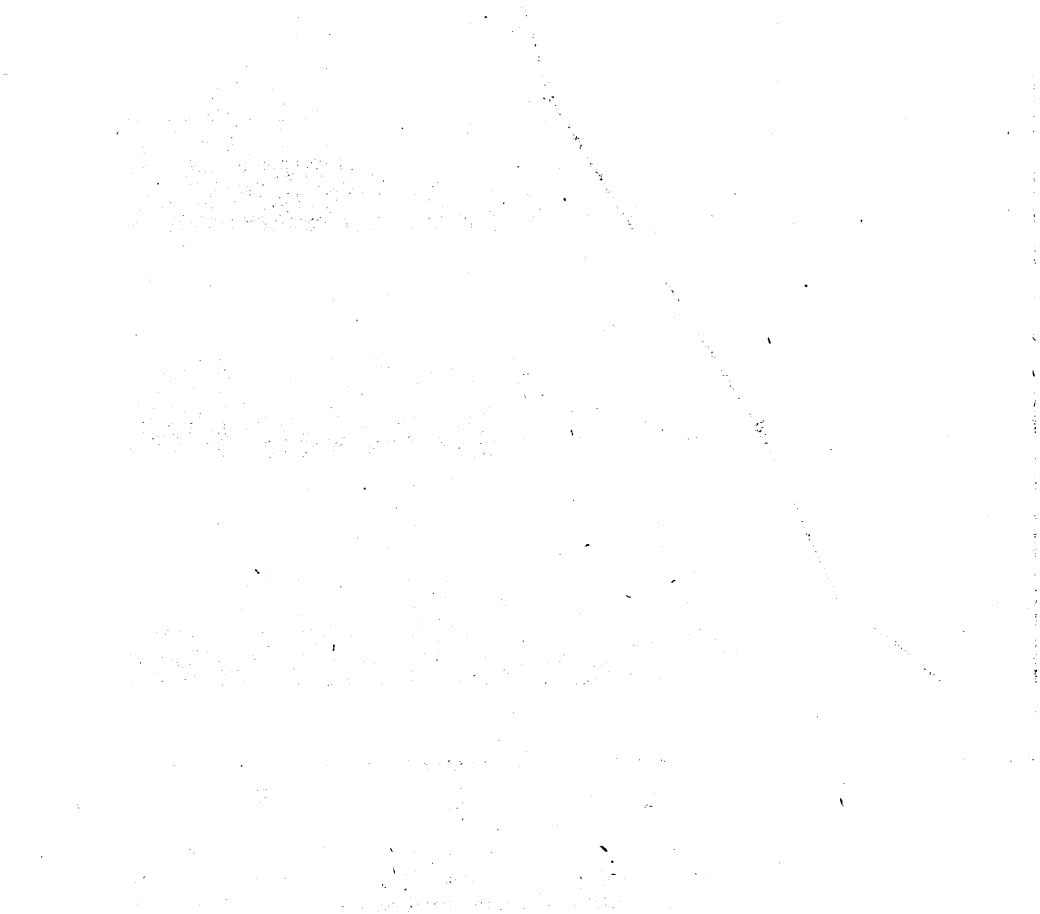

100
80
60
40
20
0
0
10
20
30
40
% Redn. in worm burdens
Dose FBZ (mg/kg)
Expt. 1
Expt. 2
Expt. 3

Figure 2.9

A mean dose titration curve plotting percentage reduction in worm burden against dosage of FBZ (mg/kg) used orally in lambs using combined data from dose titration Experiments 1-3.



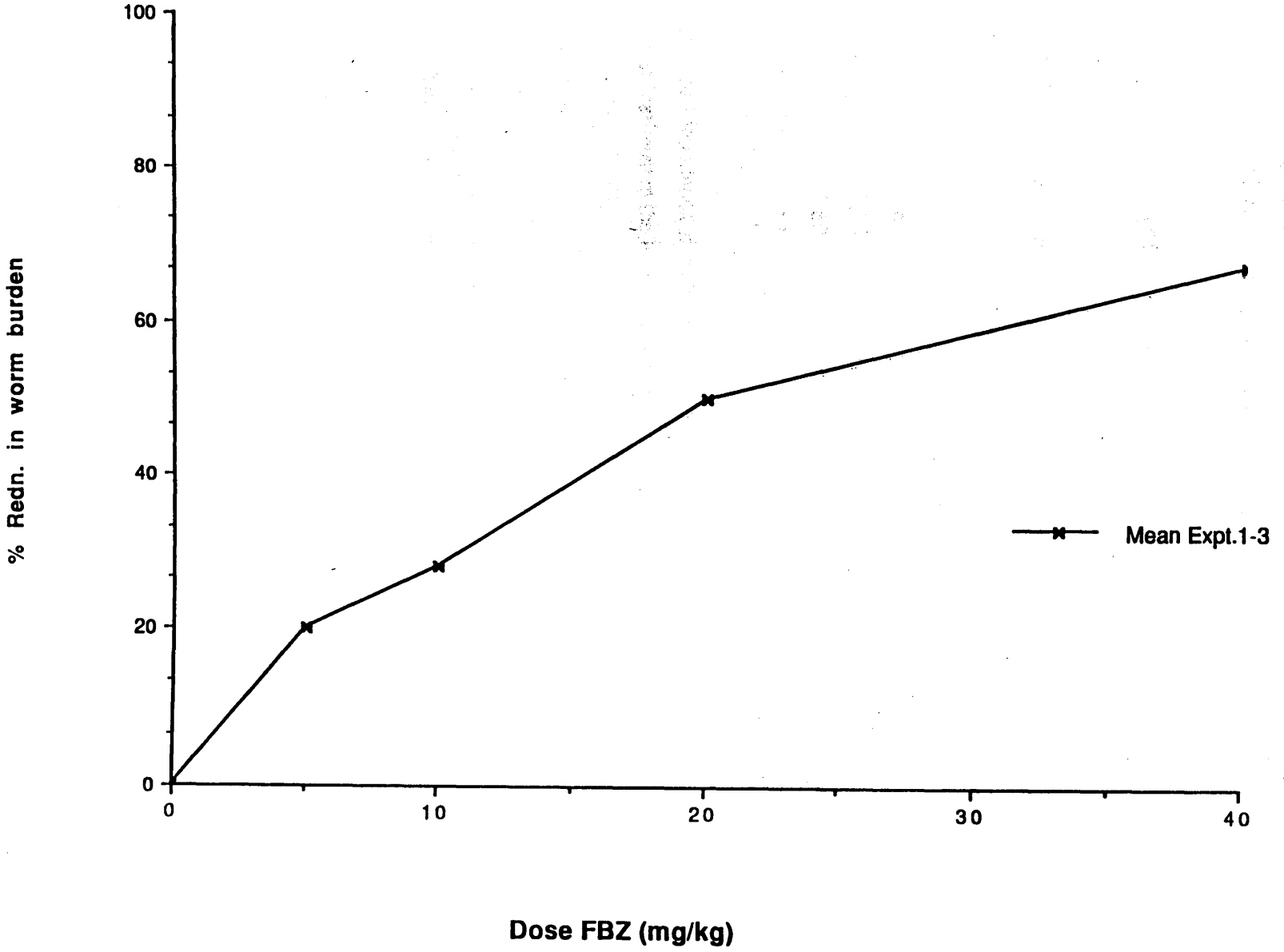

% Redn. in worm burden
100
80
60
40
20
0
0
10
20
30
40
Mean Expt.1-3
Dose FBZ (mg/kg)

Table 2.6

Results of egg hatch assays using TBZ for lambs in Experiment 2 before and after treatment with FBZ at the stated dose rates

	ED50 (ug/ml TBZ) calculated from egg hatch assays				
	Dose of FBZ used in Lambs				
Time *	control	5mg/kg	10mg/kg	20mg/kg	40mg/kg
(days)					
31	1.73	1.67	1.65	2.06	1.66
35	1.67	1.81	1.72	1.78	1.71
38	1.60	1.68	1.71	1.40	1.36
49**	1.60	1.44	1.50	1.55	1.67
51**	1.61	1.67	1.60	1.57	1.70

*Days post inoculation of lambs with 10,000 *O. circumcincta* L3 larvae.

**Post treatment of the lambs with FBZ administered orally at the stated dose rates.

Table 2.7

Results of egg hatch assays using ABZ for lambs in Experiment 2 before and after treatment with FBZ at the stated dose rates

	ED50 (ug/ml ABZ) calculated from egg hatch assays				
	Dose of FBZ used in Lambs				
Time * (days)	control	5mg/kg	10mg/kg	20mg/kg	40mg/kg
31	2.99	2.51	2.15	3.17	2.66
35	2.83	3.40	2.76	2.85	3.49
38	2.13	1.97	2.79	1.63	2.02
49**	2.88	2.66	2.64	2.62	2.82
51**	2.75	3.16	2.94	2.74	2.58

*Days post inoculation of lambs with 10,000 *O. circumcincta* L3 larvae.

**Post treatment of the lambs with FBZ administered orally at the stated dose rates.

Table 2.8

Results of egg hatch assays using TBZ for lambs in Experiment 3 before and after treatment with FBZ at the stated dose rates

	ED50 (ug/ml TBZ) calculated from egg hatch assays				
	Dose of FBZ used in Lambs				
Time * (days)	control	5mg/kg	10mg/kg	20mg/kg	40mg/kg
23	1.89	1.64	1.67	2.01	1.94
24	1 74	1 50	1 75	1 29	1 83
29**	1.41	1.47	1.51	1.72	-
35***	1.68	1.44	1.94	1.65	1.61
36***	1.49	1.59	1.59	1.54	1.50

*Days post inoculation of lambs with 10,000 *O. circumcincta* L3 larvae.

**Egg hatch assay results for the female lambs after treatment with FBZ administered orally at the stated dose rates.

***Post treatment of the lambs with FBZ administered orally at the stated dose rates.

Table 2.9

Results of egg hatch assays using ABZ for lambs in Experiment 3 before and after treatment with FBZ at the stated dose rates

	ED50 (ug/ml ABZ) calculated from egg hatch assays				
	Dose of FBZ used in Lambs				
Time * (days)	control	5mg/kg	10mg/kg	20mg/kg	40mg/kg
23	3.68	3.18	3.28	3.33	3.50
24	2.03	2.10	2.27	2.41	2.37
29**	2.00	1.87	1.78	2.64	-
35***	2.25	1.58	2.87	2.30	2.13
36***	1.88	2.20	1.88	2.02	1.60

*Days post inoculation of lambs with 10,000 *O. circumcincta* L3 larvae.

**Egg hatch assay results for the female lambs after treatment with FBZ administered orally at the stated dose rates.

***Post treatment of the lambs with FBZ administered orally at the stated dose rates.

Figure 2.10

The level of resistance measured by the ED50 with TBZ in lambs before and after treatment of the lambs with increasing doses of FBZ in dose titration Experiment 2 .

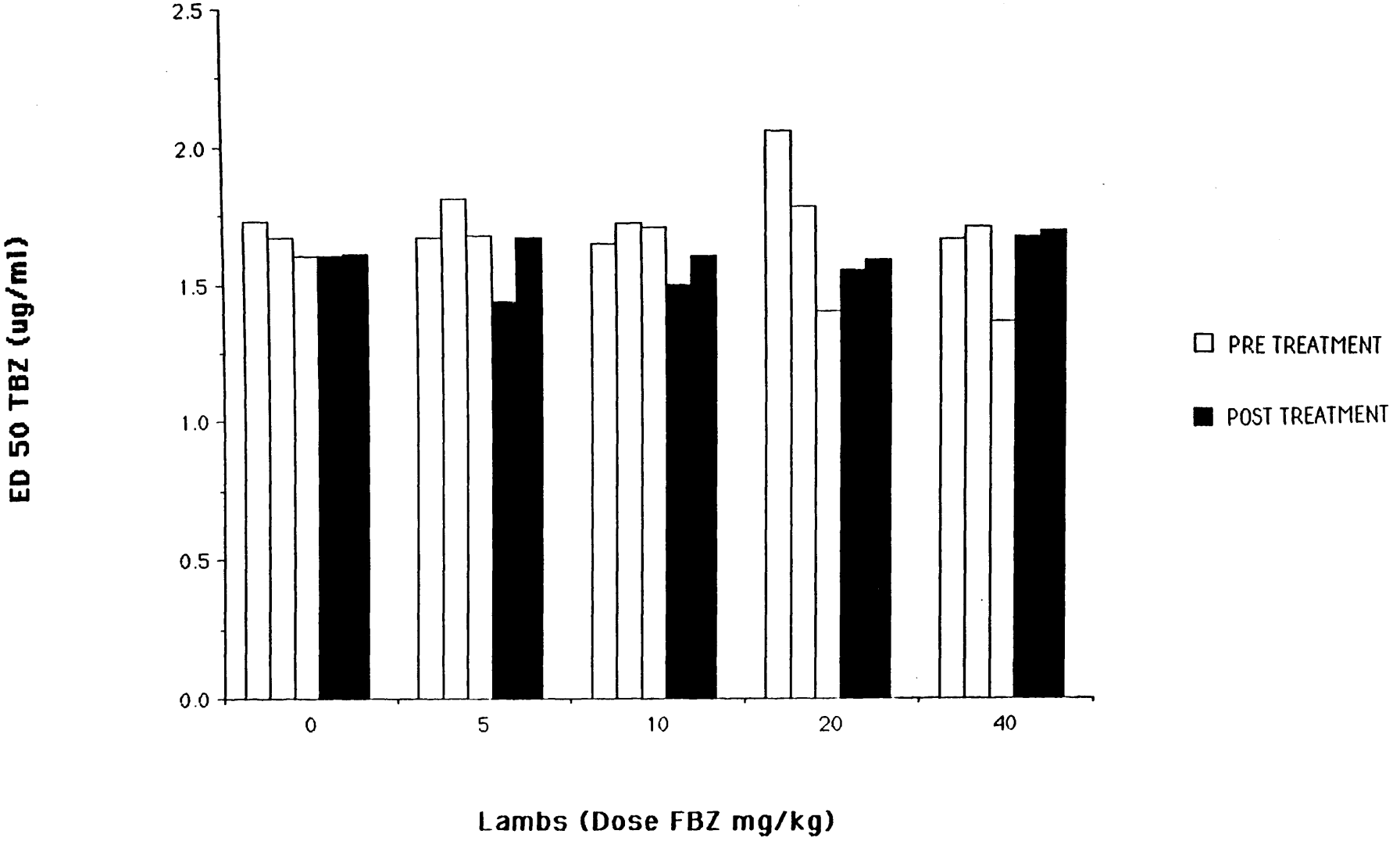
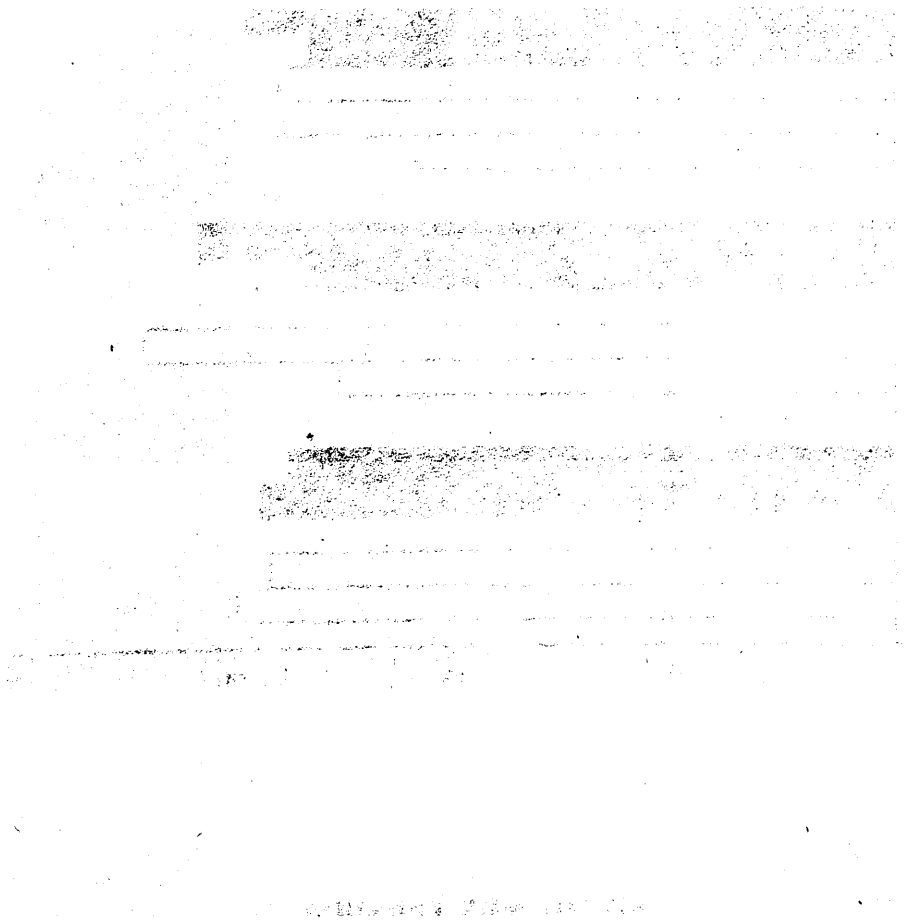

ED 50 TBZ (ug/ml)
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
20
40
Lambs (Dose FBZ mg/kg)
PRE TREATMENT
POST TREATMENT

Figure 2.11

The level of resistance measured by the ED50 with ABZ in lambs before and after treatment of the lambs with increasing doses of FBZ in dose titration Experiment 2 .



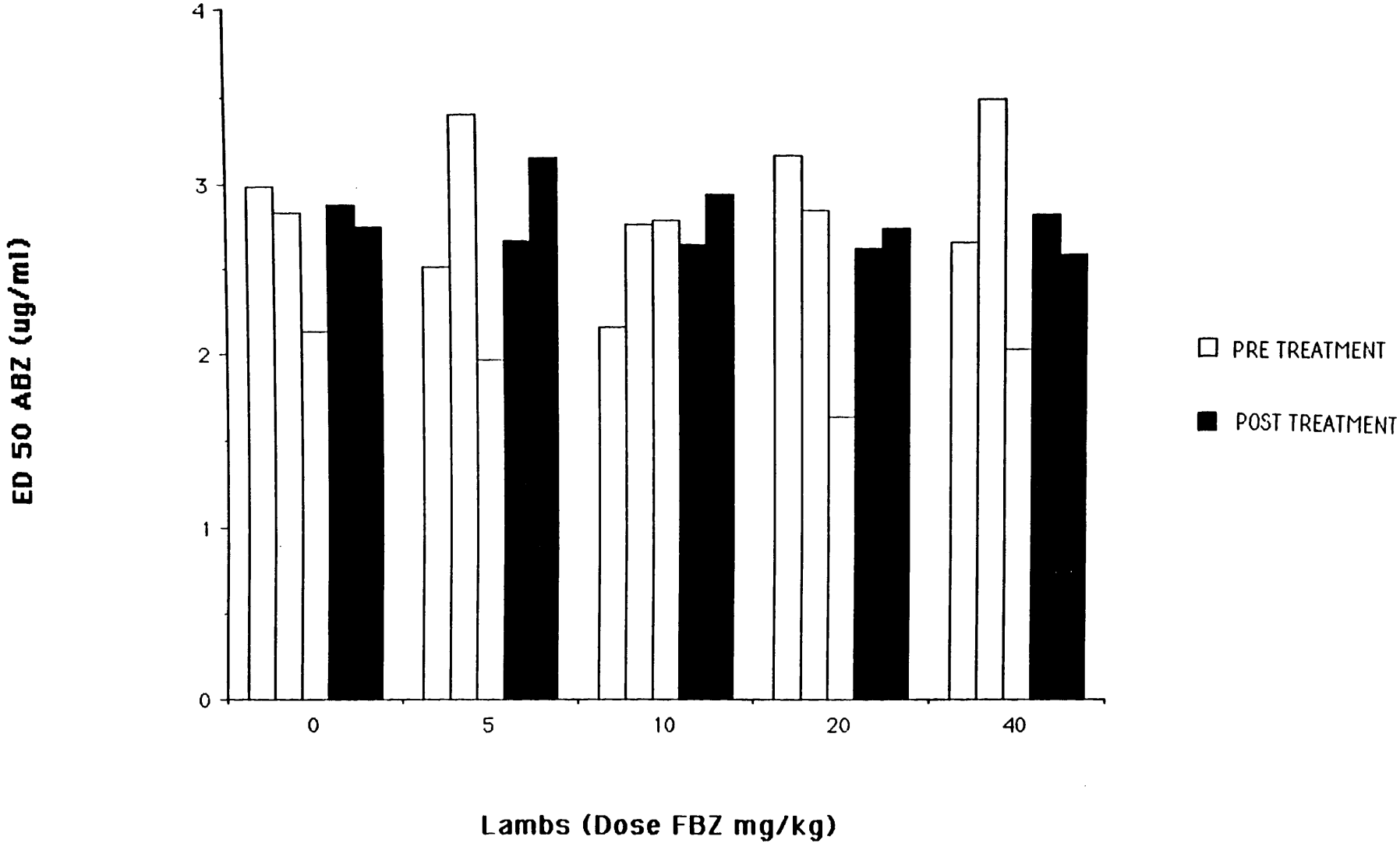
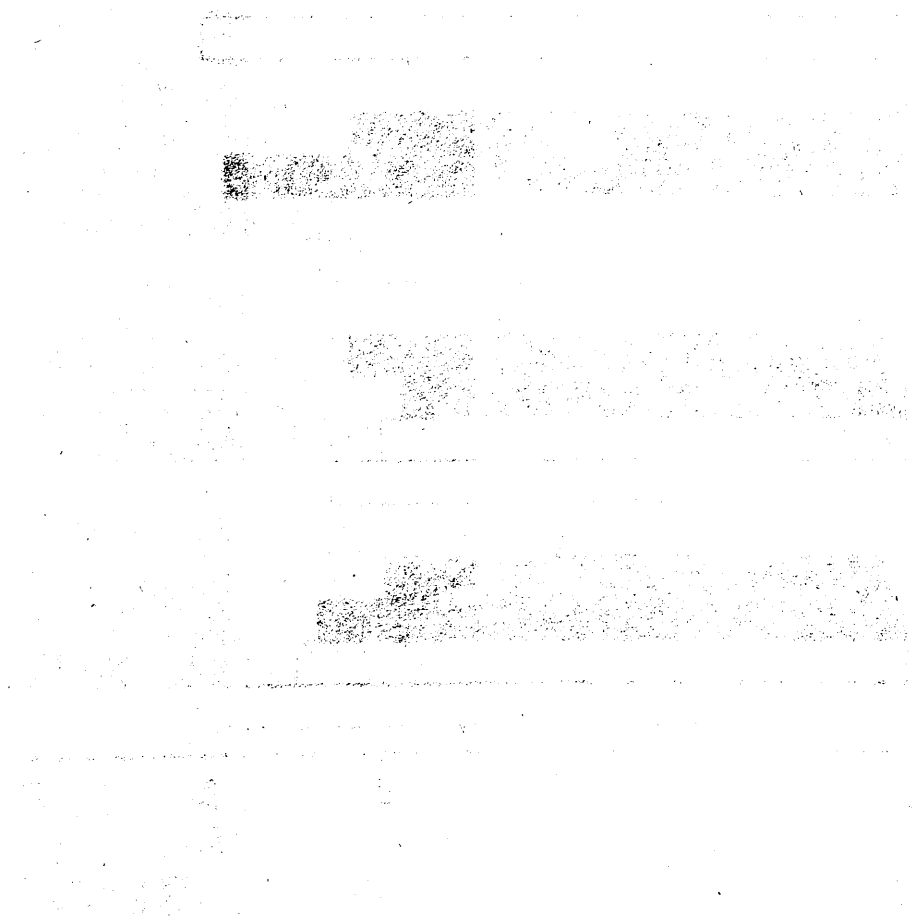

ED 50 ABZ (ug/ml)
4
3
2
1
0
0
5
10
20
40
Lambs (Dose FBZ mg/kg)
PRE TREATMENT
POST TREATMENT

Figure 2.12

The level of resistance measured by the ED50 with TBZ in lambs before and after treatment of the lambs with increasing doses of FBZ in dose titration Experiment 3.



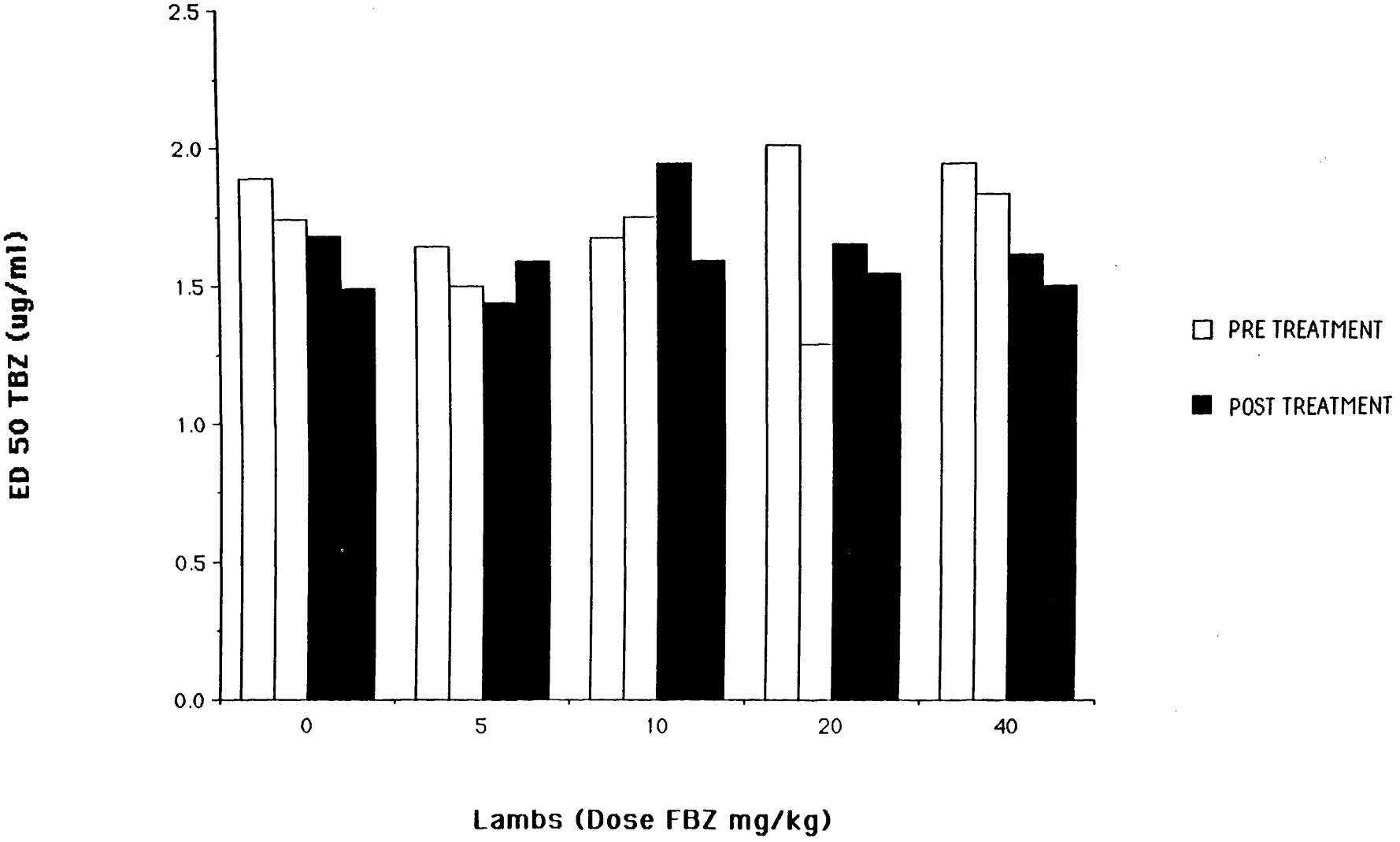
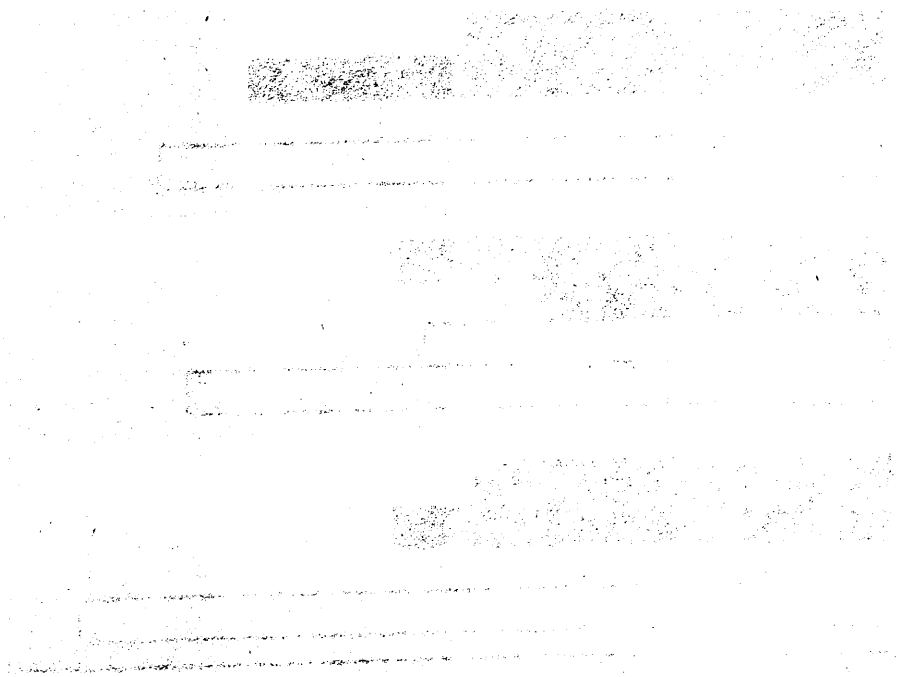

ED 50 TBZ (ug/ml)
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
20
40
Lambs (Dose FBZ mg/kg)
PRE TREATMENT
POST TREATMENT

Figure 2.13

The level of resistance measured by the ED50 with ABZ in lambs before and after treatment of the lambs with increasing doses of FBZ in dose titration Experiment 3 .



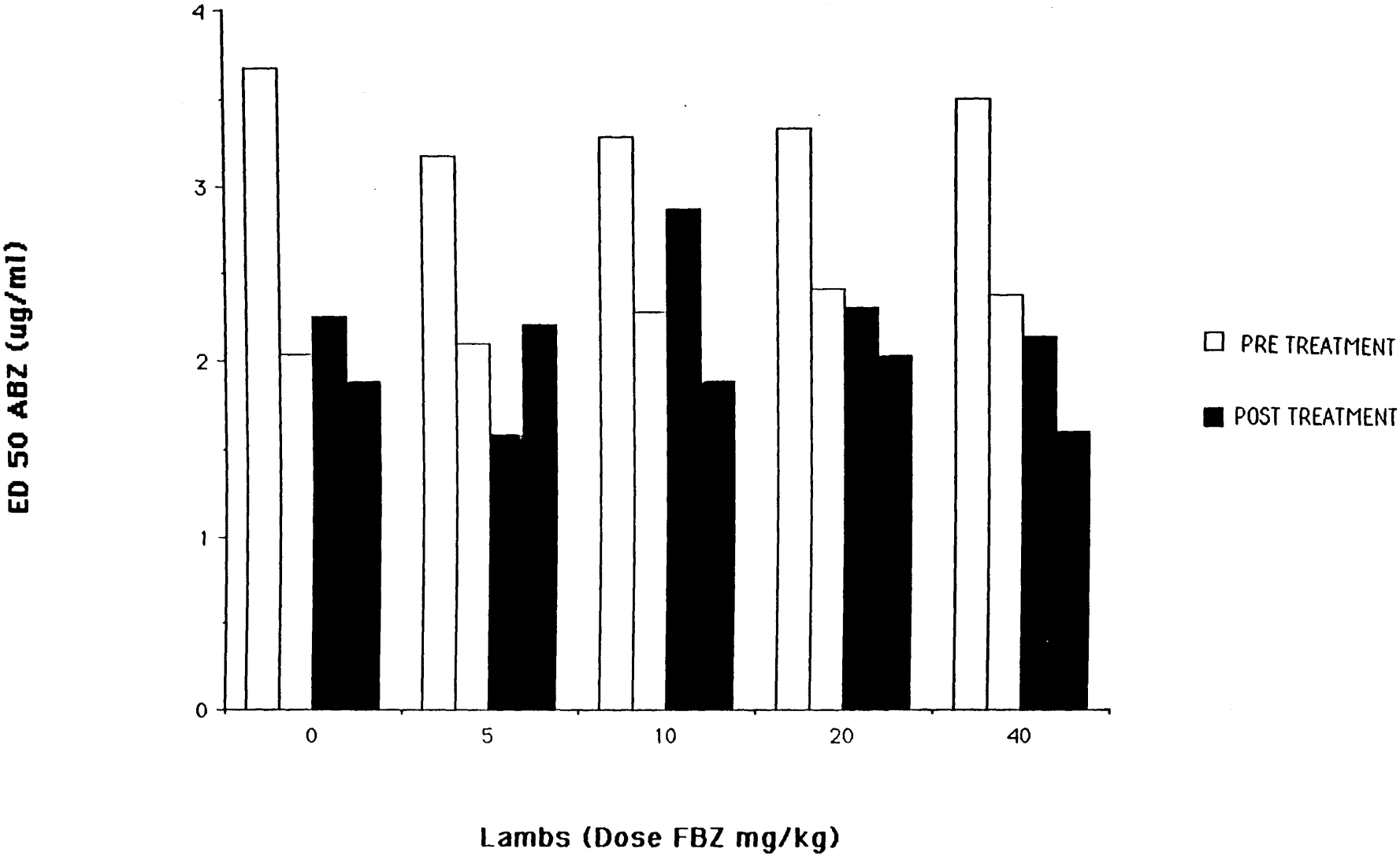
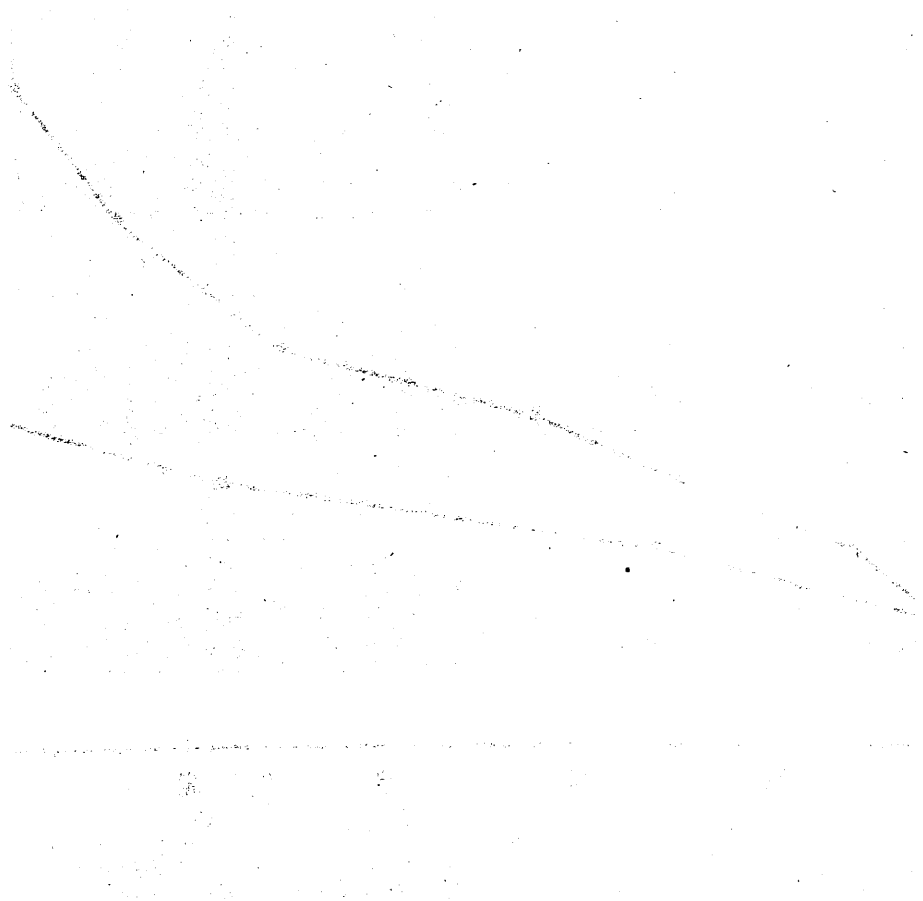

ED 50 ABZ (ug/ml)
4
3
2
1
0
0
5
10
20
40
Lambs (Dose FBZ mg/kg)
PRE TREATMENT
POST TREATMENT

Figure 2.14

Dose response curves plotting the mean percentage failing to develop of eggs collected from faeces of 5 lambs in dose titration Experiment 2, prior to treatment with FBZ and exposed to increasing concentrations of ABZ or TBZ in egg hatch assays on 3 occasions.



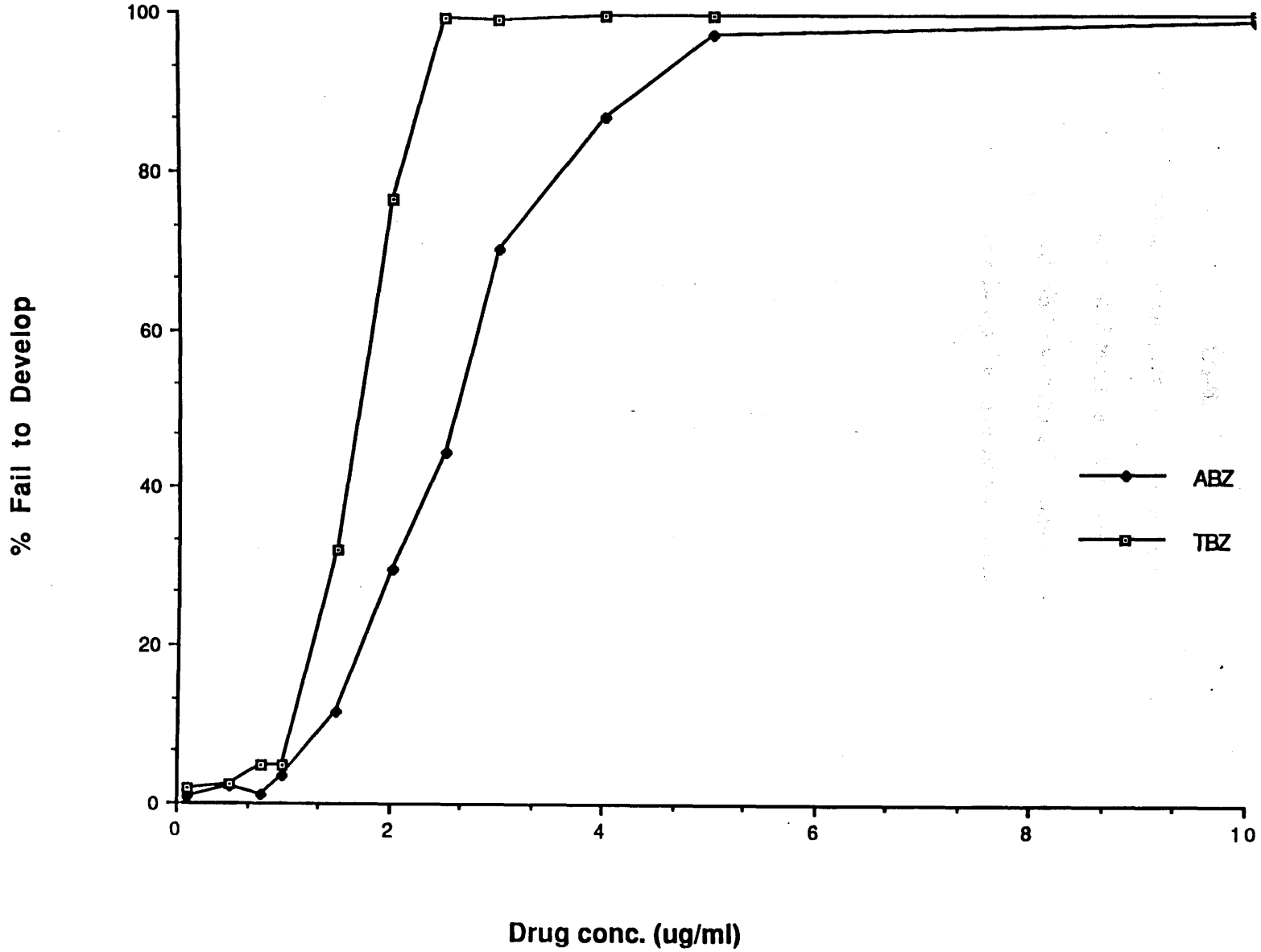
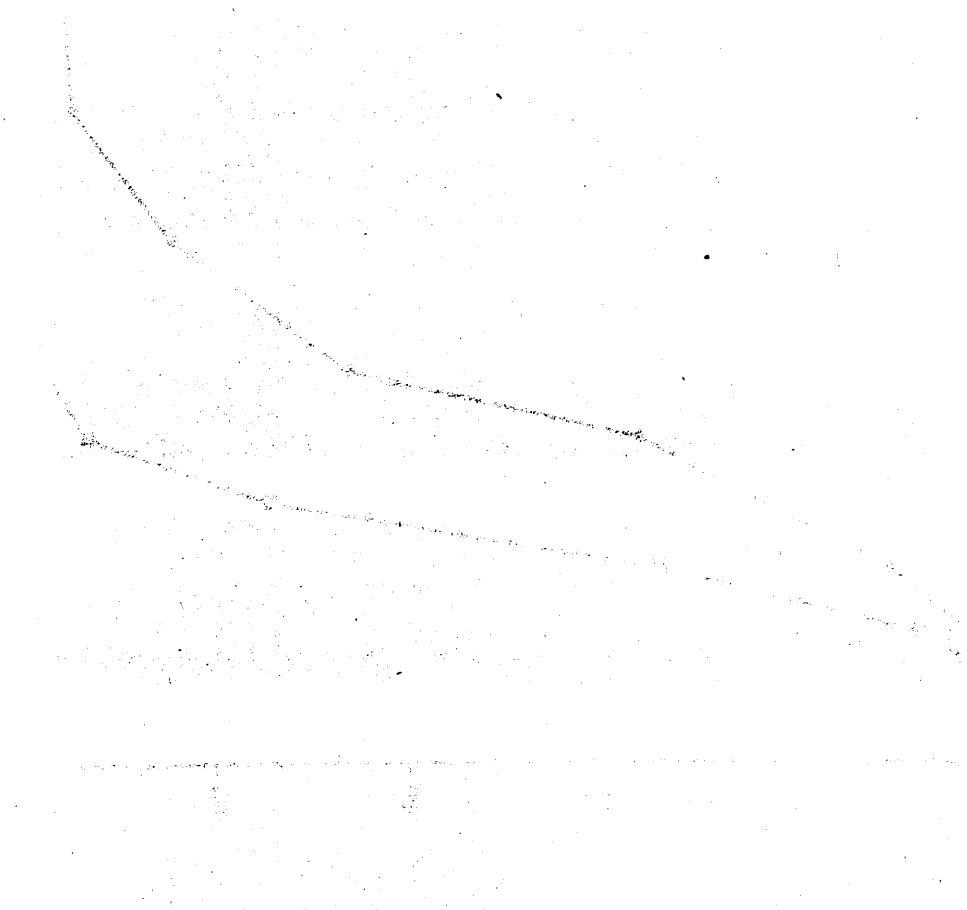
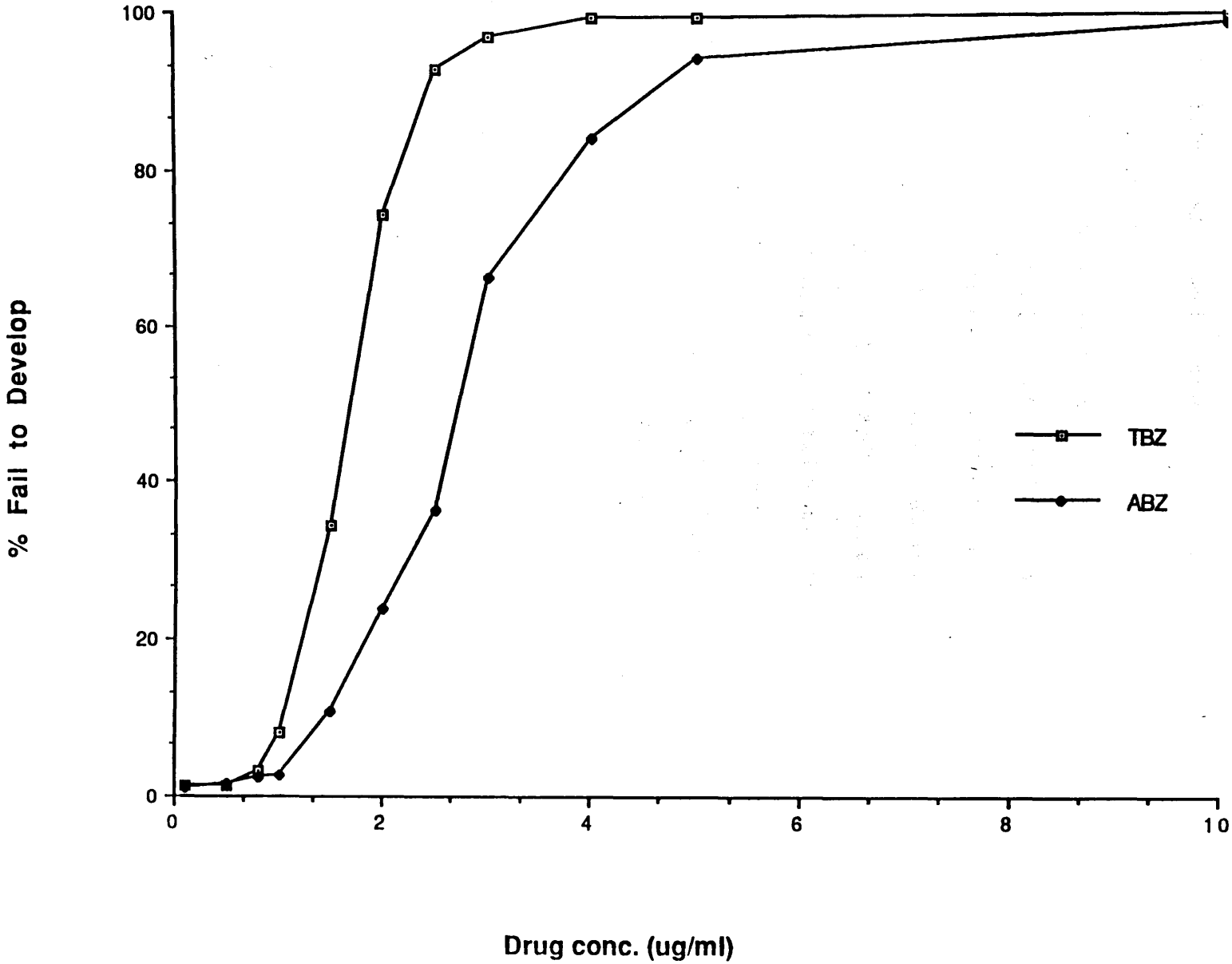

% Fail to Develop
100
80
60
40
20
0
0
2
4
6
8
10
Drug conc. (ug/ml)
ABZ
TBZ

Figure 2.15

Dose response curves plotting the mean percentage failing to develop of eggs collected from faeces of 5 male lambs in dose titration Experiment 3, prior to treatment with FBZ and exposed to increasing concentrations of ABZ or TBZ in egg hatch assays on 2 occasions.




% Fail to Develop
100
80
60
40
20
0
0
2
4
6
8
10
TBZ
ABZ
Drug conc. (ug/ml)

each dose of FBZ. In the other two experiments one and two lambs respectively were treated at each dose level. These low numbers of animals could account for the non-linear dose response curve achieved in Experiment 3. In Experiment 2 the lambs were killed at a late stage in the infection, Day 58, and the worm burdens could have been affected by natural mortality of adult parasites.

In Experiment 3, the yield of adult parasites at slaughter was greater in the female sheep than the male sheep except for the male lamb treated with FBZ at 40mg/kg which retained a high parasite burden compared with the comparably treated female lamb. The differences may reflect variation in the infection rate within the animals used in this experiment. In Experiment 1, the LD50 to adult *O.circumcincta* for orally administered FBZ calculated from the dose titration curve is 31.5mg/kg. The dose titration curve using the mean of values from all six lambs treated at each dose of FBZ (Fig. 2.9) has a similar slope to the dose titration curve drawn using results from Experiment 1 (Fig. 2.8). The LD50 calculated using the mean dose titration curve is 25.7mg/kg FBZ which is lower than that calculated in Experiment 1. The efficacy of orally administered FBZ as measured by the percentage reduction in adult worm burden was greater, but not significantly, in Experiments 2 and 3 than in Experiment 1 at dose rates of 5mg/kg and 10mg/kg. This may reflect individual variation and emphasises the disadvantages of this method for measuring anthelmintic resistance accurately in that large numbers of animals are required to overcome biological variation (Powers *et al.*, 1982). For the controlled anthelmintic efficiency test it has been estimated that 16 animals per group are required to give accurate measurement of a two fold difference in resistance status because of between animal variation in worm burdens (Martin *et al.*, 1982).

Hall *et al.*, (1978b) performed a dose titration study using resistant strains of *H.contortus* and *T.colubriformis* with 3 dose rates and 3 lambs in each group similar to Experiment 1 described above, and obtained linear log dose, log response curves. These

were used to calculate an effective dose ED95 (95% reduction in worm counts) for the compound used in their experiment. However, from Fig. 2.9 it is impossible to obtain a figure for ED95 for the HFRO strain of *O.circumcincta* because of the lower efficacy of the FBZ at the dose rates used and the high level of resistance of this strain of *O.circumcincta* Another trial in the United Kingdom to establish the resistance to orally administered FBZ of a benzimidazole resistant strain of *O.circumcincta* (Britt and Oakley, 1986) showed FBZ at dose rates of 5mg/kg and 10mg/kg to have efficacies of 74.2% and 91.3% respectively. These levels of efficacy are much higher than values obtained in the current experiments (Table 2.5).

In Experiments 2 and 3, a comparison between the egg hatch assay and the controlled anthelmintic efficiency test was investigated. In each egg hatch assay a dose titration curve is constructed for the effect of varying concentrations of TBZ or ABZ on the eggs produced by the benzimidazole resistant worms. The ED50 for the egg hatch assays is a measure of the level of resistance of the eggs produced by the population. Prior to treatment with FBZ the population of parasites in each lamb should be the same. In Figs. 2.14 and 2.15 the dose response curves for egg hatch assays carried out prior to treatment in Experiments 2 and 3 show the similarity of the populations. There is no significant difference between the slopes of the curves and the ED50s calculated for each population prior to treatment. Comparison of these dose response curves in Figs. 2.14 and 2.15 with the mean dose titration curve (Fig. 2.9) confirms that measurement of a high ED50 in the *in vitro* egg hatch assay correlates with a high LD50 in the *in vivo* controlled anthelmintic efficiency test. Good correlations among results of *in vivo*faecal egg count reduction tests , results of *in vitro* egg hatch assays and results from controlled anthelmintic efficiency tests have been found previously in Australian studies (Martin *et al*, 1982; Presidente, 1985)

These results indicate that the *in vitro* egg hatch assay gives a good indication of levels of resistance in the field. A direct comparison of ED50 in the egg hatch assay and LD50

from the *in vivo* dose titration trial would suggest that an ED50 for TBZ of 1.61-1.65 ug/ml and ABZ of 2.61-2.87 ug/ml for the egg hatch assay correlates with a dose rate of 25.7-31.5 mg/kg FBZ. This type of comparison will vary between laboratories because of the different methods used in the egg hatch assay and also which member of the group of benzimidazole anthelmintics is used in the field. In this case FBZ was used in the dose titration study but a more appropriate comparison with the egg hatch assay results could have been made if TBZ had been used.

Results of egg hatch assays show consistency amongst animals. For both Experiments 2 and 3 prior to treatment with FBZ the populations of parasites in the lambs should be similar and this is reflected in the consistent results from egg hatch assays. In previous work using the egg hatch assay technique there was day to day variation in results. However in the dose titration studies egg hatch assay results were consistent from day to day, particularly in assays carried out using TBZ. In assays using ABZ there was some day to day variability in results (Figs. 2.11 and 2.13) but assays carried out on the same day gave similar results.

After treatment of the lambs by oral administration of FBZ at different dose rates the population of the adult worms in the abomasa of the lambs was changed because there was a reduction in the worm burdens. The level of resistance was measured in egg hatch assays using eggs collected after FBZ treatment to investigate if there was any difference in the resistance after selection with anthelmintics at increasing dose rates. In both Experiments 2 and 3 there was no significant difference ($p>0.01$) between egg hatch assay results in each lamb before and after treatment with FBZ (Tables 2.6-2.9 and Figs. 2.10-2.13). The use of FBZ orally at high dose rates of 20mg/kg and 40mg/kg in the lambs reduced worm burdens compared with the control animals and was therefore selectively killing less viable parasites leaving more resistant parasites in the abomasum. From the results of egg hatch assays the level of resistance measured in the eggs of these selected parasites

was not significantly different from unpressurised control animals. Waller *et al.* (1985) found that increasing the level of benzimidazole used for selection of a resistant strain of *T.colubriformis* did not increase the ED50 in the egg hatch assays carried out on the offspring. Similarly Waller *et al.* (1986) found that selection of levamisole resistant *T.colubriformis* over 12 generations resulted in an increase in the ED50 calculated from an egg hatch assay in each generation up to 8 generations, then from generations 8-12 there was a period with little alteration in the level of resistance. The HFRO benzimidazole resistant strain of *O.circumcincta* has been pressurised over several generations with no significant increase in resistance to benzimidazoles (see previously). The parasite population reaches a high level of resistance and further pressurisation cannot increase this level. The decrease in the adult worm burden in lambs treated with high doses of FBZ suggests that less resistant adults were being removed. However, the more resistant parasites which remain do not seem to confer any increased resistance to their offspring. These more resistant parasites may have a phenotypic advantage but be genetically similar.

Alternatively, differences in resistance of the adult parasites to the effects of orally administered benzimidazoles may not be reflected as a difference in hatchability of parasite eggs in the presence of benzimidazoles. Le Jambre *et al.*, (1979) noted that it is possible that resistance in eggs and adults of *H.contortus* are not controlled by the exact same series of genes. The ability of eggs to hatch in the presence of TBZ has been shown to be related to resistance in the adult worms. However, it has been suggested that due to the possible polygenic nature of the genetic control of benzimidazole resistance there could be differences between eggs and adults (Le Jambre *et al.*, 1979). In Australia studies on the genetic inheritance of resistance (Le Jambre *et al.*, 1979) have suggested that it is inherited as a polygenic trait in *H. contortus* whereas resistance to levamisole in *T.colubriformis* is caused by a single gene (Waller *et al.*, 1986). In the case of the HFRO

strain of *O.circumcincta* continuous selection of the parasites could have produced a homogeneous population. The steepness of the slope of the dose response curves from the egg hatch assays is suggestive of a homogeneous population of resistant parasites. If this was the case pressurisation with increasing doses of FBZ would not increase the level of resistance in the parasite population. This, however, does not explain the differences in resistance of adult parasites to high doses of FBZ given orally to lambs. It appears that continuous pressurisation of the HFRO strain with benzimidazole anthelmintics has produced a population with a uniformly high level of resistance but, as with all populations, there is variation in the fitness or viability of individuals.

Transplant Experiments

The method of transferring a single male and female adult parasite of the HFRO strain of *O.circumcincta* into the abomasum of lambs was investigated to establish if adult parasites survived, mated and could produce fertile eggs from a known parentage. This method could then be used to investigate the genetic transfer of characteristics conferring resistance.

Transplantation of Adult *O.circumcincta* into the Abomasum of Lambs

Materials and Method

Materials.

Lambs - Parasite naive Dorset cross or Suffolk cross bred lambs, reared indoors.

Method.

1. Lambs were treated with Ivermectin (Oramec - M.S.D.) at the normal dose rate 200ug/ml administered orally 2-3 weeks prior to commencement of the experiment.

2. Lambs were starved for 24h prior to surgery

3. They were anaesthetised using pentobarbitone sodium (Sagatal - RMB Animal Health Ltd.) and anaesthesia was maintained using incremental doses of the same anaesthetic.

4. Using a sterile surgical technique the abomasum was located and partially exteriorised. A small fistula was produced into the abomasum into which were placed the adult parasites. Adult parasites were obtained from donor lambs freshly slaughtered (see General Materials and Methods).

5. The abomasal fistula was closed surgically as were the muscle layers and skin.

6. The lambs were treated with penicillin (Duplocillin L.A. - Gist-Brocades Animal Health) injected intramuscularly to help prevent wound infection.

7. Leather harnesses and faecal bags were placed on the lambs 5 days after surgery to collect faeces for culture of larvae (see previously).

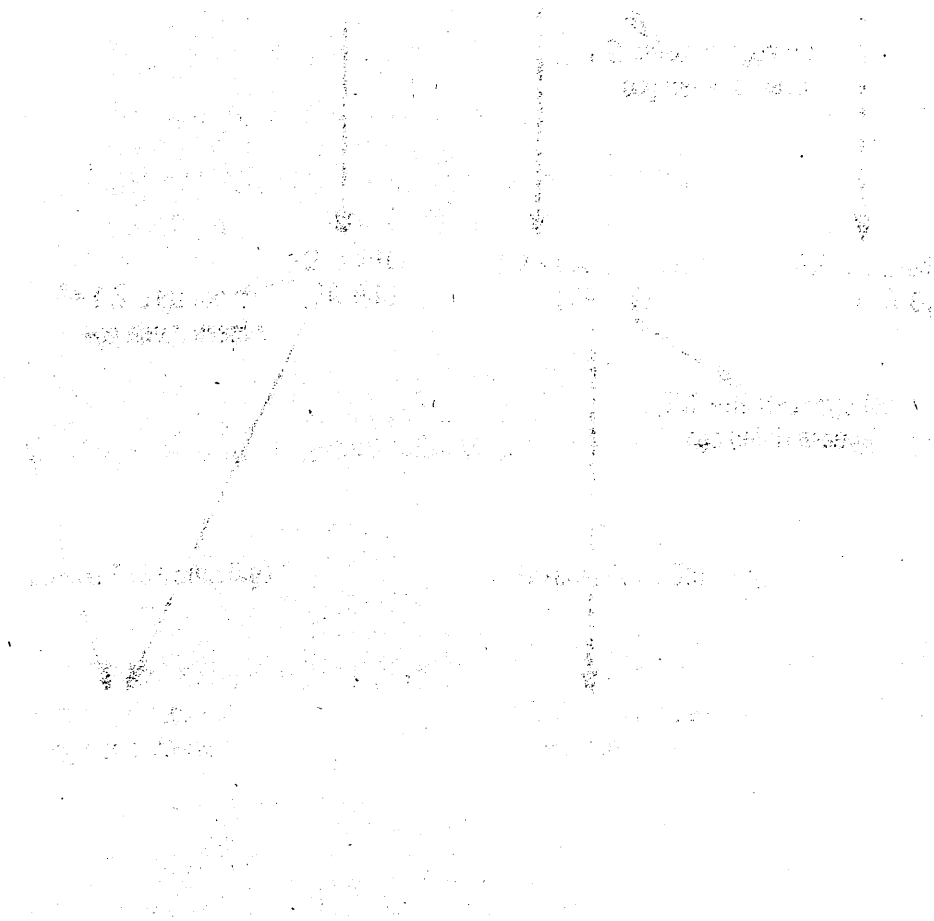
Experimental Design.

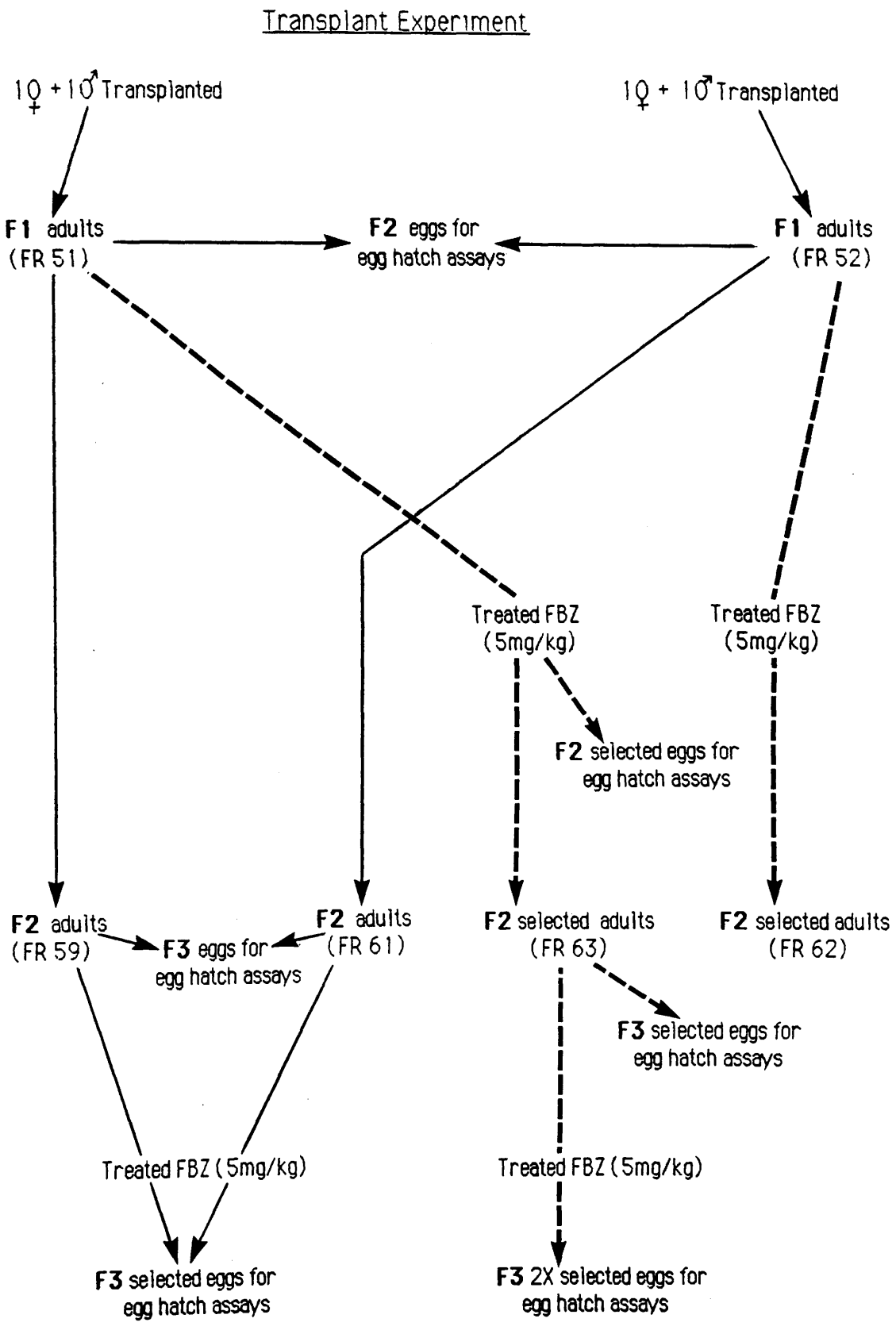
Donor lambs were infected with 10,000 infective larvae of the HFRO resistant strain of *O.circumcincta* and were killed on Days 35 and 49 post-infection respectively. Adult parasites were isolated from the abomasum of these lambs and surgically implanted into parasite free recipient lambs. Lambs 1 and 2 received adult parasites from the donor killed on Day 35 post infection and Lambs 3 and 4 adults from the lamb killed on Day 49 post infection. At the same time as the transplantation into Lambs 1 and 2 a single female parasite was transplanted into Lamb 5. Similarly Lamb 6 was implanted with a single female at the same time as transplantation of pairs of parasites into Lambs 3 and 4 . All faeces were collected from Lambs 1-6 from Day 5 post transplantation to Day 19 post transplantation and cultured to obtain parasite larvae.

Further passages of the larvae obtained from Lambs 1 and 2 were carried out and the progeny of these larvae were tested for resistance using egg hatch assays. A flow diagram of the passages and treatments of the lambs used to study the offspring of the transplanted parasites is shown in Fig. 2.16. The larvae from Lamb 1 were used to infect FR 51 and the larvae from Lamb 2 were used to infect FR 52. Both of these lambs (FR 51 and FR 52) contained adults of the F1 generation of the transplanted parents. Eggs passed in the faeces

Figure 2.16

Plan of the development of 'clones' of the HFRO strain of *O.circumcincta* after transplantation of pairs of parasites into donor lambs.




Transplant Experiment
1♀ + 1♂ Transplanted
1♀ + 1♂ Transplanted
F1 adults (FR 51)
F2 eggs for egg hatch assays
F1 adults (FR 52)
Treated FBZ (5mg/kg)
Treated FBZ (5mg/kg)
F2 selected eggs for egg hatch assays
F2 adults (FR 59)
F3 eggs for egg hatch assays
F2 adults (FR 61)
F2 selected adults (FR 63)
F2 selected adults (FR 62)
F3 selected eggs for egg hatch assays
Treated FBZ (5mg/kg)
Treated FBZ (5mg/kg)
F3 selected eggs for egg hatch assays
F3 2X selected eggs for egg hatch assays

of these lambs were used in egg hatch assays and these eggs would form the F2 generation from the transplanted parents. At the same time faeces were cultured to obtain sufficient larvae to infect lambs FR59 and FR61 with infective larvae of the F2 generation which in turn matured to F2 adults which mated producing eggs of the F3 generation. These F3 generation eggs were tested for resistance using egg hatch assays. The lambs FR59 and FR61 were treated orally with FBZ at the normal dose rate of 5mg/kg and eggs passed in the faeces after selection of the F2 adults were tested for resistance using egg hatch assays.

Lambs FR51 and FR52 which contained the F1 generation adults were treated orally with FBZ at the normal dose rate of 5mg/kg. Eggs passed in the faeces after treatment were used for egg hatch assays and were cultured to produce larvae which were used to infect lambs FR62 and FR63. These lambs FR62 and FR63 therefore contained the F2 generation of the original transplant parents which had been selected with FBZ during the F1 generation. The eggs produced by these F2 generation of adults were tested for resistance using egg hatch assays and lamb FR63 was treated orally with FBZ at the normal dose rate of 5 mg/kg and eggs produced after selection of the F2 adults were tested for resistance using egg hatch assays.

Results

The number of larvae cultured from the faeces of Lambs 1-6 are shown in Table 2.10. Lambs 1-4 were implanted with pairs of adult parasites and Lambs 5 and 6 were each implanted with a single adult female. The number of larvae cultured from faeces on the stated days is listed along with the total number of larvae collected from each lamb. Five days were allowed to elapse after implantation to ensure that eggs passed in the faeces were the result of matings between the implanted pair and were not fertilized eggs present in the female at the time of implantation (Le Jambre *et al*, 1979)

Results of egg hatch assays carried out on the F2 generation of eggs pre-treatment and post treatment with FBZ are shown in Table 2.11. Results of egg hatch assays on the F3

Table 2.10

Number of larvae collected from faeces of lambs after implantation of adult *O. circumcincta* into the abomasum

			Days after implantation									
Age* of Parasites	**Implant**	**Lamb No.**	**6**	**7**	**8**	**12**	**13**	**14**	**15**	**19**	**20**	**Total No. of Larvae**
35days	1♂+1♀	1	0	1	0	0	196	0	38	100	58	393
35days	1♂+1♀	2	50	37	6	0	65	71	48	86	62	425
49days	1♂+1♀	3	0	0	2	0	0	0	31	20	0	53
49days	1♂+1♀	4	0	0	0	0	0	11	0	3	5	19
35days	1♀	5	0	0	0	0	0	0	0	0	0	0
49days	1♀	6	0	0	0	0	0	0	0	0	0	0

*Number of days after infestation of donor lambs with L3 larvae.

Table 2.11

Results of egg hatch assays carried out on eggs of the F2 generation of *O.circumcincta* 'clones' produced by transplantation of adult parasites into lambs.

Time (Days)		ED50 (ug/ml)			
Post Infection	Post Treatment	TBZ		ABZ	
		FR51	FR52	FR51	FR52
26	-	0.51	0.37	0.61	0.28
27	-	0.74	0.43	0.74	0.70
32	-	0.95	0.43	0.49	0.31
36	-	0.96	0.79	1.00	0.75
54	14	>2.0	-	>2.0	-
60	20	1.31	-	1.23	-

Table 2.12

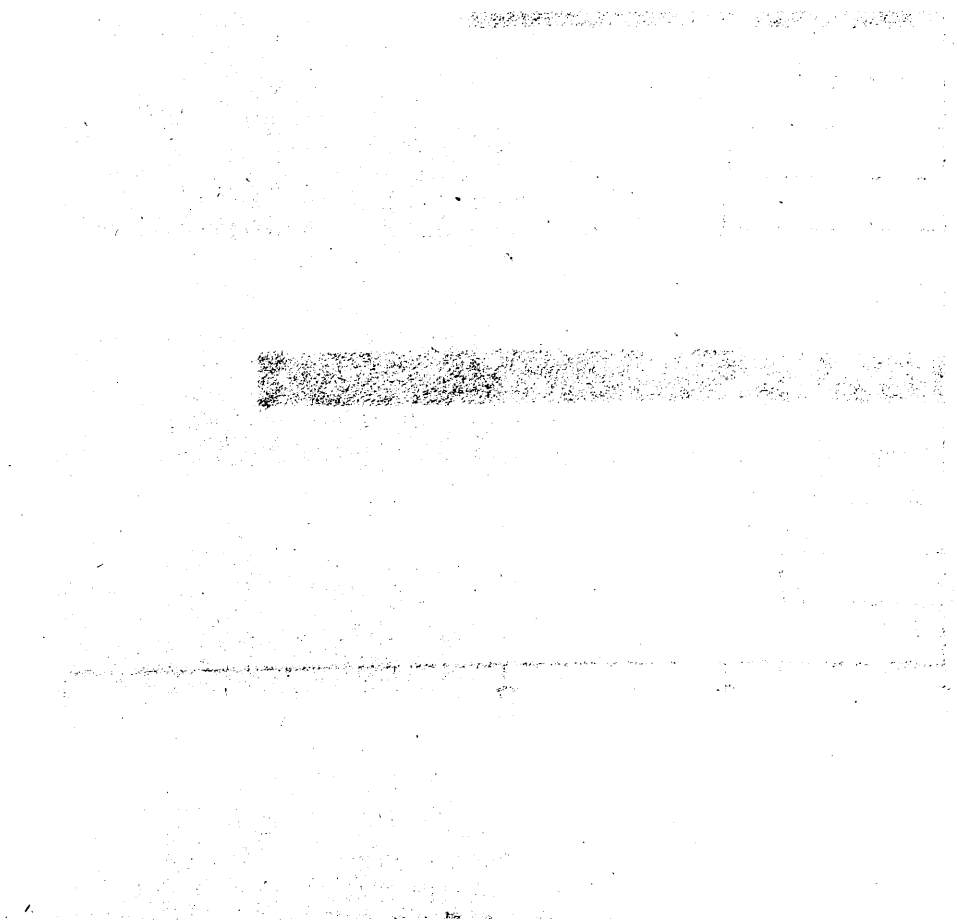
Results of egg hatch assays carried out on eggs of the F3 generation of *O.circumcincta* 'clones' produced by transplantation of adult parasites into lambs.

Time (Days)		ED50 (ug/ml)					
Post Infection	Post Treatment	TBZ			ABZ		
		FR59	FR61	FR63*	FR59	FR61	FR63*
22	-	0.37	0.50	0.99	0.74	0.50	1.95
25	-	0.36	0.50	1.43	1.05	1.33	2.36
32	-	<0.2	0.25	0.80	0.19	0.46	1.22
35	-	0.29	0.33	1.10	0.53	0.85	2.01
54	7	1.56	1.08	1.43	1.50	-	1.56

*Lamb infected with larvae cultured from faeces collected after treatment of the F1 generation (in lamb FR51) with FBZ administered orally at 5mg/kg.

Figure 2.17

Level of resistance as measured by the ED50 with TBZ for eggs collected from the faeces of Lambs FR59, FR61 and FR63 infected with 'clones' of the HFRO strain of *O.circumcincta* before and after treatment of the lambs with FBZ.



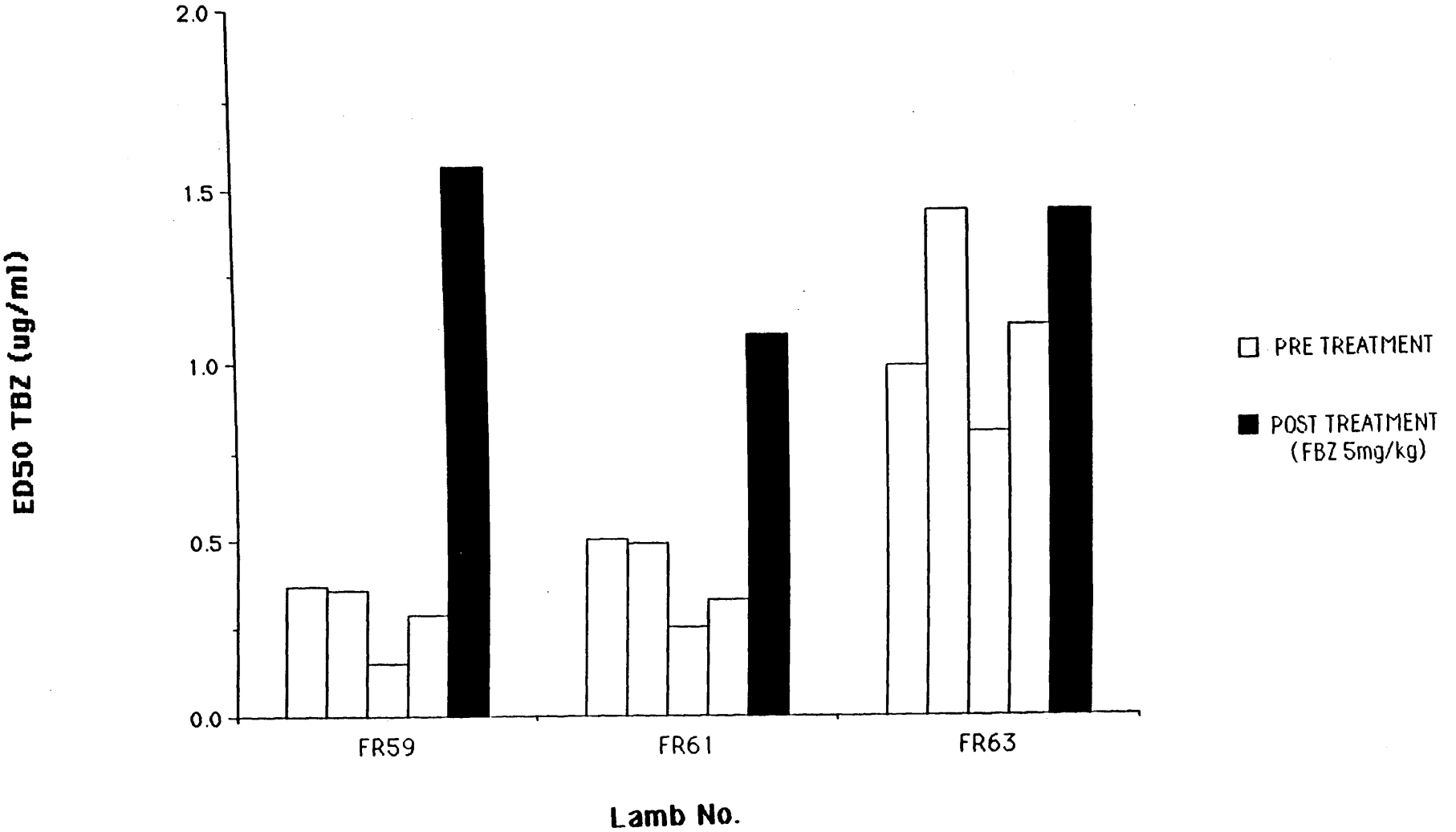

ED50 TBZ (ug/ml)
2.0
1.5
1.0
0.5
0.0
FR59
FR61
FR63
Lamb No.
PRE TREATMENT
POST TREATMENT
(FBZ 5mg/kg)

Figure 2.18

Dose response curves for eggs collected from Lamb FR59 plotting percentage of eggs failing to develop against concentration of TBZ. The lamb was treated with FBZ on Day 47 post infection.

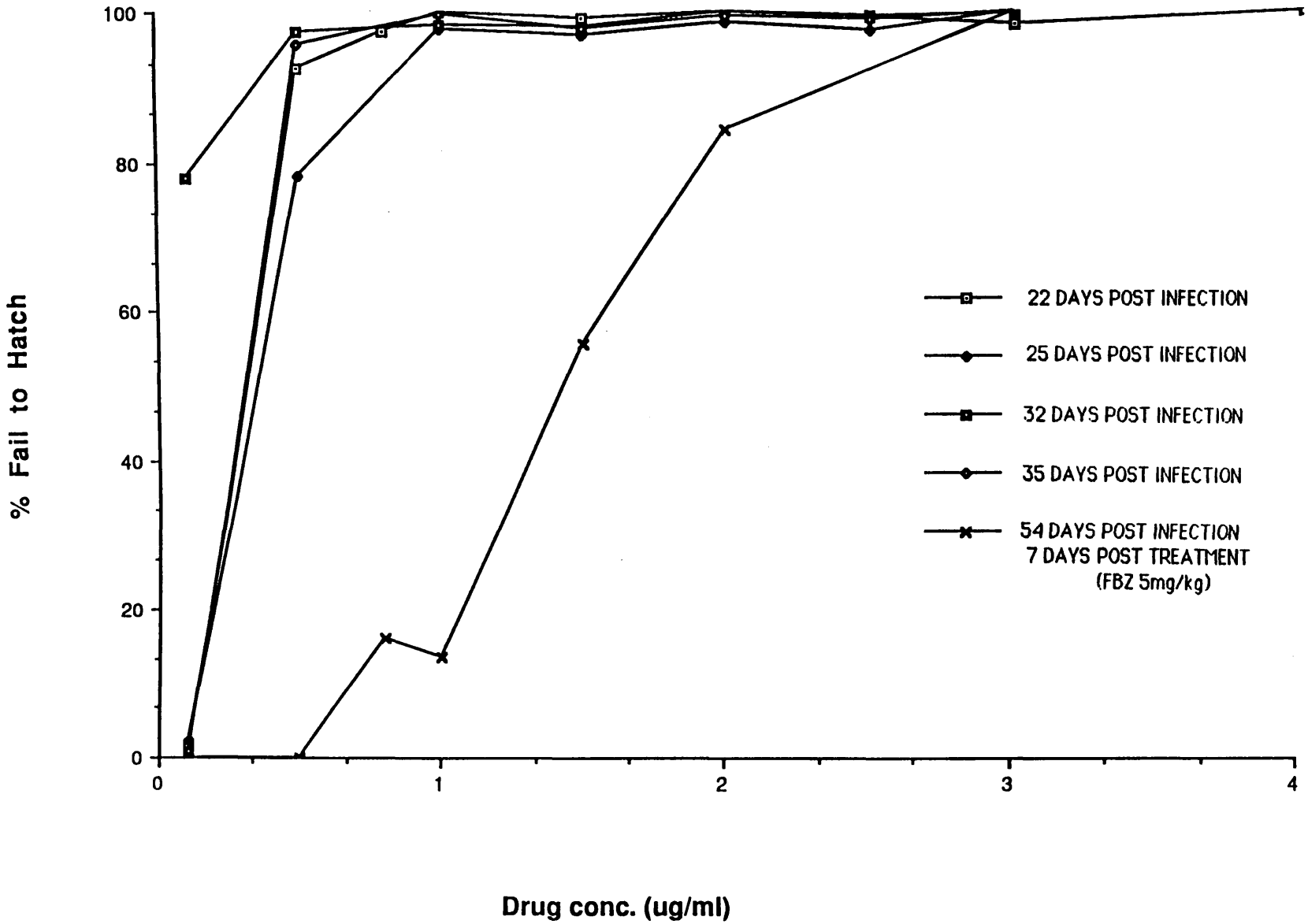
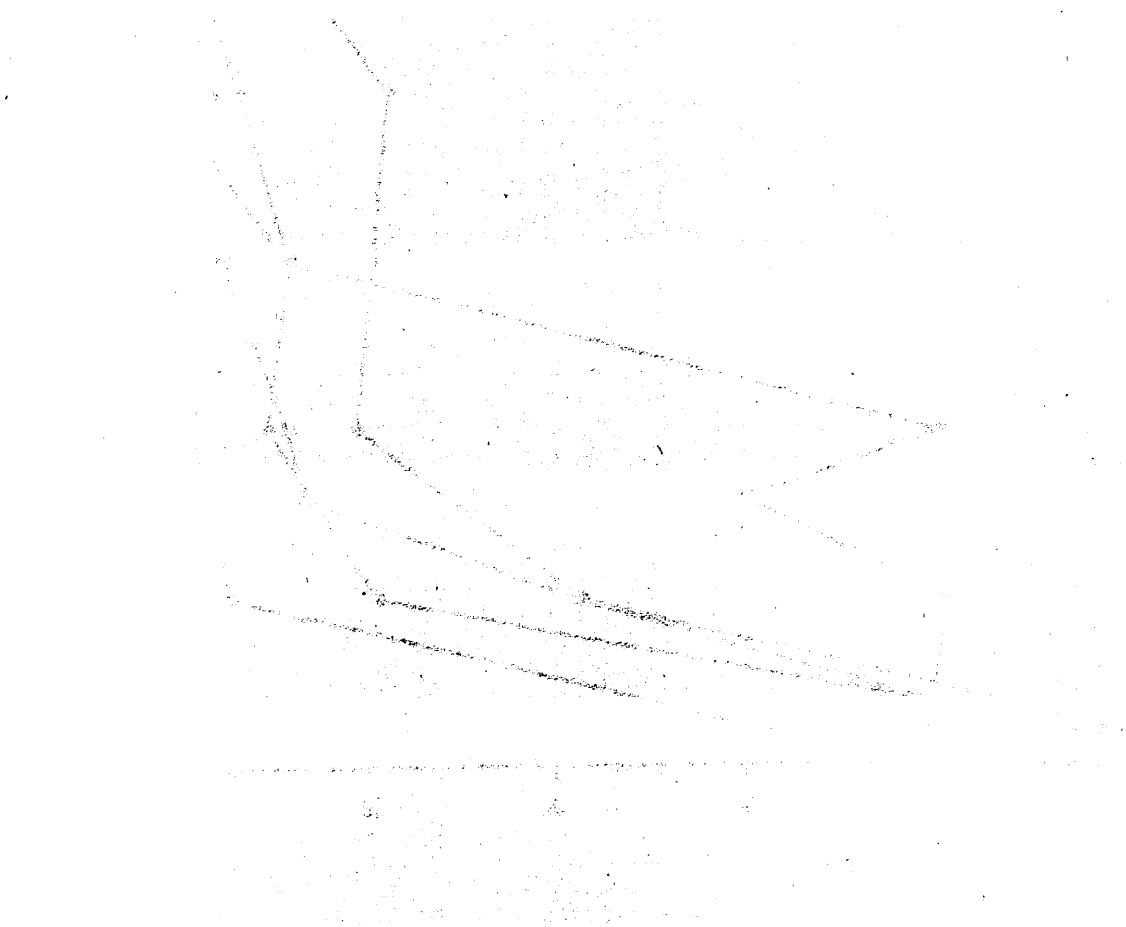

% Fail to Hatch
100
80
60
40
20
0
0
1
2
3
4
Drug conc. (ug/ml)
22 DAYS POST INFECTION
25 DAYS POST INFECTION
32 DAYS POST INFECTION
35 DAYS POST INFECTION
54 DAYS POST INFECTION
7 DAYS POST TREATMENT
(FBZ 5mg/kg)

Figure 2.19

Dose response curves for eggs collected from Lamb FR61 plotting percentage of eggs failing to develop against concentration of TBZ. The lamb was treated with FBZ on Day 47 post infection.



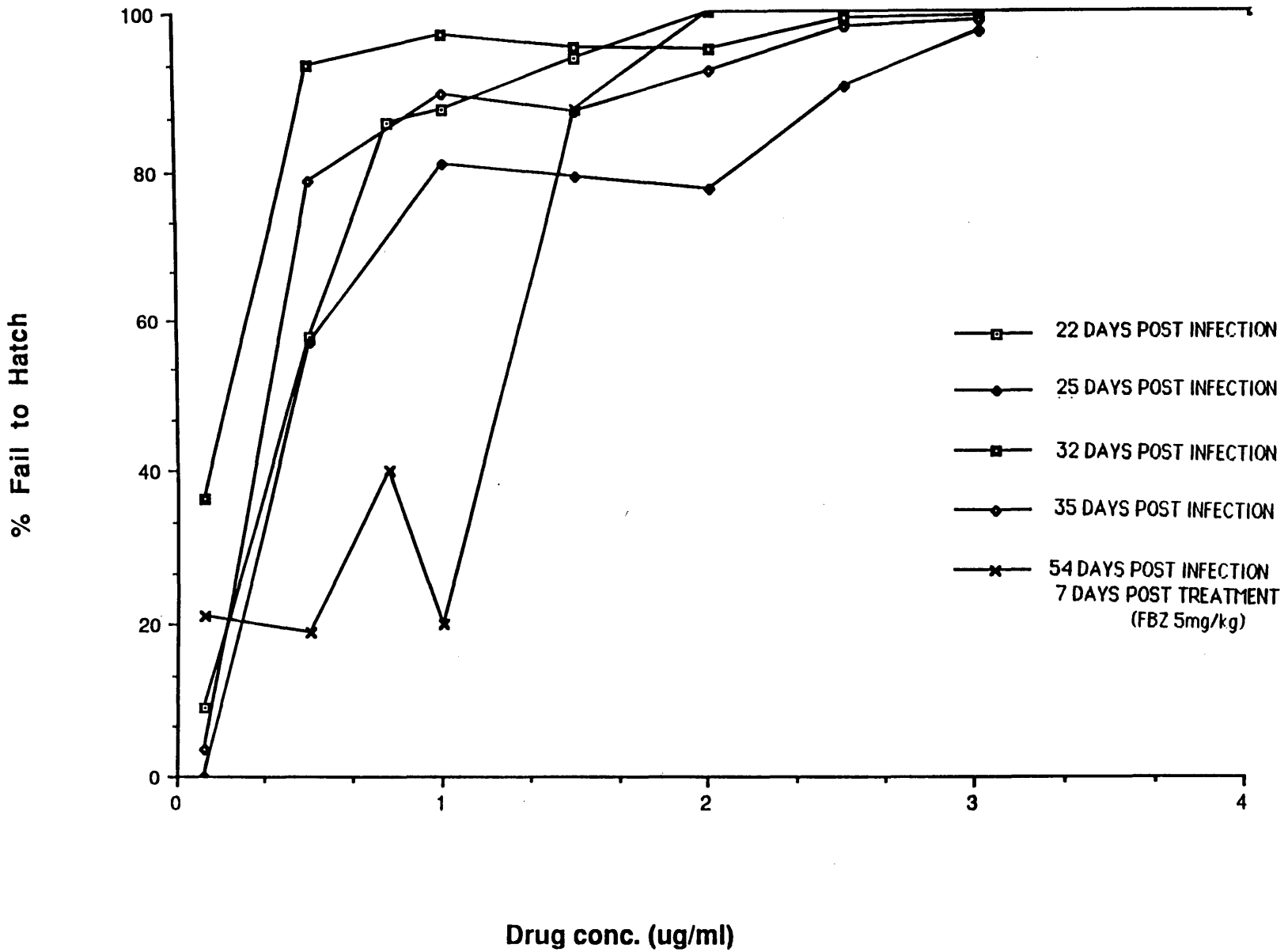
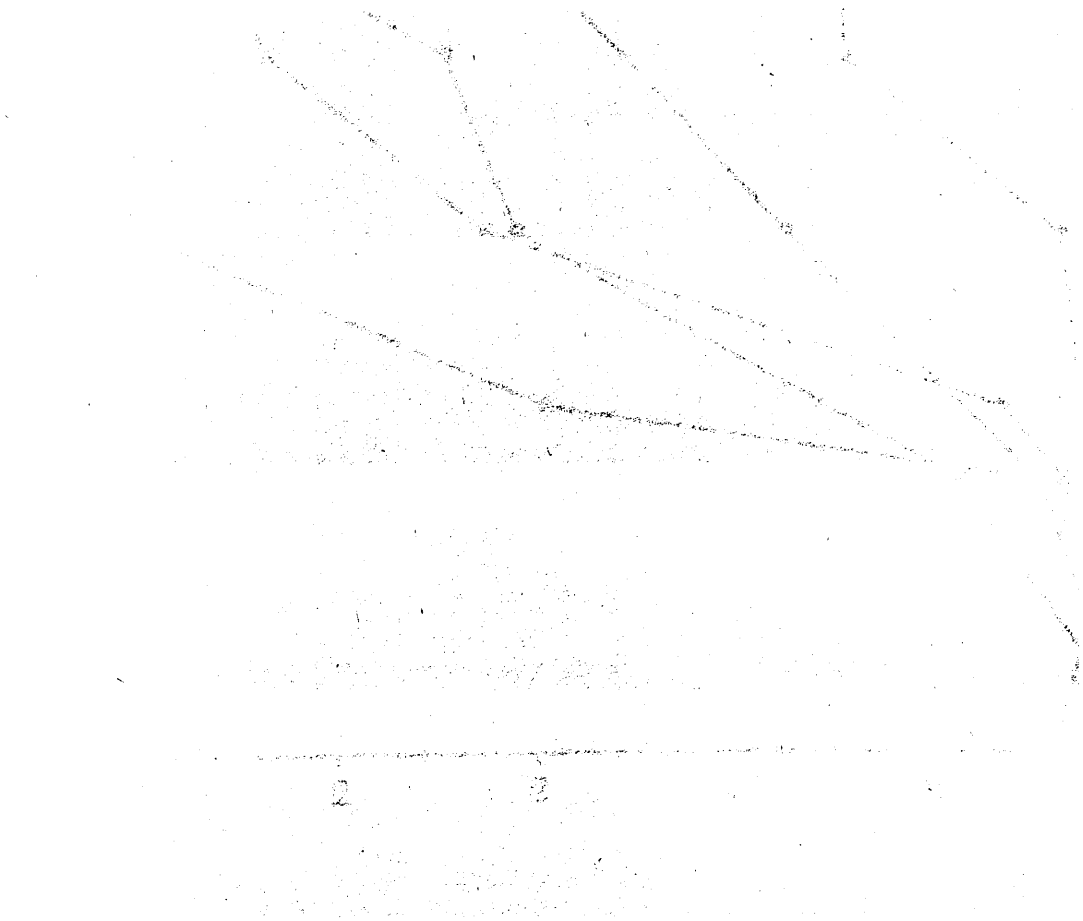
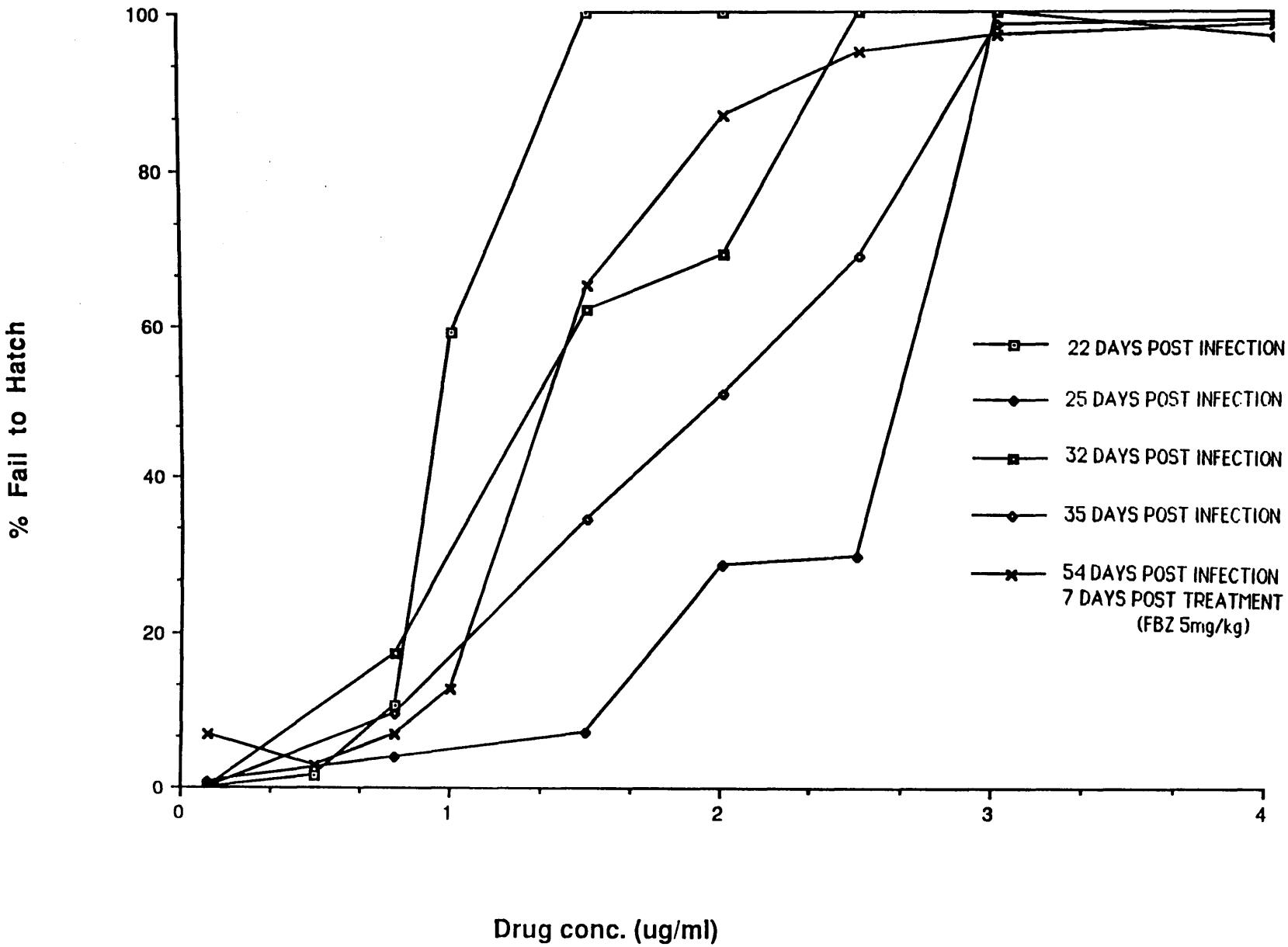

% Fail to Hatch
100
80
60
40
20
0
0
1
2
3
4
Drug conc. (ug/ml)
22 DAYS POST INFECTION
25 DAYS POST INFECTION
32 DAYS POST INFECTION
35 DAYS POST INFECTION
54 DAYS POST INFECTION
7 DAYS POST TREATMENT
(FBZ 5mg/kg)

Figure 2.20

Dose response curves for eggs collected from Lamb FR63 plotting percentage of eggs failing to develop against concentration of TBZ. The lamb was treated with FBZ on Day 47 post infection.




% Fail to Hatch
100
80
60
40
20
0
0
1
2
3
4
Drug conc. (ug/ml)
22 DAYS POST INFECTION
25 DAYS POST INFECTION
32 DAYS POST INFECTION
35 DAYS POST INFECTION
54 DAYS POST INFECTION
7 DAYS POST TREATMENT
(FBZ 5mg/kg)

generation eggs produced from Lambs FR59, FR61 and FR63 are shown in Table 2.12 and Fig. 2.17 before and after treatment of each of the lambs with FBZ. Graphs of percentage of eggs which developed against concentration of TBZ for egg hatch assays on the F3 generation eggs produced from Lambs FR59, FR61 and FR63 before and after treatment of each of the lambs with FBZ are shown in Figs. 2.18-2.20 respectively.

Discussion

In all cases where a pair of adult parasites was implanted into the abomasum of lambs fertilized eggs were passed in the faeces. Faecal egg counts using the McMaster technique carried out during the period of faecal collection for culture of larvae from Lambs 1-4 were negative on all occasions. However, from Lambs 1 and 2, 393 and 425 larvae respectively were cultured and from Lambs 3 and 4 , 53 and 19 larvae were cultured. The single female worms implanted into Lambs 5 and 6 did not produce any fertilized eggs. These single implants were used to show that mating between the male and female parasites was necessary in order to obtain fertilized eggs. In Lambs 1-4 the single male and female parasites may have had some mechanism to allow them to identify each other and come into contact within the large area of the abomasum.

The donor lamb used to provide adult parasites for implantation into Lambs 1 and 2 was killed earlier in the course of infection, Day 35 post infection, than the lamb used as a donor for adults implanted into Lambs 3 and 4 and this might explain the lower fecundity of the females in the latter lambs. The egg output by the female worms in Lambs 1 and 2 was high and may reflect increased fecundity in the absence of other parasites (Michel, 1967). The high level of larvae obtained from faecal culture of faeces from Lambs 1 and 2 allowed further passages of these single clones of parasites to be attempted (Fig. 2.16). From Lambs 3 and 4 the yields of larvae were poor and further passages were not possible.

Lambs FR51 and FR52 harboured adults of the F1 generation and faecal egg counts from

these lambs varied from day to day but were >50 e.p.g. on all occasions tested. Egg hatch assay results on the progeny of matings between adults of the F1 generation (Table 2.11) gave low ED50s with TBZ and ABZ for both lambs. These eggs were of the F2 generation. ED50s on Day 26 post infection were 0.51ug/ml TBZ and 0.61 ug/ml ABZ for FR51 and 0.67ug/mlTBZ and 0.28ug/ml ABZ which were much lower than the expected ED50 for the HFRO resistant strain of *O.circumcincta* calculated during routine passages at approximately 1.6 ug/ml TBZ and 2.5 ug/ml ABZ. In all assays the ED50s calculated for eggs passed in the faeces of FR52 were consistently lower than ED50s for eggs produced by FR51. Both of the original transplants were carried out using adults from the same population of the HFRO strain of *O.circumcincta*. Differences in the ED50 for eggs of the F2 generation may be due to variation in the genetic make-up of the two 'clones' of parasites which had been established from the original transplants. However, the differences in ED50s were small and only a single animal was infected with each 'clone' of *O.circumcincta* and therefore conclusions on minor variations in the resistance status of the offspring could be misleading in that no account could be taken of possible host influences on the parasite population.

In previous work with egg hatch assays (see Routine Passages and Dose Titration Trial) the ED50 for ABZ was higher than for TBZ for the HFRO strain of *O.circumcincta* Results of assays on the F2 generation from transplant experiments show similar ED50s for TBZ and ABZ. This could indicate variation in the level of side-resistance to different benzimidazoles or possibly different sensitivities of the egg hatch assays with individual benzimidazoles.

The disappointingly low level of ED50 measured for the F2 generation suggests that there was loss of resistance by the parasites. There could be several explanations for this including selection of less resistant members of the population for the initial transplants, loss of resistance brought about by the severe pressure on the original transplanted

parents to survive and reproduce or contamination of the recipient sheep with susceptible parasites which could mate with members of the 'clone'.

To guard against the contamination of lambs with *O.circumcincta* at the time of transplantation the lambs, which had been reared indoors, were treated with ivermectin. It was difficult to ensure that animals were totally parasite free. This would require animals to be isolated in a parasite free environment and fed material with no risk of parasite contamination. To prove that the animals were parasite free would require culture of faeces for several days to check that no larvae were obtained and during the period required for larval culture from faeces immature parasites could mature to become egg laying or the animal could have become infected with larvae from the environment. In the transplant experiment on both occasions a lamb was given a single female parasite, Lambs 5 and 6. No larvae were cultured from the faeces of these lambs (Table 2.10). This would suggest that the animals were parasite free at the start of the experiment. Contamination of the 'clones' could have occurred at the next generation , Lambs FR51 and FR52 , however, every precaution possible was taken to try to use lambs reared indoors and animals had negative faecal egg counts at the time of inoculation with infective larvae cultured from the faeces of the transplanted sheep.

Alternatively the individuals selected for the transplants may have been less resistant members of the population. Within the population of HFRO benzimidazole resistant *O.circumcincta* there will be differences in the susceptibility of individual parasites to the effects of the anthelmintic. A small proportion of the population may be fully susceptible to the benzimidazoles and have avoided removal by selection with anthelmintics. Also, members of the population showing the characteristics of resistance, which could be transferred genetically , may be heterozygous for these characteristics and therefore a proportion of the following generation could revert to susceptibility. When selecting individuals for transplantation they were picked randomly from the population

and therefore the 'clones' produced could have characteristics which vary from the mean characteristics exhibited by the population taken as a whole. However, it seems unlikely that both 'clones' which were established had a low level of resistance suggesting that one or other of the parents had a low level of resistance to benzimidazoles.

The mechanism of inheritance of resistance characteristics in *O.circumcincta* is unknown. Herlich *et al*, (1981) and Le Jambre *et al*,(1979) have suggested that the inheritance of benzimidazole resistance in *H. contortus* is not sex linked and resistance is controlled by more than one gene. Herlich *et al*.,(1981) found that in crosses between resistant and susceptible strains of *H.contortus* the F1 generation was similar to the susceptible strain as regards benzimidazole resistance. These authors suggested that resistance was therefore a recessive characteristic. Le Jambre *et al*, (1979) found similar results but these authors noted that offspring of crosses with the resistant component from the maternal side showed increased resistance on egg hatch assays. Martin *et al*, (in press) also noted a maternal influence on the resistance level in crosses with benzimidazole resistant and susceptible strains of *T.colubriformis*. They suggested that benzimidazole resistance in *T.colubriformis* was inherited as a semi-dominant trait with some increased maternal effect and overall resistance was controlled as a polygenic trait. The maternal influence on resistance noted in eggs produced by crosses of resistant and susceptible parasites could be due to the structure of the egg which is produced in the female parasite and therefore there would be a greater maternal effect on the egg (Le Jambre *et al*, 1979). However Martin *et al*., (in press) found the increased level of resistance in crosses where the resistance characteristics were of maternal origin rather than paternal origin to persist in the later parasitic stages of the nematode although differences were less than those detected in eggs.

The results of the transplant experiments with *O.circumcincta* suggested that there was a fall in the level of resistance in the F1 and F2 generations. If one resistant parasite and

one susceptible parasite were selected at the time of implantation then the offspring would have reduced resistance unless resistance to benzimidazoles in *O.circumcincta* was inherited as a dominant trait. If resistance was recessive the offspring, F1 generation, would be fully susceptible to the effects of benzimidazoles and 3/4 of the F2 generation would be fully susceptible to the effects of benzimidazoles. Martin *et al* (in press) found that with a population of *T.colubriformis* where 25% of the parasites were resistant to benzimidazoles it was impossible to detect any alteration in resistance from a fully susceptible population in controlled anthelmintic efficiency trials. Therefore with a low percentage of the *O.circumcincta* population with resistance characteristics it would be difficult to detect the presence of resistant parasites if the characteristics of the whole population were examined. This was not the case in the F2 generation of the transplanted *O.circumcincta* 'clones' where there was a low but significant level of resistance measured in the egg hatch assays. If resistance was a polygenic trait then the combination of possible crosses which would produce an F2 generation with the resistance characteristics found in eggs from FR51 and FR52 would be difficult to predict. The fact that the resistance status of the maternal component of the cross may have a greater influence on the resistance characteristics of the eggs in *O.circumcincta* as occurs in *H.contortus* (Le Jambre *et al*, 1979) and *T. colubriformis* (Martin *et al*, in press) produced further complications. It therefore was impossible to estimate the resistance status of the transplanted parents from the egg hatch assay results of the F2 generation because the genetic mechanisms involved in inheritance of resistance in *O.circumcincta* are poorly understood. The slight differences between the resistance level in the offspring of the two transplants may be suggestive of polygenic resistance with transfer of different characteristics in each 'clone'. Both transplants involved the transfer of resistant characteristics to at least some of the offspring because treatment with benzimidazoles failed to remove all the adult parasites of the F1 and F2 generations. The rapid selection

for resistance on treatment with benzimidazoles could be suggestive of a small number of genes involved in the development of resistance with possibly semi-dominant expression.

The loss of resistance in the F2 and F3 generation may reflect the pressure on the low numbers of parasites to survive and produce offspring. The egg production of nematode parasites may be controlled by the number of established parasites at the site. Michel, (1967) noted that the fecundity of parasites was limited by the size of the population. In the transplant experiments the transplanted *O.circumcincta* parents in Lambs 1 and 2 produced a surprisingly large number of larvae (Table 2.10). These larvae were then used to infect FR51 and FR52 and they became established as egg laying adults. The number of larvae of the F1 generation was low and therefore these adults in FR51 and FR52 were again under pressure to produce large numbers of offspring. The possible increased fecundity of these F1 adult parasites in FR51 and FR52 may have reduced the rate of passage of the eggs, or length of time of storage in the gravid female parasites. Therefore there could be an alteration in the tubulin content of the eggs which could have some effect on the resistance of the eggs to the effects of benzimidazoles.

After treatment of FR51 and FR52 orally with FBZ at the normal dose rate of 5mg/kg the faecal egg count from both animals was reduced compared with the faecal egg count prior to anthelmintic treatment. This suggested that the adults of the F1 generation in the lambs were less resistant to the effects of orally administered benzimidazoles. Sufficient eggs were obtained in the faeces of FR51 to allow egg hatch assays post treatment but this was not the case for FR52 where the egg count dropped to < 50e.p.g. This fall in faecal egg count possibly indicated a loss of resistance in the parasites compared with the HFRO strain of *O.circumcincta* which was used to establish the 'clones'. However sufficient larvae were obtained after faecal culture from FR51 and FR52 post treatment with FBZ suggesting that the population had some level of resistance to the effects of FBZ administered orally.

Eggs were collected from the faeces of FR51 on Days 14 and 20 post treatment with FBZ and used in egg hatch assays to measure the level of resistance. The ED50s calculated were higher than results for egg hatch assays carried out prior to treatment with FBZ (Table 2.11). Results for assays using ABZ and TBZ in the egg hatch assays were similar unlike results from routine passages where ED50s with ABZ were higher. These results suggested that there had been selection of more resistant adult parasites which produced offspring with a higher level of resistance. For the F1 generation from the transplant experiments selection of the parasites with FBZ at the normal dose rate increased the resistance of the offspring unlike results from the routine passages (Table 2.1) and the dose titration trial (Tables 2.6-2.9) where selection with FBZ at the normal dose rate and with increasing doses failed to increase the resistance measured in the progeny of the selected parasites. The increase in the level of resistance after treatment with FBZ is indicative of the presence of a mixed population of resistant and susceptible parasites and therefore the 'clones' produced by transplantation were not highly resistant to the effects of benzimidazoles.

The ED50s for eggs of the F3 generation were significantly different for the selected (FR63) and unselected lines (FR59 and FR61),(Table 2.12). The ED50s for eggs collected from FR59 were lower than the ED50s for FR61 except for Day 22 post infection in the assay using ABZ. This was the converse of results from egg hatch assays in the previous generation where eggs from FR52 showed lower ED50s than eggs from FR51. These differences could be due to host effects on the level of resistance or the dynamics of the parasite population with possible alterations in the genetic make-up of the population. These host effects may influence the percentage of susceptible and resistant parasites produced in the progeny from a mixed population.

Eggs produced from the F1 generation showed a similar ED50 for TBZ and ABZ (Table 2.11) whereas eggs produced by the F2 generation had a higher ED50 for ABZ in the egg

hatch assays in most assays although levels were rather variable for FR59 and FR61 (Table 2.12). From results in Table 2.12 the ED50s showed day to day variation with eggs collected from all the animals on Day 35 post infection having a lower ED50 than on other occasions. However the ratios of ED50s calculated by comparing ED50s from FR59 or FR61 with ED50s from FR63 on the same occasion were similar. Prior to treatment with FBZ the mean ratio of FR59/FR63 was 3.61 for TBZ and 3.77 for ABZ and for FR61/FR63 was 2.84 forTBZ and 2.67 for ABZ. The ratios of resistance are similar with TBZ and ABZ. They also indicate that there was a difference in the level of resistance between the strain which had been selected with FBZ which was used to infect Lamb FR63 and the unselected strains in Lambs FR59 and FR61.

Selection of the F2 adult parasites was carried out by treating lambs FR59, FR61 and FR63 with FBZ administered orally at 5mg/kg. Egg hatch assays were carried out on eggs passed in the faeces 7 days post treatment. For Lambs FR59 and FR61 there was a significant increase in the ED50s found post treatment compared with the ED50 pre-treatment (Table 2.12 and Fig. 2.17). For FR63 there was little difference between the ED50s pre-treatment and the ED50s post treatment (Table 2.12 and Fig. 2.17). Treatment with FBZ in Lambs FR59 and FR61 selected more resistant members of the 'clones' and the offspring of these selected parasites showed an increased level of resistance. The parasites in FR59 and FR61 had not been selected with FBZ during the previous generation. However, the parasites in FR63 had been selected with FBZ during the F1 generation and selection during the F2 generation did not increase the level of resitance in the offspring, the F3 generation eggs. Results from this selected strain in FR63 suggested that there was rapid selection for resistance by treating the F1 generation with FBZ. Comparison of the resistance measured as the ED50 for F3 generation eggs of the three transplanted strains post treatment with FBZ was carried out by calculating resistance factors using FR63 as the resistant standard. For FR59/FR63 the factor was

0.92 for TBZ assays and 0.96 for assays using ABZ. For FR61/FR63 the factor was 1.04 for the assays using TBZ and there was no assay carried out on FR61 with ABZ. These resistance factors show that there is no difference in the level of resistance measured between the strains selected on one occasion at the F2 generation (FR59 and FR61) with the strain selected twice with FBZ at both the F1 and F2 generations (FR63). These resistance factors can be compared with factors calculated prior to selection of the F2 generation (see previously) with FBZ and show that rapid selection for resistance had occurred. However, the level of resistance measured in the F3 generation eggs does not appear to be as high as the resistance measured in eggs from the routine passages of the HFRO strain of *O.circumcincta* which was the original source of the transplanted parasites. This rapid selection for resistance may have important implications when examining results of egg hatch assays after isolation of the HFRO strain of *O.circumcincta*. The level of resistance was measured by an egg hatch assay at Passage 4 prior to treatment 2 (Lamb DB74). The strain was pressurised with a single dose of FBZ during Passage 1 (Lamb DB48) and from results in the transplant experiment it would appear that this single dose could have been sufficient to select the strain which would then produce a high ED50 when first tested by the egg hatch assay during Passage 4.

The egg hatch assays may not be suitable for detection of small changes in resistance and therefore any differences in the level of resistance measured between the selected and unselected strains of transplanted 'clones' may not be identified. Larvae cultured from the faeces of Lamb FR61 post treatment with FBZ were used to produce a tubulin extract and tested for resistance using a tubulin binding assay with tritiated MBZ and tritiated ABZ (see later). Results of the tubulin binding assays are shown in Table 3.20 and comparison of resistance with other strains of *O.circumcincta* including the routinely passaged HFRO strain and the Bearsden strain is shown in Table 3.21. From the tubulin binding assay the extract from FR61 showed a low level of resistance which was similar to

the level for the Bearsden susceptible strain of *O. circumcincta* Egg hatch assay results comparing these three strains show that eggs from FR61 have an ED50 slightly lower than the highly resistant HFRO strain but much higher than the Bearsden susceptible strain. There could be differences in the level of resistance measured during different stages of development of the parasite with eggs showing a higher level of resistance than larvae. During the dose titration trial (see previously) oral dosage of lambs selected for more resistant adult parasites but there was no alteration in the level of resistance shown by progeny of the selected parasites when tested using egg hatch assays. Again these differences could be the result of possible alterations in the mechanism or genetic transfer of resistance at the different stages of development of the parasites. These changes in resistance may occur because of the possibly polygenic nature of resistance in some nematode parasites (Le Jambre *et al.*, 1979). Martin *et al* (in press) also found slight differences in the resistance level measured in *T.colubriformis* for eggs in egg hatch assays and adult parasites tested by an anthelmintic efficiency trial.

Alternatively the tubulin binding assay measures differences in binding of tritiated benzimidazoles to tubulin extracts from the parasite larvae whereas in the egg hatch assay the effect of the benzimidazole on the complete parasite is measured. If there were any differences in the action of benzimidazoles on resistant parasites these could be detected using egg hatch assays. However, the tubulin binding assay would detect changes in a specific component of the parasite which is probably involved in the mechanism of action of the benzimidazoles but may not reflect all the actions of the benzimidazoles on the parasites and alterations in the parasites causing resistance to benzimidazoles. Therefore, results from the tubulin binding assay , which indicated a low level of resistance in the F3 generation from FR61, showed that there was little alteration in the benzimidazole binding capacty of tubulin extracted from these parasites but egg hatch assay results indicated that the eggs were resistant to the effects of benzimidazoles. This could indicate

that alteration in the binding of benzimidazoles to tubulin is not the only mechanism of resistance to benzimidazoles (see later).

The effect of benzimidazoles on the F3 generation eggs from FR59, FR61 and FR63 was studied by constructing dose titration graphs for each of the egg hatch assays carried out with TBZ before and after treatment of the lambs with FBZ (Figs. 2.18, 2.19 and 2.20). For FR59 and FR61 the slope of the dose titration graph was steep and there was a shift of the graph, although little alteration in the steepness of the slope, after treatment with FBZ. For FR63 there was day to day variation in the steepness of the graphs. Graphs drawn for egg hatch assays on Days 25 and 32 post infection showed a step in the slope of the line. A step in the slope of the graph also was found in the graphs for egg hatch assays on eggs from FR59 and FR61 post treatment with FBZ. This could posssibly indicate that the population was not uniform and consisted of two sub-populations with differing resistance status (Le Jambre *et al.*, 1982). However the graphs for FR63 before treatment on two occasions did not show a step in the slope.

Graphs for egg hatch assays on eggs passed in the faeces of FR63 (Fig 2.20) showed day to day variation in the steepness of the slope of the curve There was also no shift in the curve or alteration in the steepness of the graph after treatment of FR63 with FBZ. During the early routine passages of the HFRO strain of *O.circumcincta* there was day to day variation in the dose titration curves drawn for egg hatch assays and variation in the ED50s calculated during these passages. However, in the dose titration trial there was less day to day variation in the ED50s and dose titration plots drawn for each egg hatch assay. This could indicate that during the early passages of the HFRO strain of *O.circumcincta* the parasite population contained members with variable levels of resistance whereas after several passages with selection the population became more homogeneous. If this was the case it would suggest that the F2 population within Lamb FR63 was a mixed population containing members with differing levels of resistance. The significance of changes in the

slopes of egg hatch assay dose titration curves was difficult to assess because low numbers of animals were used and changes in the resistance levels could be due to host factors. This may have an effect on the egg laying pattern of individual female worms within the population. A single animal was used for each 'clone' of parasites because low numbers of infective larvae were available to infect the animals and the nature of the egg hatch assay makes it impossible to test large numbers of animals at the same time.

Further transplant experiments were carried out to try to produce highly resistant 'clones' of *O. circumcincta* On one occasion the above transplant experiment was repeated with a single pair of adult parasites from a donor lamb being transplanted into each of two parasite free lambs. Culture of faeces of the two lambs from Day 5 post transplantation yielded low numbers of *O.circumcincta* larvae with contamination of the cultures by *Strongyloides spp.* larvae. These larvae were not used for further passages because of the possibility of contamination of the supposedly parasite free lambs. Another transplant trial was carried out where adult parasites collected from the abomasum at necropsy of one of the lambs from the dose titration trial (see previously) treated orally with FBZ at a dose rate of 40mg/kg were used to select parents which were transplanted into 2 parasite free lambs. One of the lambs was treated with FBZ administered orally at a dose rate of 5mg/kg 24h after transplantation of the adult parasites. Faeces of both lambs were cultured from Day 5 post transplantation and no larvae were obtained from the faeces of either of the lambs. The age of the adult parasites or their exposure to high levels of benzimidazoles, 8 times the normal dose rate, may have reduced the ability of the transplanted parasites to survive . The high dose level of benzimidazoles also may have reduced the fecundity of the female parasites. During passages of the HFRO benzimidazole resistant strain treatment with FBZ at the normal dose rate resulted in a reduction in the faecal egg output post-treatment for several days. This was thought to be due to reduced reproductive function in female parasites brought about by the effects of the

benzimidazole. The poor results from these transplant experiments prevented further studies on the mechanisms of genetic transfer of resistance.

LARVAL MOTILITY TESTS

Larval Motility Test using Infective Larvae of *Ostertagia circumcincta* Incubated with Anthelmintics

Materials and Method

Materials.

Infective larvae - cultured from faeces and prepared by the Method described previously (see General Materials and Methods).For the assays larvae were exsheathed (see General Materials and Methods).

Anthelmintic stock solutions - Stock solutions of various anthelmintics were prepared in methanol.

Larval culture medium (Jenkins *et al*, 1980) - 2g pancreatic digest of casein (Bacto-casitone, Difco), 1g yeast extract (Difco), 0.5g D-glucose, 0.08g di-potassium hydrogen orthophosphate, 0.08g potassium dihydrogen orthophosphate made up to 100ml with permanganate distilled water. Divided into 20ml aliquots and autoclaved for 15min. Prior to use 20mg sodium benzylpenicillin, 25mg streptomycin sulphate and 1mg natamycin was added to each aliquot.

Sterilin 16 well plastic plates.

Method.

1. Approximately 50-70 infective larvae were added to each well in the Sterilin plates. The volume in each well was made up to 1.98ml using culture medium for incubations >48h or water where incubation of approximately 24h was used.

2. In duplicate 20ul of each stock solution of the test anthelmintic was added to the well. For the blank added 20ul water and the methanol control added 20ul methanol.

3. Plates were covered and sealed with cling film to prevent evaporation of water over the period of incubation which would cause concentration of the anthelmintic. Plates were incubated at 37°C for a given time. The larvae were examined periodically under a binocular microscope and the motility assessed by observing each larva for approximately 30 seconds.

4. The percentage motility for the larvae in each well was calculated and corrected for natural mortality by subtracting the value obtained from the methanol control well.

Incubation of Infective Larvae of HFRO and Bearsden Strains of *O.circumcincta* with Thiabendazole, Albendazole and Albendazole Sulphoxide.

Results

Infective larvae were incubated in larval culture medium with the benzimidazoles TBZ, ABZ or ABSX at concentrations of 3, 5, 10 or 20ug/ml. Motility of the larvae was assessed at intervals during the incubation period and the mean percentage of parasites motile, corrected for natural mortality using the controls, at each drug concentration was calculated and is shown in Table 2.13 for the Bearsden Strain and Table 2.14 for the HFRO benzimidazole resistant strain.

Discussion

During the transplant experiments difficulty was experienced in obtaining sufficient material to test the individual clones for resistance. It was impossible to test for resistance at the F1 generation because insufficient eggs were passed in the faeces of the lambs to allow egg hatch assays to be carried out and all the faecal material was retained for culture of infective larvae. Offspring of the F1 generation adults were tested because sufficient eggs were available in the faeces to allow egg hatch assays to be performed. However, to study the genetic inheritance of resistance it would be more appropriate to

Table 2.13

Motility of infective larvae of the Bearsden strain of *O.circumcincta* incubated in various concentrations of TBZ, ABZ or ABSX. Percentage of larvae which were motile was assessed at intervals during the incubation period and results corrected for natural mortality using the controls.

Drug concentration (ug/ml)		% Motility of Larvae			
		24h	48h	72h	120h
20	TBZ	-	-	-	-
	ABZ	78.5	86.5	77.5	76.7
	ABSX	81.8	67.6	90.8	44.1
10	TBZ	100	83.8	93.0	-
	ABZ	83.0	83.1	78.1	77.8
	ABSX	88.4	99.4	79.5	74.1
5	TBZ	100	92.3	100	-
	ABZ	89.5	92.3	86.5	73.6
	ABSX	94.2	98.0	92.7	71.5
3	TBZ	100	85.6	100	-
	ABZ	94.8	83.9	86.8	73.6
	ABSX	83.3	100	92.7	62.5

Table 2.14

Motility of infective larvae of the HFRO strain of *O.circumcincta* incubated in various concentrations of TBZ, ABZ or ABSX. Percentage of larvae which were motile was assessed at intervals during the incubation period and results corrected for natural mortality using the controls.

Drug concentration (ug/ml)		% Motility of Larvae			
		24h	48h	72h	120h
20	TBZ	-	-	-	-
	ABZ	100	86.5	97.4	100
	ABSX	100	100	98.2	94.8
10	TBZ	96.5	92.0	100	-
	ABZ	99.9	98.1	95.2	100
	ABSX	100	93.2	96.0	100
5	TBZ	95.8	90.2	90.6	-
	ABZ	97.3	97.8	94.1	100
	ABSX	100	89.4	94.7	98.3
3	TBZ	95.2	93.7	100	-
	ABZ	95.4	95.8	98.0	100
	ABSX	100	91.0	95.2	100

test the resistance of the F1 generation. In order to quantify the level of resistance at the F1 generation an alternative test for resistance, possibly using low numbers of larvae, would need to be found.

Infective larvae of the Bearsden benzimidazole susceptible strain and the HFRO benzimidazole resistant strain were incubated with benzimidazoles to investigate if differences in the level of resistance could be detected using this method. The mode of action of the benzimidazoles is thought to be by binding to tubulin and therefore causing disruption of the microtubular framework in the parasite and death (Hoebeke *et al.*, 1976; Friedman and Platzer, 1978). There may also be an effect on the metabolism of the parasite by disruption of the fumarate reductase enzyme (Barrowman *et al.*, 1984). From Tables 2.13 and 2.14 it was shown that incubation in benzimidazoles had little effect on larval motility for 72h. After incubation in 20ug/ml ABZ or ABSX for 120h there was reduced motility but many of the larvae remained alive and motile after this long period of incubation with the anthelmintic drug. The infective larvae were exsheathed to try to improve uptake of the drugs from the surrounding medium. Normally the infective larvae are encased in the sheath of the previous stage of development and this could act to protect the larvae and also reduce drug uptake into the parasites. The benzimidazoles rely on their inhibitory effects on parasite metabolic processes to kill them and in the case of infective larvae metabolism is very slow. Infective larvae can remain on pasture over the winter period and therefore their survivability depends on their ability to withstand harsh climatic conditions and retain a slow metabolic rate. Therefore, it is necessary to incubate the larvae in high concentrations of the benzimidazoles for several days to reduce the motility of the larvae. Culture medium containing antimicrobials was essential to prevent overgrowth with fungi and bacteria during the incubation period and the medium provided nutrients to the exsheathed larvae. The motility of the larvae in the control wells varied over the period of the experiment but remained at >90% motile throughout. When

assessing larval motility the parasites showed periods of activity and periods of inactivity. In order to get a fairly consistent value for motility larvae which were non-motile were examined for a period of 30 sec. Larvae seemed to exhibit rest/move cycles with periods of immobility for up to 30seconds and therefore close examination of the larvae was necessary to ensure that results were consistent. From the results in Tables 2.13 and 2.14 some of the larvae which were non-motile on one occasion appeared to recover their motility by the next occasion.

The difference in motility between benzimidazole resistant and benzimidazole susceptible parasites was not significant for incubations of 24h, 48h and 72h. The percentage motile was significantly different between resistant and susceptible larvae using a Student's t-test ($p<0.01$) after 120h incubation with ABZ and ABSX. Incubation with TBZ was not carried out for >72h in the experiment. Although there was a significant difference in the motility between the two strains at 120h incubation the benzimidazole susceptible strain retained motility ranging from 73.6-77.8% for ABZ and 44.1-74.1% for ABSX. Therefore some of the benzimidazole susceptible parasites were able to survive in the presence of the anthelmintics. Both ABZ and ABSX were ineffective against infective larvae of the the benzimidazole resistant strain of *O.circumcincta* (Table 2.14). The HFRO strain of *O.circumcincta* was shown to be highly resistant to the effects of benzimidazoles with an approximately 10 fold difference in ED50s between this strain and the Bearsden benzimidazole susceptible strain when resistance was tested using egg hatch assays. Although the differences in motility were significant they were not very large between these two strains on incubation of infective larvae with benzimidazoles and therefore strains of *O.circumcincta* with a low level of resistance may not be detected using this method.

The reduction in motility of infective larvae in the presence of ABZ was not dose related. At 120h the motility of larvae was similar in 3, 5, 10 and 20ug/ml ABZ. It was

impossible to calculate an ED50 for ABZ against infective larvae. For ABSX, one of the metabolites of ABZ, the reduction in motility was greatest in a concentration of 20ug/ml but in 5ug/ml and 10ug/ml the larval motility was similar. The results for reduction in motility were similar for ABZ and its metabolite ABSX except at 120h in a concentration of 20ug/ml where the ABSX had a greater effect. Results from egg hatch assays showed that ABSX failed to prevent hatching of *O.circumcincta* eggs and therefore the metabolite had no ovicidal effect *in vitro* (see Table 2.15). However ABSX appeared to have similar, if not slightly greater activity, to the parent compound ABZ when used against larvae *in vitro*. Differences in activity of ABZ and ABSX against different stages of *O.circumcincta* may be due to differences in drug solubility or their ability to penetrate into the parasites.

Incubation of both strains of *O.circumcincta* in TBZ produced no effect on motility over a 72h period. The period of incubation may have been insufficient to cause a reduction in motility compared with controls. The concentration of TBZ used in the assay may have been too low to produce a reduction in motility. The oral dose rate of TBZ required to treat parasitised animals is higher for TBZ than the modern benzimidazoles e.g. ABZ. This is because of the greater solubility of TBZ which is excreted from the host more rapidly than the more modern, less soluble benzimidazoles and therefore TBZ has less effect on the nematode population within the host. The greater solubility of TBZ would suggest that if TBZ was used for *in vitro* tests with parasite eggs or infective larvae uptake of the drug into the parasites could be increased. This may be the case in egg hatch assays where the ED50s calculated for TBZ were lower than the ED50s for ABZ (see previously). However in the larval motility test TBZ was not as effective as ABZ at reducing larval motility (Tables 2.13 and 2.14) although these slight differences in efficacy of the two benzimidazoles against infective larvae were not significant. The reduction in motility of infective larvae in the presence of anthelmintics as a test for resistance to levamisole or morantel tartrate has been described (Martin and Le Jambre, 1979). Modification of this

method for use to test for benzimidazole resistance using infective larvae of *O.circumcincta* was not possible because of the mode of action of the benzimidazoles and possibly the limited uptake of these drugs by larvae.

In the above trial the majority of the larvae maintained motility in the presence of benzimidazoles (Tables 2.13 and 2.14). Benzimidazoles however, are effective in removing larval stages of *O.circumcincta* when used *in vivo*. Rahman *et al.*, (1977) studied the effect *in vivo* of MBZ on adult *H. contortus*. These authors found that MBZ caused inhibition of parasite energy metabolism and the parasites became detached within 9-15h of drug administration. These detached worms remained alive suggesting that in the presence of benzimidazole anthelmintics the inability of parasites to maintain their position in the host animal was not due to death of the parasites. Therefore the efficacy of benzimidazoles *in vivo* may not be linked to their ability to kill larvae or adults which can be tested *in vitro*. The host-parasite interaction is important in expulsion of benzimidazole affected parasites. In the larval motility test the ability of benzimidazoles to kill both resistant and susceptible parasites was poor and differences between the two strains were slight. However metabolic changes sufficient to cause expulsion of parasites from the host may have occurred before death of the larvae or larvae became non-motile and these changes may differ between resistant and susceptible strains but they cannot be detected using this type of assay.

Bennet and Pax (1986) described the use of a micromotility meter to measure worm movement. The micromotility meter operates on the principle that movement of worms within a tube changes the amount of light which can be transmitted though the tube. These changes in pattern of light transmitted depend on the motility of the worms and can be expressed as a motility index. Studies by these authors using *O.circumcincta* infective larvae found an 8 fold difference in the motility score between fully motile parasites and heat killed parasites. However, there are disadvantages with this technique in that a high

concentration of larval suspension is required (>500 larvae/ml) and small differences in the motility may not be detected e.g. between partially resistant and susceptible strains of parasites in the presence of anthelmintics. Folz *et al*, (1987) described the use of the micromotility meter to detect the effect of various anthelmintics on infective larvae of *H.contortus*. These authors used higher doses of ABZ than in the experiments described above 50-200ug/ml compared with 3-20ug/ml which were used in the above experiment. Folz *et al*.,(1987) also suggested the use of the micromotility meter to detect resistance to anthelmintics by comparing the motility of resistant and susceptible larvae in the presence of anthelmintics. The test would be more appropriate in the measurement of resistance to drugs which act by causing paralysis of parasites e.g. levamisole, morantel tartrate or ivermectin rather than benzimidazoles which required a prolonged period of exposure to the drug to exert their effect and therefore reduce motility of parasites.

Again a problem with the micromotility meter would be the number of parasites required to carry out a viable test for resistance. During work on the transplant 'clones' of *O.circumcincta* a test for anthelmintic resistance using low numbers of parasites was sought. Using the micromotility meter several replicates would be required at a variety of drug concentrations and approximately 250 larvae per replicate would be required (Bennet and Pax, 1986). These numbers of parasites would not be available to allow tests to be carried out on infective larvae of the F1 generation of the single transplanted parents. Work with the micromotility meter to assess the effect of drugs using a single adult parasite has been descibed (Nowak *et al*, 1987). These authors used single adult males of *Onchocerca gibsoni* to measure parasite motility in the presence of drugs. Similar tests using a single adult *O.circumcincta* are not feasible because of the much smaller size of this parasite compared with adult *Onchocerca gibsoni*. The poorer survivability of adult *O.circumcincta* outwith the host compared with survivability of

Onchocerca gibsoni would also be a limiting factor especially with the benzimidazole anthelmintics where a prolonged exposure time is necessary to cause an anthelmintic effect in parasites. In addition adult *Onchocerca gibsoni* are available from surface nodules whereas adult *O.circumcincta* are available only from the abomasum which usually requires slaughter of the host.

Studies of the Effects of Albendazole, Netobimin and the Albendazole Metabolites, Albendazole Suphoxide and Albendazole Sulphone, on Eggs and Infective Larvae of the Bearsden Benzimidazole Susceptible Strain of *O.circumcincta*.

Results

Egg hatch assays were carried out using eggs collected from the faeces of lambs infected with the benzimidazole susceptible strain of *O.circumcincta*. The percentage of eggs which failed to hatch in the presence of increasing concentrations of netobimin, ABZ, ABSX and ABSO are shown in Table 2.15.

Infective larvae of the Bearsden strain of *O.circumcincta* were exsheathed and incubated in larval culture medium containing increasing concentrations of ABZ, ABSX and ABSO. Larval motility was assessed after 24h, 72h and 96h incubation. The percentage of larvae which remained motile expressed as a percentage of the total number of larvae remaining motile in control wells was calculated and results are shown in Table 2.16.

After incubation of the larvae with the appropriate drug for 96h the material in each of the sample wells with ABZ at10ug/ml, ABSX at 10ug/ml and ABSO at 3ug/ml was collected. The larvae were filtered from the culture medium and the remaining material was extracted to isolate benzimidazoles, reconstituted in methanol and injected onto an HPLC system to determine the concentration of ABZ, ABSX and ABSO in each sample (for method see section describing the method used for analysis of purity of tritiated

Table 2.15

Development of eggs of the Bearsden benzimidazole susceptible strain of *O.circumcincta* used in egg hatch assays with netobimin, albendazole, albendazole sulphoxide or albendazole sulphone.

Drug concentration (ug/ml)	% Failing to Develop ± SD
Control	11.4 ± 0.9
ABZ (10ug/ml)	100
ABZ (5ug/ml)	100
ABZ (1ug/ml)	99.6 ± 0.5
ABSX (10ug/ml)	12.7 ± 4.2
ABSX (5ug/ml)	11.2 ± 1.6
ABSO (3ug/ml)	12.8 ± 0.8
ABSO (1ug/ml)	11.2 ± 0.7
Netobimin (20ug/ml)	8.4 ± 1.4
Netobimin (10ug/ml)	12.9 ± 3.5
Netobimin (5ug/ml)	8.7 ± 2.0

Table 2.16

Motility of infective larvae of the Bearsden strain of *O.circumcincta* after incubation in albendazole, albendazole sulphoxide or albendazole sulphone.

Drug Concentration	% of larvae motile		
(ug/ml)	24h	72h	96h
ABZ (10ug/ml)	61.2	49.2	30.7
ABZ (5ug/ml)	51.7	57.1	53.1
ABZ (0.5ug/ml)	67.3	55.0	44.0
ABSX (20ug/ml)	64.5	47.6	40.4
ABSX (10ug/ml)	59.8	40.3	48.2
ABSX (5ug/ml)	87.7	57.7	54.5
ABSO (3ug/ml)	98.3	72.0	63.4
ABSO (1ug/ml)	97.0	97.6	93.3
ABSO (0.5ug/ml)	96.9	100	90.8

Table 2.17

Concentration of albendazole, albendazole sulphoxide and albendazole sulphone expressed as a percentage of the total drug concentration measured by HPLC in standard solutions and extracts from larval culture medium after incubation of *O.circumcincta* infective larvae in albendazole (10ug/ml), albendazole sulphoxide (10ug/ml) or albendazole sulphone (3ug/ml) for 96h.

Sample	Drug Concentration (%)		
	ABZ	ABSX	ABSO
Standard ABZ	98.54	1.24	0.22
Post-Culture ABZ	98.51	1.17	0.32
Standard ABSX	0.46	97.56	1.98
Post-Culture ABSX	ND	95.35	4.65
Standard ABSO	ND	0.12	99.88
Post-culture ABSO	ND	ND	100

ND- drug not detected in samples

benzimidazoles). The standard solutions of ABZ, ABSX and ABSO which were added to each well in the larval motility assay were also injected onto the HPLC system and the levels of ABZ, ABSX and ABSO in these standards was calculated. The concentration of benzimidazoles analysed by HPLC in the samples from the larval motility test is shown in Table 2.17.

Discussion

Albendazole is oxidised *in vivo* to form the metabolites ABSX and ABSO (Gyurik *et al.*, 1981). The sulphide form (ABZ) and the sulphoxide (ABSX) are both thought to have anthelmintic activity *in vivo*(Marriner and Bogan, 1980) but the sulphone form (ABSO) is thought to be inactive (Averkin *et al*, 1975). This group of benzimidazoles are available commercially as netobimin the pro-drug which is metabolised to ABZ (McDougall *et al.*, 1985), the parent sulphide ABZ and ricobendazole which is ABSX. The activity of these compounds against various stages of *O.circumcincta* was studied *in vitro*

Egg hatch assays were carried out on eggs of the Bearsden benzimidazole susceptible strain of *O.circumcincta* using netobimin , ABZ, ABSX and ABSO (Table 2.15). ABZ was shown to be ovicidal preventing egg development at all concentrations tested and this drug was used in routine egg hatch assays to measure the level of resistance in eggs of *O.circumcincta* (see previous experiments). When ABZ was administered orally to sheep it was converted to ABSX and this compound was thought to have anthelmintic activity (Marriner and Bogan, 1980). When ABSX was used in the egg hatch assays at concentrations of 10ug/ml and 5ug/ml egg development was not inhibited and there was no significant difference between egg development in controls and eggs exposed to ABSX. Similar results were obtained with ABSO and netobimin with neither of these drugs reducing egg development. Therefore of this group of benzimidazoles only the sulphide, ABZ, has ovicidal activity and the pro-drug netobimin and metabolites ABSX and ABSO have no ovicidal activity *in vitro*.

Netobimin requires metabolism to ABZ *in vivo* in order to have anthelmintic activity

and therefore it was not surprising that this pro-drug failed to inhibit egg development in egg hatch assays. The metabolites of ABZ, ABSX and ABSO were inactive against eggs of *O.circumcincta* This may be due to poor uptake of these metabolites into the parasite eggs. During the egg hatch assay penetration of the eggs by the benzimidazoles is thought to be by passive diffusion from the surrounding medium (Weston *et al*, 1984; Sangster and Prichard,1984) and therefore depends on the solubility of the drug, the available concentration of the drug and the ability to penetrate the outer coating of the egg. ABSX is slightly more soluble in aqueous solution than the parent ABZ. However the uptake of the benzimidazoles into eggs may depend on the lipid solubility of the drug and the lipid concentration within the egg (Weston *et al*, 1984; Sangster and Prichard, 1984). The partitioning coefficient for ABSX between the solution and the eggs may be disadvantageous with a very low concentration of ABSX entering the eggs which was insufficient to prevent development. The further metabolite ABSO is insoluble in aqueous solution and therefore for the egg hatch assays the highest concentration which could be reconstituted in methanol was 3ug/ml. At higher concentrations when the drug was added to the egg suspension a precipitate formed. This metabolite also failed to prevent development of eggs possibly because of its inactivity as an anthelmintic and inability to penetrate the eggs.

Incubation of ABZ, ABSX or ABSO with infective larvae of *O.circumcincta* for 96h caused a decrease in larval motility, with ABZ showing a greater effect than ABSX at a concentration of 10ug/ml but similar activity at 5ug/ml (Table 2.16). ABSO at a concentration of 3ug/ml reduced larval motility after 72h and 96h incubation but at concentrations of 1ug/ml and 0.5ug/ml ABSO had little effect. ABSO is insoluble therefore a concentration of 3ug/ml was the highest whch could be achieved without the ABSO precipitating from solution. In a previous experiment to study the effect of benzimidazoles, ABZ, ABSX and TBZ on the motility of infective larvae of *O.circumcincta* (Table 2.13) there was little reduction in larval motility after incubations of 24h, 48h

and 72h. After 120h incubation in ABZ and ABSX there was a reduction in larval motility. In the present experiment the observed reduction in larval motility occurred more rapidly with significant reduction in motility in ABZ and ABSX after 24h. With ABSO a reduction in larval motility was not observed until the larvae had been incubated in the drug at a concentration of 3ug/ml for 48h. With ABZ and ABSX the reduction in larval motility was similar after 72h and 96h incubation at drug concentrations of 10ug/ml and 5ug/ml (Table 2.16) and this reduction in motility was much greater than that observed with ABZ and ABSX in the previous experiment (Table 2.13). These discrepancies in the effect of ABZ and ABSX on the larvae may be due to differences in the ages of larvae tested. For the present experiment the infective larvae had been stored at 4^{0}C for a longer period of time prior to their incubation with the anthelmintics than larvae used in the previous experiment. The differences in age of the larvae may affect the metabolic stores of the larvae and the partially depleted stores in older larvae may make them more susceptible to the effects of benzimidazoles.

The metabolite of ABZ , ABSX was inactive against eggs of *O.circumcincta* (Table 2.15) but appeared to have similar activity *in vitro* to the parent compound against infective larvae of *O.circumcincta* This differential activity against the various stages of *O.circumcincta* suggested that the ABSX has nematocidal activity but is prevented from exerting this effect in eggs because of poor uptake of the drug by the eggs. The other metabolite of ABZ, ABSO is thought to have poor activity as an anthelmintic (Averkin *et al.*, 1975). From the above experiments it had no effect against *O.circumcincta* eggs but caused a reduction in larval motility when incubated at a concentration of 3ug/ml with infective larvae for >72h. After oral administration of ABZ at a dose rate of 10mg/kg to sheep the peak concentrations of ABZ, ABSX and ABSO in the abomasum were 6ug/ml, 26ug/ml and 4ug/ml respectively (Marriner and Bogan, 1980). The concentrations of ABSX obtainable *in vivo* are therefore higher than the concentrations of ABZ. From

results of *in vitro* larval motility tests (Tables 2.13 and 2.16) both the parent compound ABZ and ABSX have anthelmintic activity which appears to be comparable. However, in displacement assays using tubulin extracts from *H.contortus* and tritiated ABZ to compare the tubulin binding ability of ABZ, ABSX and ABSO (see later) the binding of the metabolites ABSX and ABSO to tubulin was lower than the binding of the parent ABZ. These results would suggest that if the mode of action of the benzimidazoles was due to tubulin binding the metabolites ABSX and ABSO should have much poorer activity than the parent sulphide, ABZ.

These conflicting results of *in vitro* tests to measure the activity of ABZ and its metabolites ABSX and ABSO confirm that many factors play a role in determining the anthelmintic effect of this group of drugs. The concentration of the drug which is achieved in the host and therefore the availability of the drug to the parasites is important. Aligned to this is the solubility of the drug which influences its kinetics within the host and also the ability of the parasites to absorb the drug. The exact mode of action of the benzimidazoles is also important with ABZ binding more strongly to nematode tubulin than ABSX but ABSX may have other effects on the parasites e.g. effect on parasite metabolism.

Within the host the ABZ is converted to ABSX and this oxidation reaction is reversible. The possibility that parasites could convert ABZ to ABSX and ABSO or *vice versa* was investigated by measuring the concentration of ABZ and its metabolites in the sample medium surrounding the larvae after incubation for 96h. Comparison of the proportions of each of the metabolites in the original standard solutions with the proportions of each of the metabolites in the samples indicated that there was negligible interconversion of the metabolites over the period of incubation of the *O.circumcincta* larvae (Table 2.17). These results suggested that the larvae cannot convert ABZ to its metabolites ABSX and ABSO. The reduction in larval motility observed in the previous experiments (Tables 2.13 and 2.16) was therefore due to the compound which was added originally. The solution of

ABSX contained a low level of ABZ (0.49% which equates with 0.049ug/ml in a 10ug/ml solution) and at this low concentration it is unlikely that the ABZ was responsible for the reduction in motility. The metabolite ABSX therefore has anthelmintic activity.

From results of the larval motility assays ABZ and ABSX had similar efficacy against infective larvae of *O.circumcincta* with ABSO having lower efficacy. The larvae do not appear to convert ABZ to ABSX or ABSO and therefore an increased rate of drug metabolism to the less active metabolite ABSO is not likely to be a mechanism by which the parasites have become resistant to the effects of benzimidazoles.

Detection of Benzimidazole Resistance in *O.circumcincta* using a Larval Paralysis Assay with Infective Larvae Incubated in Physostigmine (Eserine) (Method from Sutherland *et al*, 1988)

Materials and Method

Materials.

Larvae - *O.circumcincta* infective larvae of the Bearsden benzimidazole susceptible strain and HFRO benzimidazole resistant strain.

Physostigmine (Eserine) - Obtained from Sigma and used at a concentration of 1mM. The solution was made up freshly for each assay by dissolving 28g of physostigmine in 0.5ml 100% aqueous acetone + 0.5ml distilled water and the volume was made up to 100ml using distilled water.

Sterilin 16 well plastic plates

Method.

1. Approximately 30 infective larvae of the appropriate strain were placed in each well of the plastic plate and excess water was removed with a fine glass pipette. The experiment was carried out in triplicate or quadruplicate.

2. To each well was added 1ml of the 1mM physostigmine solution and plates were

maintained at room temperature.

3. The motility of the larvae was assessed at 15 min intervals for 1h using a binocular microscope.

4. The percentage of larvae motile in each well was calculated at each interval and the replicates were meaned. The mean percentage of larvae which were motile in the two strains of *O.circumcincta* was compared at each time interval using the Student's t-test.

Results

Larval paralysis assays using physostigmine were carried out on four occasions with infective larvae of the Bearsden and HFRO strains of *O.circumcincta* and the motility of infective larvae after incubation in 1mM physostigmine was calculated at 15min intervals and results are shown in Table 2.18. Using a Student's t-test the percentage motility for each strain of *O.circumcincta* was compared in Assays 1-4 at each time interval and those assays where the difference in motility was significant are shown in Table 2.18. Two assays were carried out using infective larvae of *O.circumcincta* Bearsden and HFRO strain which had been exsheathed (for method see General Materials and Methods) and results are shown in Table 2.19.

Discussion

Biochemical tests carried out on infective larvae of strains of *H.contortus* have revealed differences in the non-specific esterase activity between benzimidazole resistant and susceptible strains of the parasites (Sutherland *et al.*, 1988). During studies with isoelectric focussing of larval homogenates bands for esterases were found for resistant strains which were absent in susceptible strains and therefore there was a quantitative increase in the overall esterase staining in the resistant strain. A simple colorimetric assay (Ellman *et al.*, 1961) was modified to measure quantitative differences in the esterase activity of strains of parasites and differences were found to correlate with resistance levels in strains of *H.contortus* (Sutherland *et al.*, 1988). The larval

Table 2.18

Motility of larvae measured during larval paralysis assays with infective larvae of *O.circumcincta* Bearsden and HFRO strains incubated in physostigmine at a concentration of 1mM

Incubation Time	% of Infective Larvae Motile							
(min)	Bearsden Strain				HFRO Strain			
Assay No.	1	2	3	4	1	2	3	4
15	100	97.9	100	99.3	100	100	100	97.7
30	97.2	97.8	94.3	99.2	100	100	100	90.6
45	69.1	88.6	70.3	58.1	88.5*	87.8	91.3*	75.7*
60	53.6	55.1	48.6	46.5	77.4*	62.9	77.3*	78.0*

* Motility significantly different ($p<0.01$) from value obtained for susceptible larvae in the comparable assay at the same time interval.

Table 2.19

Motility of larvae measured during larval paralysis assays using exsheathed infective larvae of *O.circumcincta* Bearsden and HFRO strains in the presence of 1mM physostigmine.

Incubation Time (min)	% of Infective Larvae Motile			
	Bearsden Strain		HFRO Strain	
Assay No.	1	2	1	2
15	100	80.5	100	91.4
30	98.0	50.7	97.0	44.7
45	69.5	27.2	58.5	21.6
60	29.5	25.2	30.6	29.9

paralysis test developed for the detection of resistance to levamisole and morantel (Martin and Le Jambre,1979) was modified to detect differences in the level of acetylcholinesterase activity between benzimidazole resistant and susceptible parasites by incubating infective larvae in the presence of physostigmine (Sutherland *et al*, 1988).

Physostigmine is an alkaloid which can be extracted from the Calabar or Ordeal bean, the dried ripe seed of *Physostigma venosum Balfour*, a perennial plant grown in West Africa. The pure alkaloid physostigmine was isolated from the plant by Jobst and Hesse in 1864 and the same alkaloid was isolated by Vee and Leven in 1865 and named eserine. Physostigmine has a carbamyl ester linkage which can be hydrolysed by acetylcholinesterase and acts as an alternative substrate to acetylcholine for acetylcholinesterase. When physostigmine interacts with the enzyme acetylecholinesterase it produces the carbamylated form of the enzyme which is much more stable than the acetyl form of the enzyme and therefore sequestration of the enzyme in its carbamylated form prevents the enzyme acting to hydrolyse acetylcholine for periods of up to 3-4h. Consequently, there is accumulation of acetylcholine at receptors in the nervous system with resulting overstimulation at receptor sites. In nematode parasites this accumulation of acetylcholine causes paralysis.

Physostigmine can be used to detect differences in the esterase activity between benzimidazole resistant and susceptible parasites because resistant parasites which contain higher levels of esterases are capable of withstanding the effects of physostigmine for a longer period than susceptible parasites. Reduction in the levels of esterase after physostigmine treatment causing paralysis of the larvae due to a build up of acetylcholine will be greater in the susceptible parasites than in the resistant parasites.

Results for the larval paralysis assays with physostigmine using the Bearsden and HFRO strains of *O.circumcincta* showed significant differences between the two strains ($p<0.01$) in Assays 1, 3 and 4 after 45min and 60min incubation in the drug (Table

2.18). In Assay 2 there was a reduction in the motility of the susceptible strain compared with motility of the resistant strain at 60min incubation but this was not significant. The test was therefore not reproducible on all occasions with Assay 2 giving different results from the other three assays. From results of egg hatch assays using eggs of these strains of *O.circumcincta* the HFRO strain was found to be highly resistant to the effects of benzimidazoles. In the larval paralysis test using infective larvae of these strains of *O.circumcincta* incubated in physostigmine there were significant differences between motility of benzimidazole resistant parasites and benzimidazole susceptible parasites but the magnitude of variation was not large. The difference in motility between a partially resistant strain of *O.circumcincta* and the susceptible strain may be small and therefore not significant when trying to detect resistance using the larval paralysis assay with physostigmine. Similar problems were encountered when incubating infective larvae with benzimidazoles and measuring motility after 24, 48 and 72h (see Tables 2.13 and 2.14). However, the larval paralysis assay with physostigmine can be performed using low numbers of larvae, down to 10 larvae per well, but there may be a reduction in the reproducibility of the assays with very low numbers of larvae.

In an effort to try to increase the difference between motility of the resistant and susceptible larvae in the presence of physostigmine, the assay was repeated using a higher concentration of physostigmine. At a physostigmine concentration of 5mM larvae of both the resistant and susceptible strains of *O.circumcincta* were paralysed within 15min. Therefore, for the larval paralysis test with physostigmine to differentiate between resistant and susceptible strains of *O.circumcincta* the concentration of physostigmine in the assay appeared to be critical with the test functioning optimally when physostigmine was used at a concentration of 1mM.

Exsheathment of the infective larvae prior to their use in the larval paralysis assay with physostigmine reduced the ability of larvae of both the resistant and susceptible

strains to withstand the paralysing effect of physostigmine (Table 2.19). For exsheathed larvae there was no significant difference between the motility of larvae of the susceptible and the resistant strains of *O.circumcincta*. The sheath may therefore play a protective role in preventing the uptake of physostigmine by the larvae and this effect may be more pronounced in the resistant strain. Alternatively the sheath may contain esterases which are capable of binding to physostigmine and also degrading acetylcholine. A higher level of esterases in the sheath of the resistant larvae compared with the susceptible larvae would lead to a decreased effect of physostigmine on motility of the resistant larvae compared with the susceptible larvae.

Although the larval paralysis assay using physostigmine was found to differentiate between benzimidazole resistant and susceptible strains of *O.circumcincta* on most occasions the rational behind using this assay to detect benzimidazole resistance in nematode parasites is not clear. Measurement of the level of esterases using a colorimetric assay with alpha-naphthyl acetate has been used as a method to detect resistance to organophosphorous compounds in arthropods. The organophosphorous compounds which are used as insecticides act as anti-cholinesterases causing accumulation of acetylecholine in the insects. Resistance to organophosphorous compounds by development of higher esterase activity in strains of arthropods and measurement of the higher esterase levels in resistant insects seems a logical approach to detection of resistance to organophosphorous compounds in insects. However in nematode parasites the role of increased esterase activity which was detected in strains of parasites which are benzimidazole resistant is unclear. Kelly *et al.*, (1978) using *H.contortus* found that in strains where resistance to anthelmintics had developed there was a concomitant rise in the 'fitness' of the parasites as measured by increased infectivity and pathogenicity . This alteration in the 'fitness' of the benzimidazole resistant parasites could be due to intense selection pressure altering the genetic characteristics of the population resulting in a resistant phenotype which has

similar or superior 'fitness' to the susceptible parasites even in the absence of the selecting agent. This phenomenon has been noted in studies on insecticide resistance (Georghiou and Taylor, 1976; McKenzie and Purvis, 1984). The possible alteration in the 'fitness' of benzimidazole resistant nematodes may be expressed as increased levels of enzymes, among other changes in the parasites. Therefore, the detection of increased levels of esterases in larvae of benzimidazole resistant parasites could be a measure of the increased 'fitness' of the parasites rather than a direct measurement of the level of benzimidazole resistance developed by the strain of parasites.

Alternatively, the increase in esterases in benzimidazole resistant *O.circumcincta* may be an early biochemical mechanism along the pathway of development of resistance to benzimidazoles. Studies on a CBZ resistant strain of *H.contortus* (Rew *et al.*, 1982) showed that in the presence of benzimidazoles adult parasites were able to increase their metabolism of glucose to volatile end-products whereas catabolism of glucose was unaffected in a benzimidazole susceptible strain of *H.contortus*. It is possible that increased metabolism in resistant parasites is linked with increased levels of enzymes including esterases and these may play some role in the development of resistance to benzimidazoles in addition to the possible changes in affinity of tubulin in resistant parasites for benzimidazoles (Lacey and Prichard, 1986).

Studies on the Uptake of Tritiated ABZ or Tritiated FBZ by Infective Larvae of the Bearsden Benzimidazole Susceptible and HFRO Benzimidazole Resistant Strains of *O.circumcincta*.

Materials.

Tritiated ABZ - 3.77uM, specific activity 360dpm/pmole

Tritiated FBZ - 8.36uM, specific activity 110dpm/pmole

Infective larvae of HFRO and Bearsden strains of *O.circumcincta* cultured as described

previously (see General Materials and Methods).

Sterilin 16 well plastic plates

Method.

1. Approximately 100 larvae of the appropriate strain were placed in each well of the plate.

2. There were two control wells containing drug without larvae and two blank wells with larvae and no drug.

3. Drug was added to the plates with triplicates of each strain of *O.circumcincta* exposed to either ABZ or FBZ added at a rate of 10ul or 20ul.

4. The volume in each well was made up to 1ml using distilled water.

5. Plates were incubated at 37°C for 24h.

6. On removal from the incubator the contents of each well of the plate, except the controls, were placed in glass tubes and the wells washed thoroughly with water and washings collected in the tubes.

7. The tubes were centrifuged at 2,000g for 5mins and the supernatant removed. The larvae remaining were washed with water, centrifuged and supernatant removed 3 times to ensure that any radioactive counts were associated with the larvae and that all unbound drug was removed.

8. The larvae were transferred into vials containing 10ml Ecoscint. The contents of the control wells were placed in vials with Ecoscint. The vials were counted in a Packard counter for 1min.

9. The amount of drug taken up by the larvae, expressed as cpm/100 larvae, was calculated by subtracting the value for the blanks from each of the samples and calculating the mean of the results.

Results

Infective larvae of both strains of *O.circumcincta* were incubated with tritiated ABZ or

tritiated FBZ for 24h and the uptake of each of the drugs by the larvae was measured by counting the radioactivity in the larvae. Results expressed as cpm/100 infective larvae are shown in Table 2.20.

Discussion

The uptake of the benzimidazoles ABZ and FBZ by infective larvae of the two strains of *O.circumcincta* was studied to determine if there was a significant difference in the amount of the anthelmintics absorbed by benzimidazole resistant or benzimidazole susceptible parasites. From results in Table 2.20 the *O.circumcincta* infective larvae of the resistant strain retained a significantly higher concentration of ABZ and FBZ after incubation with the drugs for 24h compared with infective larvae of the Bearsden benzimidazole susceptible strain. With both ABZ and FBZ the larvae of the benzimidazole resistant strain of *O.circumcincta* contained approximately two times the level of drug than larvae of the susceptible strain. A two fold increase in the amount of drug added to the larvae resulted in a 2.5-3 fold increase in the amount of drug taken up by the infective larvae of both strains of parasites. This suggests that the amount of drug absorbed by the parasites was related to the drug concentration to which the larvae were exposed during incubation. Weston *et al.*, (1984) found the uptake of TBZ into *H.contortus* eggs was related to the concentration of the drug in the culture medium. The uptake of benzimidazoles into nematode eggs was believed to be by passive diffusion from the surrounding medium (Weston *et al*, 1984; Sangster and Prichard,1984). The uptake of benzimidazoles into adult nematode parasites has been studied with various species. Sangster and Prichard (1984) studied the uptake of [^{14}C]TBZ by adult *T.colubriformis* using benzimidazole resistant and susceptible strains. These authors found that uptake of the drug was rapid with maximium levels achieved after 30mins. The parasites accumulated TBZ to a concentration approximately 4-5 times that in the surrounding medium. Similar results were found by Kohler and Bachman (1980) who studied the

Table 2.20

Uptake of tritiated ABZ or FBZ expressed as cpm/100 larvae by *O.circumcincta* infective larvae of the Bearsden benzimidazole susceptible and HFRO benzimidazole resistant strains

Strain	Volume of Tritiated Drug Added	cpm/100 larvae ABZ	cpm/100 larvae FBZ
Bearsden	10ul	53.5±2.5	359.5±30.5
Bearsden	20ul	133.5±22.5	910.5±26.5
HFRO	10ul	92.5±19.5	625.0±41.2
HFRO	20ul	283.0±12.6	1841.0±8.5

uptake of MBZ in adult *A.suum*. Sangster and Prichard (1984) compared uptake of TBZ in parasites and red blood cells and found the levels to be similar. These authors concluded that uptake of the drug was by passive diffusion from the surrounding medium and therefore the differential toxicity of benzimidazoles to nematode parasites compared with host toxicity was not due to higher uptake of the benzimidazoles by nematodes.

The possibility that an alteration in the uptake of the benzimidazoles could be responsible for development of resistance to benzimidazoles by nematodes has been investigated. Sangster and Prichard (1984) found that eggs of benzimidazole resistant and benzimidazole susceptible strains of *T.colubriformis* contained similar levels of TBZ after incubation in the drug. Comparison of uptake of TBZ into adults of the resistant and susceptible strains of *T.colubriformis* showed that the rate of uptake of TBZ measured over 30mins was greater in the resistant strain but this difference between the resistant and susceptible strains was not significant. However, the final concentration of TBZ which accumulated in the adult parasites over 22h incubation was significantly greater in the resistant worms compared with the susceptibles. These results were similar to those found with infective larvae of the benzimidazole resistant and susceptible strains of *O.circumcincta* (Table 2.20). Rew *et al.*, (1982) measured the uptake of TBZ by adult parasites of a CBZ resistant strain of *H.contortus* and found the resistant parasites accumulated 15% less TBZ than a benzimidazole susceptible strain of *H.contortus*. These results were at variance with those of Sangster and Prichard (1984) and results given in Table 2.20. However, all these results indicated that a reduction in the uptake of benzimidazoles by parasites is unlikely to be the mechanism by which these parasites have developed resistance to the effects of benzimidazoles.

The increase in the uptake of benzimidazoles observed for the resistant strain of *O.circumcincta* (Table 2.20) was unexpected. The significant difference in uptake of both ABZ and FBZ by the resistant and susceptible larvae may have been due to differences in

the age of the larvae. The larvae which were used in the experiment were stored at 4°C after collection from faecal culture over a similar time period but there could have been a small difference in the age of the larvae. The age of the larvae may affect the levels of stored metabolites within the larvae which could affect the uptake of benzimidazoles. Weston *et al.*, (1984) suggested that the uptake of TBZ into *H.contortus* eggs was proportional to the lipid content of the eggs. There could be a similar effect in larval forms with larvae containing a higher level of lipid absorbing a greater quantity of benzimidazoles. The differences in uptake of benzimidazoles between the two strains may be due to biological variation which could be a reflection of the differing sources and characteristics of the two strains of parasites. The Bearsden strain of *O.circumcincta* has been a laboratory strain for many years whereas the HFRO strain has been selected in the laboratory for 3 years. Because of their different sources the strains could contain totally different gene pools and therefore they may differ in many biological characteristics in addition to the differences in susceptibility to benzimidazoles. This in turn may account for marked differences in the uptake of benzimidazoles by the two strains.

The mode of action of the benzimidazoles is thought to involve binding to tubulin (Friedman and Platzer,1978) and a reduction in the binding of benzimidazoles to tubulin from resistant nematodes has been suggested as a possible mechanism for development of resistance to the effects of benzimidazoles (Lacey and Prichard, 1986). From Table 2.20 larvae of the benzimidazole resistant strain of *O.circumcincta* contained a higher concentration of ABZ and FBZ. However, this may not relate to the capacity of the nematode tubulin to bind benzimidazoles because tubulin constitutes a very small percentage of the total protein in the parasite. Differences in the binding of benzimidazoles to tubulin extracts from the Bearsden and HFRO strains of *O.circumcincta* were observed (see later). The observed alteration in drug uptake in resistant larvae was probably due to differences in other proteins or lipids present in the larvae which may have no relevance

to the level of benzimidazole resistance of the parasites.

GENETICS OF RESISTANCE

Studies on Genetic Material Extracted from Benzimidazole Resistant and Susceptible Strains of *O.circumcincta*

Materials and Methods

Extraction of DNA from *O.circumcincta* infective larvae.

Materials.

Larvae - Infective larvae of the Bearsden susceptible strain of *O.circumcincta* were cultured from the faeces of an experimentally infected lamb. Infective larvae of the HFRO benzimidazole resistant strain of *O.circumcincta* were cultured from the faeces of Lamb DB3 and during the period of culture the faeces were exposed to TBZ at a concentration of 0.01ug/ml to ensure that the eggs which hatched were resistant to the effects of benzimidazoles. Larvae were isolated from faecal culture by Baermanisation (see General Materials and Methods).

Washing buffer - Tris-HCl 5mM, EDTA 100mM, pH8 Lauryl sarcosyl 0.5%

TE buffer - Tris-HCl 10mM, EDTA 0.5mM , pH8

Method.

1. Larval suspensions were centrifuged and excess water removed. Larvae were washed twice in washing buffer and centrifuged with removal of excess buffer.

2. Larvae were weighed and approximately 0.5g of each strain were used in the extraction procedure.

3. The larvae were ground in a mortar and pestle in the presence of liquid nitrogen producing a fine white powder which was resuspended in 15ml of washing buffer.

4. Proteinase K was added at a rate of 50ug/ml and the samples were incubated at 50°C for 2h.

5. The samples were cooled on ice and an equal volume of phenol was added and the samples were mixed gently on a roller for 15min.

6. The samples were centrifuged for 10min at 7,000 r.p.m. (MSE High speed 25 centrifuge).

7. The aqueous phase was removed gently using a wide-mouthed pipette and steps 5 and 6 were repeated using this fraction.

8. Approximately 14ml of aqueous phase was obtained for each sample. To this was added 1.4ml 3M salt solution (final concentration of salt solution 10%v/v) and 2 volumes of absolute alcohol. The samples were mixed gently.

9. The DNA in the samples formed clumped strands which were removed carefully using a glass rod. The strands were washed in 70%ethanol/TE buffer (70%v/v ethanol in 30%v/v TE buffer).

10. The washed DNA was redissolved in 500ul of TE buffer and rolled gently.

11.RNA-ase(10ug/ml) was added to the extracted DNA and incubated at 37°C for 30min.

12. Proteinase K (1 part Proteinase to 200 parts DNA extract) was added and incubated at 37°C for 30mins.

13. An equal volume of phenol was added and mixed gently on a roller for 15min. Samples were centrifuged for 2 min at low speed (< 500r.p.m.) in an Eppendorf bench top centrifuge.

14. The aqueous layer was removed carefully using a wide mouthed pipette and to this was added 1/10 the volume of 3M salt solution and 2 volumes of absolute alcohol.

15. The DNA formed clumped strands and was removed carefully using a glass rod. The DNA was resuspended in 500ul TE buffer and mixed gently.

16. The extract was stored at 4°C.

17. The purity of the DNA extract was assessed by agarose gel electrophoresis.

Segmentation of DNA Extracted from *O.circumcincta* Bearsden and HFRO Strains using Restriction Endonucleases.

Materials.

Restriction endonucleases - Pst I , Hind III and EcoR I.

Core Buffer - 50mM Tris HCl, pH 8, 10mM MgCl and 50mM NaCl (B.R.L.- React 2 Buffer) diluted 1 in 10 with distilled water for use.

Sterile distilled water

Method.

1. DNA extracts prepared by the method described above from infective larvae of the HFRO and Bearsden strains of *O.circumcincta* were used. With each restriction endonuclease used 20ul of DNA extract from Bearsden susceptible strain and 25ul of DNA extract from HFRO strain.

2. The tubes were set up as follows; Tubes 1, 3 and 5 contained 20ul DNA from the Bearsden strain and Tubes 2, 4 and 6, 25ul of DNA from the HFRO strain. To tubes 1 and 2 added 2ul Pst I, tubes 3 and 4 , 2ul Hind III and to tubes 5 and 6, 2ul EcoR I. Core buffer 3.5ul was added to each tube and the volume in the tubes was made up to 35ul using sterile distilled water. Two standard tubes containing standard DNA plus Hind III were also used. The material in the tubes was mixed, centrifuged at low speed in an Eppendorf bench-top centrifuge for 1 min and incubated overnight at 37°C.

3. The tubes were removed from the incubator and heated to 70°C for 10min then stored at 4°C.

4. The DNA fragments were separated using agarose gel electrophoresis.

Agarose gel electrophoresis

Materials./

Materials.

Ethidium bromide - Stock solution 10mg/ml

TBE buffer - 0.089 M Tris borate, 0.089M Boric acid, 0.008M EDTA.

FSB - Final sample buffer - 15% ficoll, 0.25% bromophenol blue, 0.25% xylene cyanol S.F. and 0.25% orange G in TBE buffer.

Agarose.

Method.

1. A solution of 0.8%w/v agarose was constituted in TBE buffer and microwaved for 2 min to boil the solution. The agarose was allowed to cool, ethidium bromide (0.5mg/ml) was added and then the material was poured into the gel apparatus. The end of the gel was left to seal and then the remainder of the solution was added and the gel allowed to set.

2. To asses the purity of DNA isolated from *O.circumcincta* (see above) the gel was loaded with 5ul DNA sample plus 3ul FSB and the gel was surrounded by TBE buffer containing ethidium bromide (0.5mg/ml). For assessment of DNA fragments after digestion with restriction endonucleases 25ul of each sample with 5ul FSB was loaded onto the gel and it was surrounded by TBE buffer containing ethidium bromide (0/5mg/ml). The gels were connected to the power supply and run at 150 volts for 15-30 min then 20 -30 volts overnight.

3. The gels were examined under ultra violet light.

Characteristics of DNA Extracted from Infective Larvae of *O.circumcincta* Bearsden and HFRO Strains

Results

Gel electrophoresis carried out on DNA extracts from infective larvae of *O.circumcincta*, Bearsden and HFRO strains, revealed that the DNA was pure with no contaminating RNA present and no other bands were identified on the gel. The purified DNA of each strain was

subjected to restriction enzyme digestion and the resulting DNA fragments were separated using agarose gel electrophoresis. Figure 2.21 shows the separation of DNA fragments in the gel. Marker DNA which divided into fragments with known numbers of base pairs after incubation with Hind III was used in the experiment to ensure that the restriction enzymes were functioning and there was adequate separation of the DNA fragments in the gel. A graph of the number of bases in each DNA fragment against the distance travelled by the fragments in the gel for the marker DNA was constructed (Fig. 2.22)

Discussion

Pure samples of DNA were isolated from infective larvae of *O.circumcincta* Bearsden and HFRO strains. To ensure that all the larvae cultured from the faeces of Lamb DB3 were resistant to the effects of benzimidazoles the faeces were cultured in the presence of TBZ at a concentration of 0.01ug/ml. Initial studies to culture larvae in the presence of TBZ were carried out. During egg hatch assays on the eggs of the HFRO strain of *O.circumcincta* greater than 90% of the eggs hatched in the presence of TBZ at a concentration of 0.1ug/ml. However, when faeces containing eggs of the HFRO strain of *O.circumcincta* were cultured in the presence of TBZ at a concentration of 0.1ug/ml and the larvae were collected after 12 days of culture at 28°C less than 2% of the eggs had hatched and developed to infective larvae compared with control cultures not exposed to the TBZ. There was a significant difference between hatchability of eggs of the resistant strain of *O.circumcincta* when exposed to TBZ at a concentration of 0.1ug/ml in the egg hatch assays for 48h and the ability of eggs of the same strain to develop to infective larvae in the same concentration of TBZ when incubated for 12 days. These differences could be due to differences in the susceptibility of the different stages of larvae to the effects of benzimidazoles. During development the infective larvae, L_4 larvae and adult parasites are subjected to anthelmintics in the host animal. However the L_1 and L_2 forms of the parasite

Figure 2.21

Results of agarose gel electrophoresis of DNA from Bearsden and HFRO strains of *O.circumcincta* Lanes 1 and 8 contained marker DNA, Lanes 2 and 3 DNA from Bearsden and HFRO strains respectively after incubation with Pst I, Lanes 4 and 5 DNA from Bearsden and HFRO strains respectively after incubation with Hind III and Lanes 6 and 7 DNA from Bearsden and HFRO strains respectively after incubation with EcoR I.

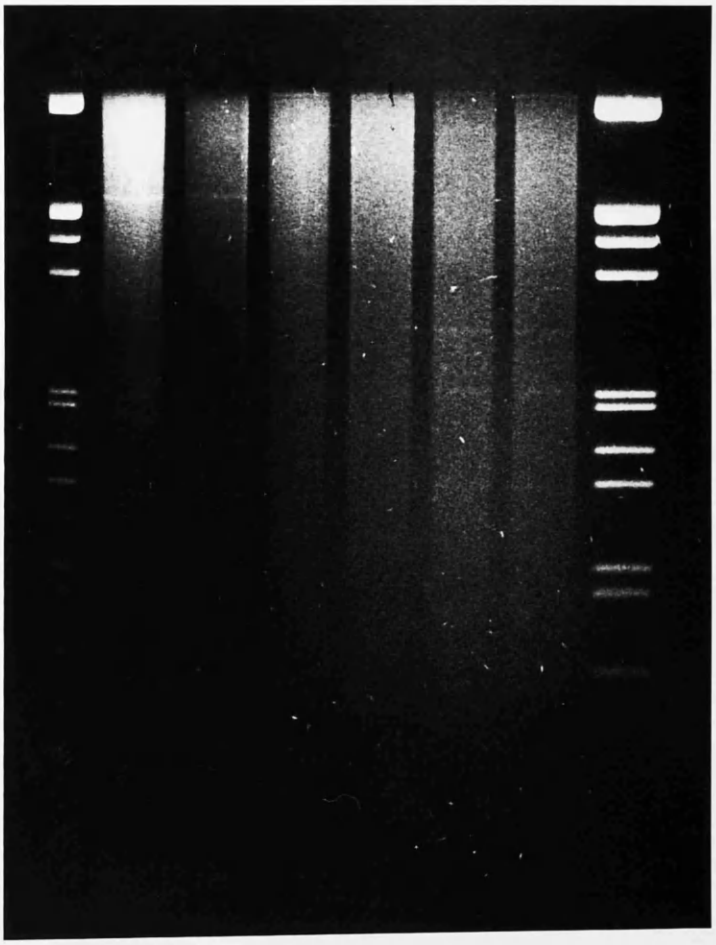
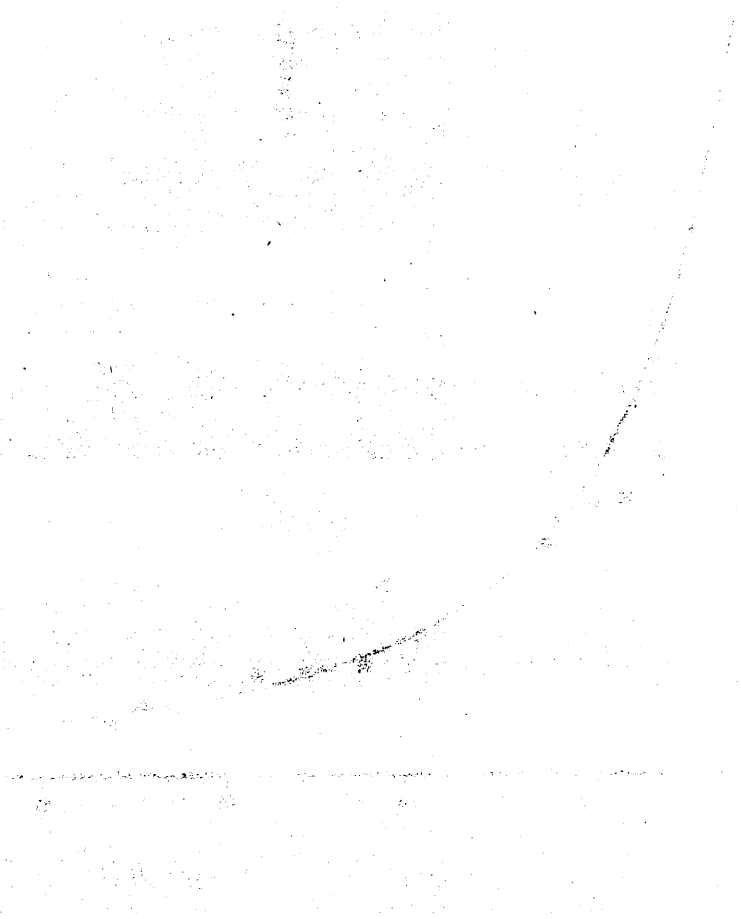
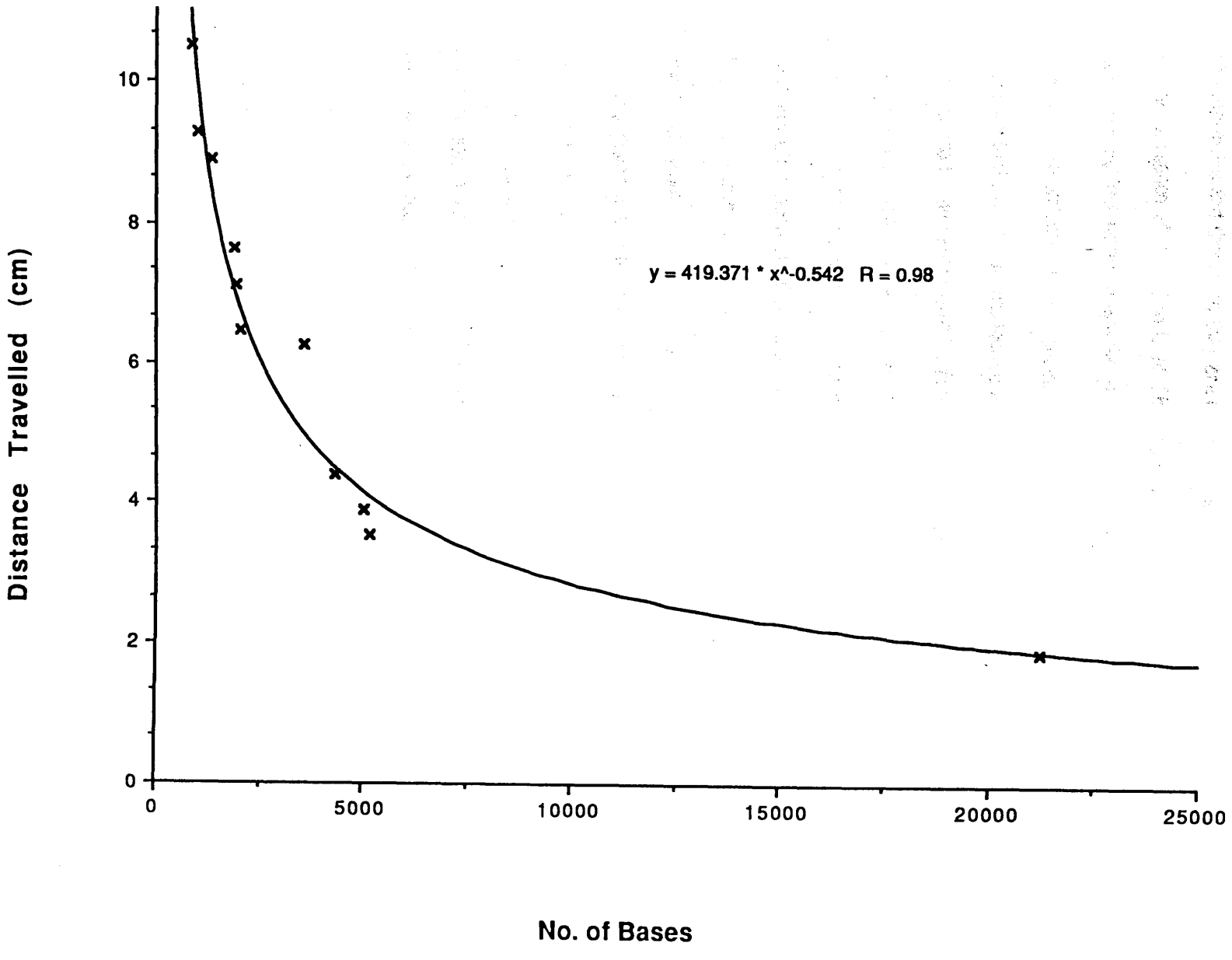


Figure 2.22

Graph of distance travelled during agarose gel electrophoresis by DNA fragments containing a known number of bases.




Distance Travelled (cm)
10
8
6
4
2
0
y = 419.371 * x^-0.542 R = 0.98
0
5000
10000
15000
20000
25000
No. of Bases

are present on the pasture and are therefore not subjected to the effects of anthelmintics. These stages of the parasite may be less resistant to the effects of the benzimidazole anthelmintics and therefore they are unable to develop to infective larvae when cultured in the presence of high levels of TBZ. Alternatively the resistance to benzimidazoles exhibited by the HFRO strain of *O.circumcincta* may be because these parasites can overcome the effects of the benzimidazoles for a short period of time but long term exposure to the drug can be effective in killing the parasites. Within the host the parasites are exposed to the benzimidazoles for a short period of time (approximately 72h for the modern benzimidazoles) and if they can remain *in situ* during this period of exposure they will resist the effects of the drug. However, these parasites may be unable to maintain their position if exposure to the drug was more prolonged. Culture of faeces in TBZ at a concentration of 0.01ug/ml yielded sufficient infective larvae for extraction of DNA to carry out the experiments.

Organisms are able to resist the effect of drugs by various mechanisms including reduction in the uptake of the drug, increased excretion of the drug, increased metabolism of the drug and decreased effect of the drug at the site of action by e.g. changes in the drug receptor sensitivity. All these changes in the phenotype of the organisms occur due to alterations in the genetic characteristics of the organism. Mutations occur within populations and these can be advantageous to the particular organism or detrimental to its survival. Within a population there will be a range of genes any of which can be selected depending on the advantages which they confer on their offspring. Most resistance in organisms to the effects of drug is thought to be due to mutations and the mutant forms become dominant in the population after selection with the particular drug. These mutations can take several forms e.g. change of a single base or several bases on the DNA, deletion or insertion of a portion of the DNA, gene amplification or uptake of extrachromosomal DNA. Benzimidazoles are thought to act by binding to nematode tubulin

(Friedman and Platzer, 1978) and tubulin plays a role in formation of the mitotic spindle. Benzimidazoles have been shown to be teratogenic in some species (Schreiner and Holden, 1983) but there is conflicting evidence relating to the mutagenic effect of the benzimidazoles (Seiler, 1972; Kappas *et al*, 1976; Carere *et al*, 1978; Weir, 1987). If benzimidazoles have a mutagenic effect this could have played a part in stimulating changes in the genetic code of the parasites with a possible influence on the development of resistance.

In some cancer cells resistance of these cells to the effects of chemotherapeutic agents was found to be due to amplification of the genes responsible for the production of the enzyme against which the drug was targeted (Bostock, 1986). Therefore, the cancer cells produced high levels of this enzyme and were able to evade the action of the chemotherapeutic agents. This gene amplification occurred in stepped increments with the base sequences involved in production of the relevant proteins multiplied several times within the one strand of DNA. This type of resistance was found to vary in its stability (Schimke, 1984). Instability of the resistant phenotype can often be linked with cases where there is gene amplification but the gene amplification can remain stable in some cases. The possibility that resistance in nematodes to the effects of benzimidazoles could be due to gene amplification was investigated in studies on *O.circumcincta* DNA from benzimidazole resistant and susceptible strains.

Nematode endoparasites in animals can show a high level of genetic variability within the population (Nadler, 1987). The degree of variability is high for field strains of nematode parasites because of the large population and therefore the large available gene pool. In strains of parasites which have been maintained in a laboratory there is less genetic variability because the gene pool is conserved from one generation to the next. Also new genetic material is not usually introduced into the strain and the genetic pool in parasites used to initiate the strain may represent only a portion of the available genetic

pool. Parasites maintained in a laboratory have a tendency to adapt to the prevailing situation with loss of genetic variability in the strain. Therefore studies on DNA from laboratory maintained strains of parasites may give some indication of the genetic make up of the parasites but cannot reflect the full spectrum of genes available in the entire population of the parasites.

In order to investigate possible differences in the DNA derived from benzimidazole resistant and susceptible strains of *O.circumcincta* the DNA from each strain was fragmented using restriction endonucleases. Each of these restriction enzymes, which are derived from bacteria, bind to a specific sequence of bases found on the DNA strand which is termed the recognition sequence. The enzyme then lyses the DNA at a cleavage site which is usually located within the recognition sequence. In the experiments with DNA from *O. circumcincta* of the Bearsden and HFRO strains restriction enzymes termed 6-cutters were used. These recognise a sequence of 6 base pairs and in a strand of DNA cut randomly using one of these enzymes fragments containing 4^6 bases , 4096 bases, will be produced. The enzymes which were used in the experiments are listed in Fig. 2.23 along with the recognition sequences and cleavage sites for each of the enzymes. When the DNA is digested by the endonuclease, fragments of DNA are produced which can be separated by agarose gel electrophoresis with the rate of migration on the gel inversely proportional to the log of the weight of the fragment.

The DNA from the Bearsden and HFRO strains of *O.circumcincta* was divided by 3 individual nucleases and the resulting fragments were examined. In order to detect differences in the fragments between the two strains there would have to be a change at one of the recognition sites for the endonuclease. Detection of a point mutation using this method is very unlikely because the statistical chance of this type of mutation occurring at the recognition site is very low. However, gross changes in the DNA e.g. gene amplification may be recognised. With gene amplification an area of the DNA is repeated and therefore

Figure 2.23

Recognition sequences and cleavage sites for restriction endonucleases.

Restriction Endonucleases

Pst I ------ recognises C-T-G-C-A↓-G

Hind III ---- recognises A↓-A-G-C-T-T

EcoR I------ recognises G↓-A-A-T-T-C

↓ bond lysed by restriction endonuclease

after cleavage with restriction enzymes there will be a large number of identical fragments from the amplified area. This increase in production of a particular fragment can be recognised as a darker staining band after agarose gel electrophoresis.

Agarose gel electrophoresis was used to separate the fragments produced after cleavage of the DNA from *O.circumcincta* Bearsden and HFRO strains. Examination of the agarose gel (Fig. 2.21) revealed that there were no differences in the DNA fragments obtained from the two strains of *O.circumcincta* This suggested that gross differences in the DNA between benzimidazole resistant and benzimidazole susceptible strains of *O.circumcincta* do not exist. Gene amplification as a mechanism for the development of resistance is therefore unlikely. Mutation of a short sequence of bases would not be identified using this method except in the extreme situation where a change occurred at an endonuclease recognition site. Resistance to benzimidazole in *H.contortus* is thought to be polygenic (Le Jambre *et al.*,1979; Herlich *et al.*, 1981). If resistance was polygenic in *O.circumcincta* the DNA of the resistant parasites may be modified at several sites but these areas of change or mutation may not be recognised by the method described above.

Lacey (1985) described a reduction in the binding of benzimidazoles to tubulin from resistant nematodes compared with the level of binding to tubulin of susceptible parasites as the possible biochemical basis for resistance to benzimidazoles. This would imply that an alteration has taken place in the genes of the resistant parasites which are responsible for the production of tubulin molecules. Foster *et al.*, (1987) found a mutant strain of *Physarium polycephalum* which was resistant to benzimidazoles and produced a β tubulin molecule with altered electrophoretic mobility compared with β tubulin from a non-resistant strain. In addition to the β tubulin with altered electrophoretic behaviour normal β tubulin was produced by the resistant organism. The mechanism controlling the production of the different forms of β tubulin and the genetic basis for development of these tubulin molecules is unknown.

In order to identify components of the genome of *O.circumcincta* the DNA fragments on the agarose gel were used to make a Southern blot. To try to identify components of the genome, DNA probing for known gene sequences e.g. the tubulin gene would be required. Suitable probes are becoming available but further investigation on the DNA from the Bearsden and HFRO strains of *O.circumcincta* has not yet been carried out.

General Discussion and Conclusions

Resistance in sheep nematodes to the effects of benzimidazole and other anthelmintics is of economic importance in Australia, New Zealand, South Africa and countries in South America. However, the incidence of benzimidazole resistance is low in the U.K. Reports of anthelmintic resistance in sheep parasites in the U.K. tend to be from research establishments and in Southern England where *H.contortus* infections occur (Michel *et al.*, 1982). A strain of *O.circumcincta* from premises owned by the HFRO was isolated and studied. Management and climatic differences between the U.K and Australia, New Zealand, South Africa and countries in South America may be important influencing the incidence and the development of nematode resistance to the effects of anthelmintics (Waller, 1987). Also, the prevalence and economic effects produced by *H.contortus* in Australia, New Zealand, South Africa and some countries in South America are of significantly greater importance than in the U.K where *H.contortus* occurs only in the southern parts of the country. Early identification and prevention of widespread development of resistance to a range of anthelmintics is important to ensure that anthelmintic resistance does not become a major problem to the sheep industry in the U.K.

Various tests are available to detect resistance including the egg hatch assay (Le Jambre, 1976; Coles and Simpkin, 1977), the faecal egg count reduction test, larval motility assays for levamisole and morantel (Martin and Le Jambre, 1979), tubulin binding assays (Lacey and Snowdon, 1988) and the controlled anthelmintic efficiency test

(Powers *et al*, 1982). Each of these tests has various drawbacks, however, the *in vitro* egg hatch assay is used most commonly to test for resistance against benzimidazoles. In egg hatch assays using TBZ and ABZ results were paralleled with resistance levels for ABZ higher than with TBZ in most cases. This suggested that TBZ penetrated the eggs better and lower concentrations were required to exert an ovicidal effect on the eggs. However, differences in the resistance levels measured in the eggs of parasites may not reflect differences in the resistance of adult parasites (Le Jambre *et al*, 1979). This may be due to differences in the genetic control of resistance at different stages in the development of the parasites. In the dose titration trial selection of adult parasites using high dose rates of fenbendazole failed to increase the resistance in the eggs produced by these adult parasites. Also, during 14 generations of the HFRO strain of *O.circumcincta* with 12 treatments using FBZ the level of resistance measured by egg hatch assays showed a negligible increase. Similar results have been found (Waller *et al*, 1985; Borgsteede, personal communication, 1987) with failure to show a rise in resistance in egg hatch assays after selection of parasites. With the HFRO strain of *O.circumcincta* although variations in the level of resistance found in parasite eggs were detected during an infection the results of the dose titration trial and the egg hatch assays suggested that the strain was highly resistant to the effects of benzimidazoles and the population appeared to be relatively homogeneous.

During tests with infective larvae of *O.circumcincta* incubated in the presence of benzimidazoles differences in the motility of larvae of the susceptible and resistant strains were detected. Because of the mode of action of the benzimidazoles a long period of incubation was required before motility of the larvae was affected and differences between resistant and susceptible strains of parasites were small. Low numbers of infective larvae can be used for a larval motility test with physostigmine to detect differences in the acetylcholinesterase activity between benzimidazole resistant and susceptible strains of

nematodes (Sutherland *et al*, 1988). The relationship between acetylcholinesterase activity and resistance to benzimidazoles has not been elucidated and therefore this test may not be suitable to detect low levels of resistance in populations of nematode parasites.

There have been reports of reduction in the level of resistance in populations of *T.colubriformis* and *H.contortus* on withdrawal of anthelmintic selection (Simpkin and Coles, 1978) but later reports (Hall *et al*, 1978b) suggested that withdrawal of selection pressure failed to reduce resistance in *T. colubriformis* and *H.contortus* to TBZ. In studies with the HFRO strain of *O.circumcincta* removal of pressurisation with FBZ over four generations did not alter the level of resistance measured in egg hatch assays. Counterselection using levamisole can reduce the level of benzimidazole resistance in strains of *O.circumcincta* (Donald *et al*, 1980; Waller *et al*, 1983; Martin *et al*, 1988) but resistance levels rose rapidly on reintroduction of the benzimidazoles.

In studies on 'clones' of the HFRO strain of *O.circumcincta* established after transplantation of a pair of adult parasites into the abomasum of a lamb there was loss of resistance in the eggs produced by the second generation after transplantation. However, selection with benzimidazole anthelmintics caused a rapid rise in the level of resistance which suggested that genes conferring resistance were present in the parasites and could be selected readily by pressurisation with benzimidazole anthelmintics. Results of these studies suggested that resistance could be a polygenic trait but controlled by a small number of genes

The main problem with genetic studies in parasites is that breeding experiments using single adult parasites *in vitro* are very difficult to perform (Douvres and Malakatis, 1977) and backcrosses to the original parents are impossible because of the short lifespan of the adult parasites. Some breeding experiments using benzimidazole resistant and susceptible *T.colubriformis* have been carried out (Martin *et al*, in press) using a guinea pig model. Resistance was controlled by more than one gene and inherited as a

semi-dominant character with a strong maternal influence. A similar mechanism of inheritance possibly could occur in *O.circumcincta*

Another problem with studies on the genetic inheritance of resistance was testing the level of resistance when small numbers of parasites were avaliable. In order to carry out egg hatch assays for benzimidazole resistance with *O.circumcincta* a faecal egg count of >100 eggs per gram was necessary.

Changes in the level of resistance in strains of nematodes maintained in the laboratory and used in experimental infections may not reflect changes in field strains of parasites. In the field parasites on the pasture which are not exposed to the effects of anthelmintics play a role in the development of resistance within the whole population. The number of parasites *in refugia* was found to influence the rate of development of resistance in benzimidazole resistant strains of *H.contortus* (Martin *et al*, 1981).

The frequency of anthelmintic treatment and the dose rate of anthelmintics used have been implicated as factors which influence the development of anthelmintic resistance in nematodes (Martin *et al*, 1982; Martin, 1987). In the dose titration experiment treatment of adults of the HFRO strain of *O.circumcincta* with increasing doses of fenbendazole failed to increase the level of resistance measured by egg hatch assays. Resistance in a population of parasites may reach a high level and further selection with anthelmintics cannot increase the level of resistance. In areas of Australia for parasite control schemes implemented on sheep farms it is recommended that an adequate dose of anthelmintic should be used to remove a high proportion of the parasite burden within the animals and therefore the low numbers of parasites surviving anthelmintic treatment are not allowed to predominate in the parasite population (Dash *et al*, 1985).

Side - resistance with benzimidazoles is found and egg hatch assays using TBZ and ABZ produced similar patterns of results. The metabolites of ABZ, ABSX and ABSO and the pro-drug netobimin were not ovicidal when used in egg hatch assays. However, ABSX was

effective in reducing larval motility when infective larvae of *O.circumcincta* were incubated in this metabolite. Within the host the modern benzimidazole anthelmintics exist in a chemical equilibrium with the suphoxide form of the drug predominating and low levels of the sulphide form found in plasma and tissues (Marriner and Bogan, 1980, 1981a and 1981b). The close relationship between the sulphide and sulphoxide structures may allow both forms to bind to parasites and have an anthelmintic action and the failure of ABSX to exert an ovicidal effect in egg hatch assays may be due to its inability to penetrate the egg shell of *O.circumcincta* For egg hatch assays TBZ, which has greater water solubility than the modern benzimidazoles, is the drug used most commonly.

Isolation of DNA from *O.circumcincta* HFRO and Bearsden strains was achieved and there were no obvious differences in the DNA after separation with some restriction enzymes. This implied that gene amplification was not involved in the development of resistance to benzimidazoles but examination of the DNA using this method would not identify small changes in the genetic code unless they occurred at an area where the restriction enzymes had their action.

The mechanism of action of the benzimidazole anthelmintics has not been described fully and therefore it is difficult to identify the mechanisms by which parasites can become resistant to the effects of these anthelmintics. Exposure to the benzimidazoles over a prolonged period is necessary to kill the parasites. Therefore, parasites which have the ability to withstand the effects of the benzimidazoles for the short period of time of exposure to the drug in the host are resistant. This may be achieved by alterations in binding of the drug or parasite metabolism. An increase in the uptake of ABZ and FBZ into resistant compared with susceptible infective larvae of *O.circumcincta* (HFRO and Bearsden strains) was found. Similar differences in drug uptake were described for benzimidazole resistant and susceptible *T.colubriformis* adults (Sangster and Prichard, 1984) but adults of a resistant strain of *H.contortus* accumulated a lower concentration

of benzimidazoles than a susceptible strain (Rew *et al.*, 1982). The significance of these differences in drug uptake by resistant and susceptible parasites is unknown as is the importance of these differences to the ability of the parasites to resist the effects of the benzimidazole anthelmintics. Some of these differences could be due to population variation in the strains of *O.circumcincta* which were obtained from different sources and therefore would have little or no relevance to the mechanisms of development of resistance to benzimidazoles.

The genetic pool in a population may influence the rate of development of resistance to different classes of drugs. *H.contortus* populations in Australia developed resistance to benzimidazoles rapidly but resistance to levamisole and morantel was slower to develop (Waller, 1985). With *T.colubriformis* and *O.circumcincta* resistance to levamisole and morantel along with benzimidazole resistance developed rapidly (Waller, 1985; Waller 1987). Before resistance to an anthelmintic is identified in the field a high level of resistance to the appropriate drug has developed (Waller, 1986). From results in the dose titration trial treatment with increased doses of benzimidazoles did not increase the level of resistance in eggs of the surviving parasites but equally once a population of parasites resistant to anthelmintics is established removal of selection pressure does not guarantee a return to anthelmintic susceptibility even after a prolonged period. These findings have important implications for parasite control in the sheep industry especially in areas of Australia where parasite populations showing multiple resistance to anthelmintics are established and livestock rearing in these areas is now dependent on the use of ivermectin and closantel with benzimidazoles and levamisole/ morantel proving useless for the control of nematode parasites. In the U.K. the situation regarding anthelmintic resistance is much less serious possibly due to differences in the predominance of parasite species, climate and management systems but the genetic ability to develop resistance to anthelmintics is present in some populations of parasites.

SUMMARY

A benzimidazole resistant strain (HFRO strain) and a benzimidazole susceptible strain (Bearsden strain) of the sheep abomasal parasite *Ostertagia circumcincta* were obtained and passaged through parasite naive lambs. Using egg hatch assays to detect resistance day to day changes in the susceptibility of the eggs from adult *O.circumcincta* of these strains to the effects of the benzimidazoles, thiabendazole and albendazole were noted. Treatment of the lambs which were parasitised with the HFRO strain with fenbendazole at the normal dose rate caused a temporary increase in the resistance level measured by egg hatch assays. However, 12 treatments with fenbendazole over a 14 generation period with the HFRO strain produced a negligible rise in benzimidazole resistance for this strain.

A dose titration trial to determine the effectiveness of fenbendazole against adult parasites of the HFRO strain of *O.circumcincta* confirmed that the parasites were highly resistant to the effects of the benzimidazole anthelmintics. Treatment with increasing doses of fenbendazole used in the dose titration trial failed to increase the level of resistance in the eggs produced by the adult parasites remaining after treatment.

The establishment of 'clones' of the HFRO strain of *O.circumcincta* by implantation of pairs of adult parasites into the abomasa of lambs was investigated. The offspring of these parents showed a reduced level of resistance to the benzimidazoles measured by egg hatch assays but after treatment with fenbendazole selection for resistance occurred rapidly.

The DNA from the two strains of *O.circumcincta* was isolated and compared after sectioning with restriction endonucleases. The banding obtained after gel electrophoresis was similar for the two strains.

Studies were carried out on the motility of infective larvae of the HFRO and Bearsden strains of *O.circumcincta* in the presence of benzimidazole anthelmintics, their metabolites and physostigmine. Prolonged incubation in the presence of benzimidazoles was required to reduce motility. Differences in the motility in the presence of

physostigmine between benzimidazole resistant and benzimidazole susceptible strains of parasites were found. The activity of the benzimidazole metabolites against larval stages and eggs of *O.circumcincta* was studied and discussed.

TUBULIN AND MICROTUBULES

INTRODUCTION

Microtubules are tubular structured polymers of protein which form part of the cytoskeleton within cells composed of subunits of the protein tubulin . They are associated with the mitotic spindle, centrioles, neurotubules , cilia and flagella and are necessary for the maintenance of cell shape, transport of material within cells and mitosis (Dustin, 1984). Tubulin has been isolated from all groups of vertebrates and many invertebrates e.g. fungi, helminths and plants. The tubulin molecules isolated from these diverse sources are closely related but not identical.

The tubulin molecule is a dimer composed of two closely related subunits α and β tubulin. These subunits have approximate molecular weights of 50,000 (Valenzuela *et al.*, 1981). The α and β peptides are thought to have derived from a common protein because of close similarities in their amino acid sequences with approximately 42-50% homology of bases (Krauhs *et al.*, 1981; Ponstingl *et al.*, 1981). Slight differences in the structure of the tubulin molecules have been found among species and also within a single animal e.g. in the mouse 7 α tubulin and 14 β tubulin isotypes of the subunits have been identified which are produced in varying quantities during development (Denoulet *et al*, 1982). However, antibodies prepared against tubulin in the rabbit can be used in immunological techniques to identify tubulin in a wide range of species indicating that the antigenic determinants are similar (Dales ,1972)

The tubulin dimer binds two molecules of guanosine nucleotide either GDP or GTP In the dimer form one molecule of the nucleotide is fixed but the nucleotide attached to the β tubulin unit can exchange with GTP in solution and the presence of GTP is thought to play a fundamental role in the assembly of dimers into the microtubule structure (Geahlen and Haley,1979) Other proteins are associated with microtubules including enzymes necessary for assembly, high molecular weight microtubule associated proteins (MAPs)

and *tau* proteins. MAPs and *tau* proteins play a role in the assembly of tubulin into microtubules and the stability of the formed polymer (Murphy *et al*, 1977).

Tubulin molecules can assemble into microtubules both *in vivo* and *in vitro* and an equilibrium can be set up between tubulin dimer and microtubules. The concentration of tubulin dimer in the cell *in vivo* may have a regulatory effect on the rate of formation of microtubules and the rate of production of tubulin mRNA (Cleveland and Kirschner, 1981). In vertebrate cells about 50% of the tubulin in cells is present in the free dimer form (Hiller and Weber, 1978).

There are many conditions which are necessary to facilitate assembly of microtubules *in vitro* (Weisenberg, 1972) including presence of GTP, adequate concentration of tubulin, a low level of calcium ions, presence of magnesium ions (Lee and Timasheff, 1977), presence of glycerol (Shelansky *et al*.,1973) and suitable temperature.

Studies on the assembly of microtubules suggest that the helical structure which is produced has polarity. The microtubule is in a dynamic relationship with free tubulin dimers and assembly and disassembly occurs at either end of the microtubule but at differing rates. "Treadmilling" occurs with an overall predominance of addition of tubulin and GTP to one end of the helical structure and loss of tubulin and GDP at the other end. In addition to assembly into microtubules other polymer forms can be identified including rings, ribbons, sheets, stacks of rings, cylinders and arrays (Burton, 1981).

In vitro the process of tubulin assembly into microtubules under suitable conditions can be measured spectrophotometrically as an increase in turbidity (Gaskin *et al*, 1974). This effect can be utilised in the study of inhibitors of microtubule formation. Many drugs are known to act as inhibitors with the cytotoxic drug colchicine the first to be discovered and its inhibitory actions described (Borisy and Taylor,1967). Distinct binding sites have been identified on the tubulin molecule where inhibitors are thought to bind. The colchicine binding site is shared by podophyllotoxin (Cortese *et al*.,1977) and the

benzimidazoles (Hoebeke *et al*, 1976). A wide range of benzimidazoles has been tested as inhibitors of tubulin polymerisation and is thought to bind to the same site on the tubulin molecule (Barrowman *et al*, 1984). The vinca alkaloids bind at a different site (Bryan, 1972).

Binding of colchicine to tubulin and the incorporation of this complex into the growing microtubule decrease the rate of assembly of the microtubule by blocking further addition of tubulin dimers to the microtubule. The colchicine tubulin complex was shown to occur at a ratio of 1 molecule of colchicine to 1 tubulin dimer (Bryan, 1972). Work with pure mammalian tubulin and parbendazole suggested that the benzimidazoles bind to the tubulin dimer in a ratio of 1:1 (Havercroft *et al*, 1981). It was suggested that colchicine binding to mammalian tubulin is not easily reversible whereas that of benzimidazoles is more readily reversible (Wilson *et al*,1974)

One of the possible modes of action of the benzimidazole anthelmintics is binding to tubulin and preventing the formation of microtubules in helminths (Hoebeke *et al*.,1976; Friedman and Platzer, 1978). Using electron microscopy ultrastructural changes in the microtubular network in cells in nematodes and cestodes have been identified (Borgers and De Nollin, 1975; Verheyen *et al*, 1976). In addition to ultrastructural studies, because colchicine and benzimidazoles bind to tubulin at the same site, the amount of benzimidazole binding to tubulin can be measured indirectly by competition with colchicine binding (Friedman and Platzer, 1978).

Like colchicine the benzimidazoles bind to tubulin from a wide variety of sources including mammalian, fungal and helminth tubulin. This widespread activity does not explain the selective toxicity of the benzimidazoles to helminths and fungi.

Isolation of mammalian tubulin to study inhibition of assembly into microtubules in the presence of the benzimidazoles is relatively simple because of the high level of tubulin in the extracts from mammalian brain. This tissue contains about 20-40 % of tubulin in

the soluble protein fraction. In helminths tubulin constitutes a low proportion of the soluble protein fraction and therefore isolation and purification of sufficient quantities of tubulin is difficult (Gull *et al.*, 1987). Several methods have been used to isolate and purify tubulin from mammalian brain with the principle methods being ammonium sulphate precipitation followed by DEAE chromatography (Weisenberg *et al.*, 1968) and cycles of assembly and disassembly (Shelansky *et al.*, 1973). To isolate tubulin from helminths crude tubulin extracts can be prepared by ultracentrifugation (Barrowman *et al.*,1984) and purified by ammonium sulphate fractionation or prepared by rapid bench top centrifugation (Lacey and Snowdon, 1988). Preparation of pure extracts from helminths using column chromatography has been described (Gull *et al.*,1987; Lacey and Snowdon , in preparation).

Studies on the inhibition of tubulin polymerisation using nematode and mammalian tubulin showed that one group of benzimidazole anthelmintics including FBZ, PBZ and MBZ had equal potency against both types of tubulin whereas OFZ and TBZ had greater efficacy as inhibitors of polymerisation of nematode tubulin with little effect on mammalian tubulin (Gull *et al*, 1987)

Further studies on microtubules in helminths (Davis and Gull, 1983) revealed that the number of protofilaments in microtubules in helminth cells differed from the number predominating in mammalian cells. There are usually 13 protofilaments in microtubules in mammalian cells and microtubules with 11, 12 and 14 protofilaments were identified in helminth cells.These ultrastructural differences may play a role in the differential toxicity of benzimidazoles to helminths.

The synthesis of tritiated benzimidazoles allowed the direct measurement of binding of these compounds to tubulin extracts *in vitro* (Lacey and Prichard, 1986). Binding of benzimidazoles to mammalian tubulin was unstable to charcoal extraction (Lacey, personal communication,1988) unlike binding of benzimidazoles to tubulin extracted

from nematodes which was stable to charcoal extraction (Lacey and Prichard, 1986). Using tubulin extracts from the nematode parasite *H.contortus* the benzimidazoles were ranked on the affinity of the interaction to tubulin and ABZ had the strongest affinity followed by FBZ, PBZ, OBZ, MBZ and OFZ. This does not seem to reflect the efficacy of these drugs as anthelmintics when used *in vivo*. Lacey and Prichard (1986) suggested that resistance in parasites to the effect of benzimidazoles was due to alteration in the degree of binding of benzimidazoles to tubulin in these strains. These authors showed that tubulin extracted from benzimidazole resistant strains of *H.contortus* bound significantly less benzimidazole than extracts from benzimidazole susceptible strains. The differential binding of benzimidazoles to tubulin extracts from nematodes was suggested as a biochemical method to detect resistance to benzimidazoles in nematode parasites (Lacey and Snowdon, 1988)

PREPARATION OF TUBULIN EXTRACTS

Materials and Method

Preparation of Tubulin Extracts from *Ascaris suum*

(Method modified from Barrowman *et al*, 1984)

Reagents.

Parasites - Adult *Ascaris suum* were obtained from the gastrointestinal tract of pigs slaughtered at the Glasgow abattoir.

Homogenisation Buffer -10uM potassium dihydrogen orthophosphate/dipotassium hydrogen orthophosphate (KH_2PO_4/K_2HPO_4) containing 1mM magnesium sulphate ($MgSO_4$), 0.1mM guanosine 5'-triphosphate (GTP), 25%(v/v) glycerol and 1uM phenylmethane sulphonyl fluoride (PMSF), pH 7.

Method.

1. Intestines were isolated from *A.suum* by dissection.

2. Intestinal material was homogenised in homogenisation buffer for 5 min using a hand-held glass homogeniser.

3. The homogenate was centrifuged at 100,000g for 1h using a SW 41 rotor and Beckman ultracentrifuge (L8-55).

4. Supernatant was retained as the crude tubulin extract and was stored at -20°C.

5. An aliquot of the crude tubulin extract was assayed for protein using a Lowry protein assay (see later).

6. Purity of the crude extract was assessed using sodium dodecylsulphate-poly acrylamide gel electrophoresis (SDS-PAGE) (see later).

Further purification of Crude Tubulin Extract.

1. Crude extract was adjusted to a final concentration of 200g/l ammonium sulphate added over 15 min at room temperature.

2. Precipitate was allowed to collect for 30 min at room temperature.

3. The mixture was centrifuged at 2,000g for 1h and pellet discarded.

4. Supernatant was dialysed overnight against 3 changes of modified homogenising buffer (GTP added at a concentration of 1uM).

5. Purified extract was stored at -20°C.

6. The purity of the extract was checked using SDS-PAGE (see later).

Preparation of Tubulin Extracts from Infective larvae of *Ostertagia circumcincta* and *Haemonchus contortus* (Method A).

Reagents.

Washing buffer - 0.025M 2(N-morpholino)ethanesulphonic acid (MES) buffer, pH6.5 containing 1mM ethylene glycol bis-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA) and 0.5mM $MgSO_4$.

Homogenising buffer - Washing buffer plus 0.1M GTP.

Method.

1. Infective larvae were obtained after Baermanisation and counted. The larvae were washed in distilled water and centrifuged at 1,000g for 5min and the supernatant removed.

2. The larvae were washed in 10ml washing buffer, centrifuged as above and supernatant removed.

3. This was followed by a wash in homogenising buffer, centrifugation as above and removal of the supernatant.

4. The pellet of larvae was transferred to a glass hand-held homogeniser in a small volume of homogenising buffer (approximately 0.5ml per 100,000 larvae).

5. The larvae were homogenised for 3min and an aliquot was checked under a dissecting microscope to ensure thorough disintegration of the larvae.

6. The homogenate was transferred to a 1.5ml Eppendorf tube and the homogeniser was rinsed using a small volume of homogenising buffer and rinsings combined with the homogenate. The overall volume in the Eppendorf tube was adjusted to approximately 0.7ml for 100,000 larvae up to 1.5ml for 400,000 larvae.

7. Tubes were placed in a bench-top Eppendorf centrifuge and centrifuged at 10,000 r.p.m. for 10min.

8. After centrifugation the supernatant was removed and stored on ice. This fraction contained the crude tubulin extract.

9. A protein assay was carried out on an aliquot of the crude extract using either a Lowry protein assay (see later) or a BioRad protein assay (see later).

Alternative Method for the Preparation of Tubulin Extracts from Infective Larvae of *O. circumcincta* (Method B).

Reagents./

Reagents:

Buffer - 10uM KH_2PO_4/K_2HPO_4 containing 1mM $MgSO_4$, 0.1mM GTP, 25%(v/v) glycerol and 1uM PMSF, pH 7.

Method.

1. Infective larvae of *O.circumcincta* were obtained after Baermanisation and counted under a dissecting microscope. A known number of larvae were washed in distilled water and centrifuged at 2,000g for 5min and the supernatant removed.

2. The larvae were homogenised in a small volume of buffer in a glass hand-held homogeniser for 3min and an aliquot examined under a dissecting microscope to ensure adequate homogenisation.

3. The homogenate was transferred to a 0.8ml ultracentrifuge tube making up the volume to 0.8ml using buffer

4. Tubes were centrifuged in a Beckman ultracentrifuge (L8-55) using a SW 50 rotor at 4°C and 100,000g for 1h.

5. The supernatant was retained as the crude tubulin extract and an aliquot used in a Lowry protein assay (see later)

Preparation of Tubulin Extracts from eggs of *O. circumcincta*

Reagents.

Buffer - 10uM KH_2PO_4/K_2HPO_4 containing 1mM $MgSO_4$, 0.1mM GTP, 25% (v/v) glycerol and 1uM PMSF, pH 7.

Method.

1. Eggs were collected from fresh faeces using salt flotation (see previously) and washed on a 38micron sieve. The egg suspension was centrifuged, the supernatant removed and the eggs resuspended in buffer. Aliquots of the egg suspension were counted under a dissecting

microscope and an estimate of the total egg number was made.

2. The eggs were homogenised for approximately 5min in a hand-held glass homogeniser and an aliquot was examined under a dissecting microscope to ensure thorough disintegration of the eggs.

3. The homogenate was transferred to a 0.8ml ultracentrifuge tube and ultracentrifuged at 4°C for 1h at 100,000g (SW 50 rotor, Beckman ultracentrifuge - L8-55).

4. The supernatant was retained and a Lowry protein assay (see later) carried out on an aliquot of the extract. The extract was used in a tubulin binding assay (see later)

Lowry Protein Assay (from Lowry *et al*, 1951)

This protein detection method employs the principle that a copper-tartrate complex reacts with the protein in alkaline solution and this complex reduces phosphomolybdate to form a blue substance which can be measured quantitatively by measuring its absorbance at 500 or 750nm.

Reagents.

Solution A -2% (w/v) Sodium carbonate in 0.1M Na OH.

Solution B -1% (w/v) Copper sulphate .$5H_2O$.

Solution C - 2%(w/v)Sodium potassium tartrate.

Solution D - Equal volumes of solutions A and B mixed.

Solution E - 1ml of solution D mixed with 50ml solution C.

Solution F - Folin and Ciocalteu's phenol reagent diluted 1:1 (v/v) with deionised water.

Standard protein solutions - A range of concentrations of bovine serum albumin (BSA) in distilled water from 0.05-2mg/ml.

Method.

1. Duplicates of 0.1ml of each sample, standard or water (blank) were placed in glass test tubes.

2. To each was added 1ml solution E vortexed and allowed to stand for 10 min at room temperature.

3. Added 0.1 ml solution F whilst vortexing and allowed to stand for 30 min at room temperature.

4. Absorbance was measured at 500nm (or 750nm) in a spectrophotometer (Pye Unicam SP8-500 UV/VIS Spectrophotometer)

5. A standard curve for protein concentration against absorbance was plotted using the results obtained with the BSA standard solutions and from this the protein concentration in the test samples was calculated after correction using the blank.

BioRad Protein Assay

This is a dye-binding assay based on the colour change of an acidic solution of Coomassie Brilliant Blue G-250 in response to binding of different protein concentrations, measured spectrophotometrically at 595nm .

Reagents.

BioRad dye reagent concentrate - Solution of dye, phosphoric acid and methanol.

Buffer - 0.025M MES buffer, pH 6.5 containing 1mM EGTA, 0.5mM $MgSO_4$ and 0.1M GTP.

Standard solutions - A range of solutions of BSA 5-50 ug/ml in distilled water

Method.

1. The dye reagent was diluted 1 in 5 (v/v) using distilled water.

2. Duplicates of a known volume(0.02-0.05) of sample, standard or buffer (blank) were placed in a glass test tube.

3. 5ml of the diluted dye reagent was added to each tube whilst vortexing and allowed to stand at room temperature for at least 5 min.

4. The absorbance was measured at 595nm.

5. A standard curve of protein concentration against absorbance at 595nm was drawn

using results of the BSA standards. From this standard curve the protein in the samples was estimated after correction using the absorbance value of the blank

Sodium Dodecylsulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) with Discontinuous Buffers (Disc gel system described by Laemmli,1970)

Reagents.

Acrylamide stock solution - 30g acrylamide and 0.8g N' -N methylenebisacrylamide made up to 100ml with distilled water Stored in dark at $4^{0}C$

Resolving gel buffer - 0.25M Tris-SDS stock solution, pH6.8 (39.4g Trizma-HCl and 2g SDS, pH adjusted to 6.8 and made up to 1,000ml with distilled water).

Stacking gel buffer - 0.75M Tris-SDS stock solution, pH8.8 (71g Trizma base, 47.2g Trizma -HCl and 2g SDS, pH adjusted to 8.8 and made up to 1,000ml with distilled water).

Electrode buffer - 0.025M Tris, 0.192M glycine buffer (15.15g Trizma base, 72g glycine and 5g SDS adjusted to pH8.8 and made up to 5,000ml with distilled water).

Sample buffer - 0.0625M Tris-SDS buffer(25ml resolving gel buffer, 2g SDS, 10ml glycerol, 5ml 2-mercaptoethanol, 0.1ml 1%bromophenol blue made up to 100ml with distilled water).

Ammonium persulphate solution - 1%(w/v) ammonium sulphate in distilled water. Made up freshly

Staining solution - 0.1% Coomassie Brilliant Blue in methanol/acetic acid/ water 5:4:1 by volume.

Destaining solution - Methanol/acetic acid/water 1:1:8 by volume.

Method

1. Glass plates 8cm by 8cm were sealed at the sides using plastic tape and placed in an oven overnight at $60^{0}C$ to ensure adequate sealing.

2. Resolving gel was prepared using 19.8ml acrylamide stock solution and 15ml separating gel buffer made up to 60ml with distilled water and degassed thoroughly. To this was added 25ul N,N, N',N' - tetramethyl-ethylenediamine (TEMED). This mixture was divided into two equal portions.

3. To half of the resolving gel mixture was added 0.75ml ammonium persulphate solution and this mixture was poured between the prepared plates and allowed to set for 15min to seal the base of the gel.

4. 0.75 ml ammonium persulphate solution was added to the remainder of the resolving gel and this was introduced between the plates.

5. On the top of the gel was placed a layer of 20% methanol to ensure a flat surface was maintained during setting of the gel for 15-20 mins. The methanol was poured from the gel surface and the gel stored at $4^{0}C$ overnight.

6. Stacking gel was prepared using 3ml stock acrylamide and 5ml stacking gel buffer made up to 20ml with distilled water and degassed thoroughly. To this was added 20ul TEMED and 0.5ml ammonium persulphate solution. This was poured onto the surface of the resolving gel and allowed to set.

7. Sample preparation involved mixing 50ul sample with 50ul sample buffer and boiling for 5min. A mixture of standard proteins of molecular weights 12,300-78,000 was boiled in sample buffer. After boiling the mixtures were left to cool for 10min.

8. Duplicates of 30ul of each sample or standard were loaded onto the top of the stacking gel.

9. The gel was placed in a Pharmacia gel electrophoresis system surrounded by electrophoresis buffer. The gel was run at 60 volts and 30 amps for 15 min then 120 volts and 65 amps with circulation of the buffer for approximately 2h.

10. The gel was removed from the plates and stained in staining solution for 1h followed by overnight destaining in the destaining solution.

11. The gel was examined and a graph of molecular weight against distance travelled in the gel by proteins was constructed for the protein standards.

Results and Discussion

Results of SDS-PAGE of Tubulin Extracts from *Ascaris suum*

A crude tubulin extract from *A. suum* which contained 15.3mg/ml protein and a purified crude extract with protein content 10.7 mg/ml were prepared. Results of SDS-PAGE using these samples are shown in Fig. 3.1. The distance travelled by the standards and their molecular weights is shown in Table 3.1 and a standard graph drawn of distance travelled against the log of molecular weight (Fig. 3.2). The distance travelled by bands in the tubulin samples is shown in Table 3.2 and their approximate weights calculated using the standard graph (Fig. 3.2).

Preparation of Tubulin Extracts - Discussion

Analysis of extracts from *A.suum* by SDS-PAGE produced a wide protein band of approximately 52,000 molecular weight corresponding to tubulin (Fig. 3.1) Molecular weights of α and β tubulin were found to be approximately 53,000 and 56,000 (Dentler *et al*, 1975). In the SDS-PAGE of extracts from *A. suum* the wide band at approximately 52,000 molecular weight could incorporate the combined bands of the α and β tubulin molecules which have failed to separate on the gel. There was no evidence of a band at 100,000 molecular weight corresponding to the molecular weight of the tubulin dimer. Other lower molecular weight constituents, possibly smaller enzyme molecules, were present on the gel (Fig. 3.1 and Table 3.2) but they were not identified accurately. The band at approximately 67,000 molecular weight may correspond to *tau* proteins which are low molecular weight microtubule associated proteins with molecular weights 55,000-70,000 (Weingarten *et al*, 1975). There was no difference on SDS-PAGE between the crude tubulin extract and the purified crude extract. Ammonium sulphate fractionation failed to remove contaminating small proteins. However, for both extracts

Figure 3.1

Diagram of SDS PAGE showing separation of protein standards, crude tubulin extract and partially purified extract from *A. suum* intestinal material in columns 1,2 and 3 respectively.

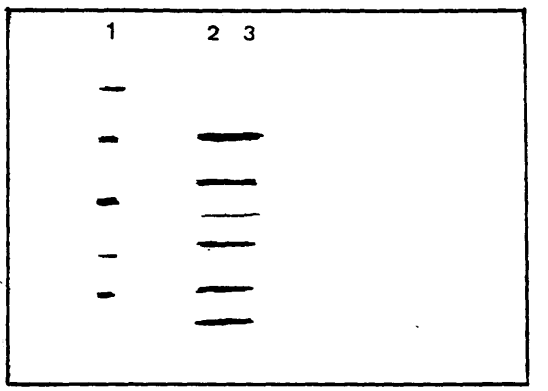

1
2 3

Table 3.1

Distance travelled and molecular weights of standard proteins used in SDS-PAGE

Protein	Molecular weight	Distance Travelled from base of gel (mm)
Phosphorylase b	94,000	11
Albumin	67,000	17
Ovalbumin	43,000	26
Trypsin Inhibitor	20,100	38

Table 3.2

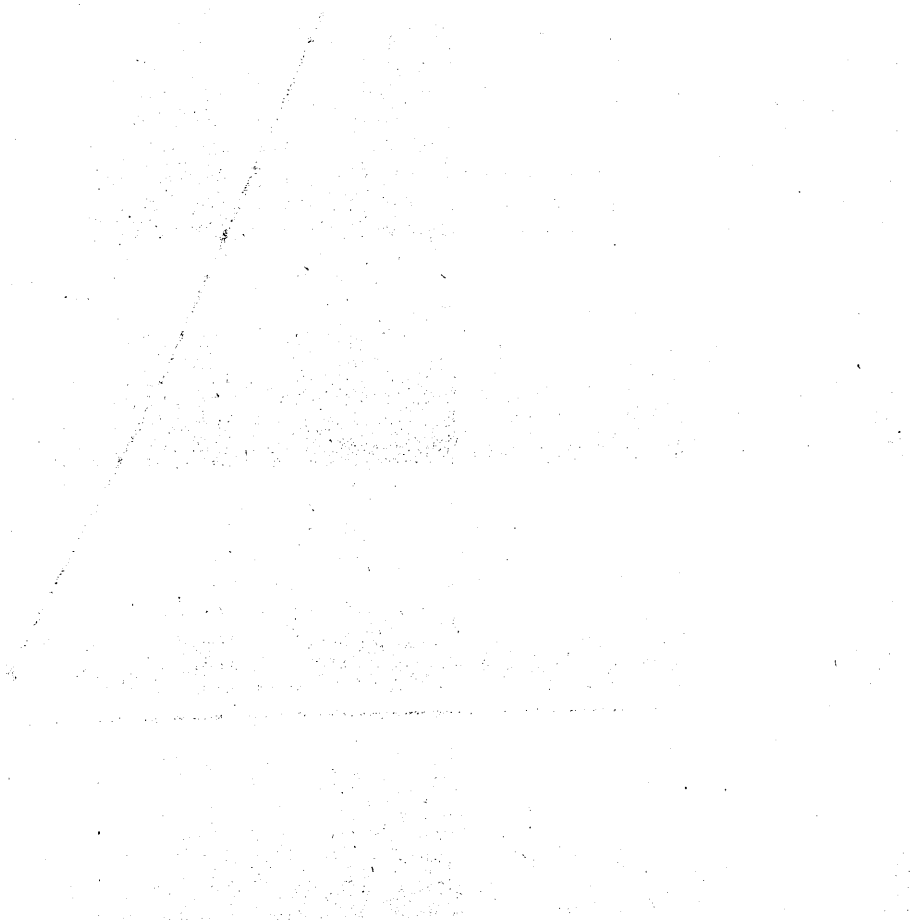
Distance travelled from the base of the gel and molecular weights of proteins in tubulin extracts from *Ascaris suum*

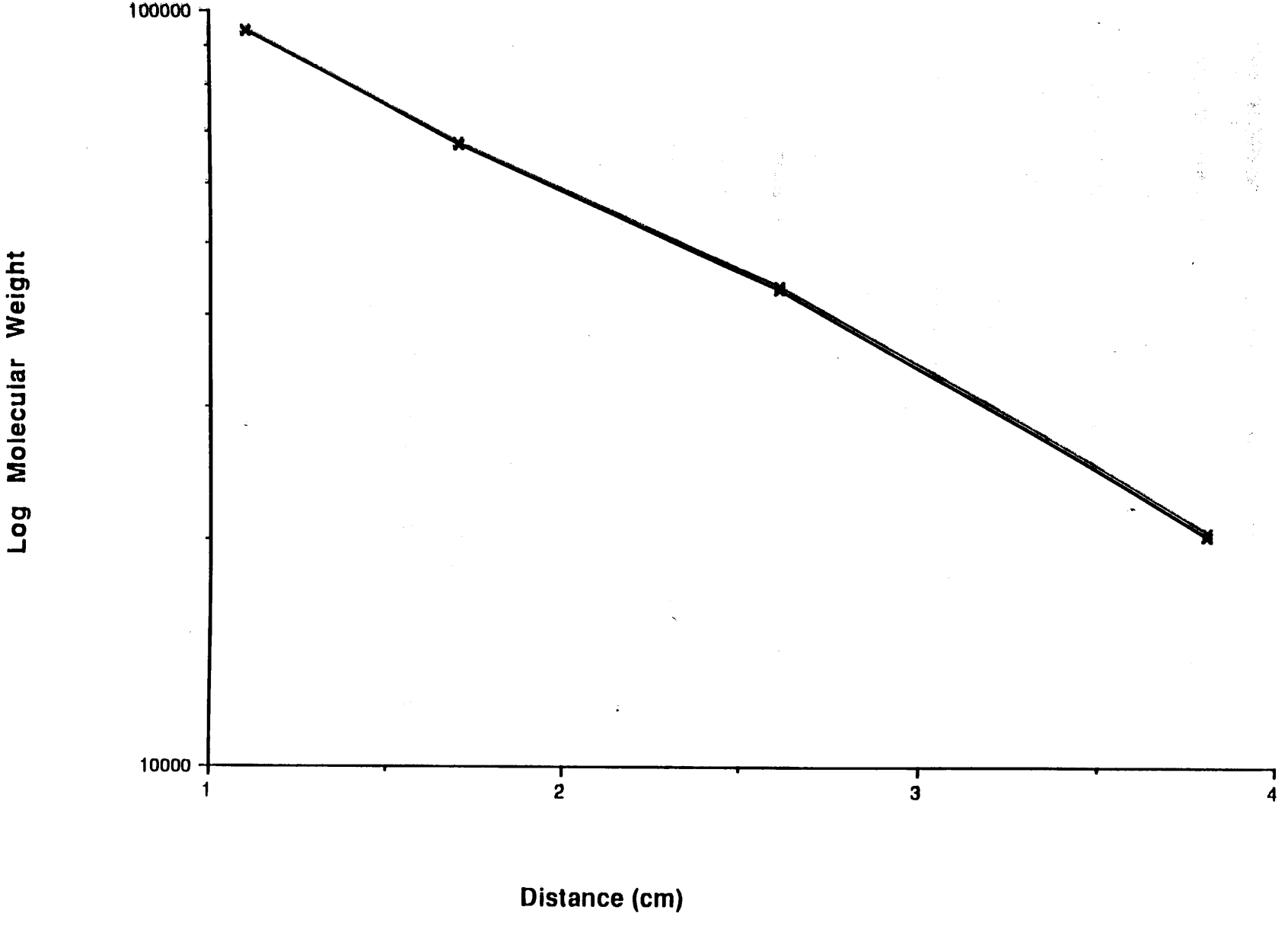
Band	Distance Travelled from base of gel (mm)	Molecular Weight*
A	17	67,000
B	22	52,000
C	27	39,000
D	31	32,000

*Values calculated using standard graph plotting distance travelled against log molecular weight of proteins (Fig 3.2)

Figure 3.2

Graph of distance travelled by protein standards in SDS PAGE against log molecular weight.




100000
10000
Log Molecular Weight
1
2
3
4
Distance (cm)

the tubulin band was darker staining than other bands suggesting that relatively high concentrations of tubulin were available after extraction from the parasites. For binding assays the crude tubulin extract without further ammonium sulphate fractionation to try to purify the extract was considered suitable.

Extracts of tubulin prepared from mammalian brain tissue contain much higher concentrations of tubulin at 25-40% of the total soluble protein as compared with nematodes where the tubulin fraction is less than 1% of the total soluble protein content. For *A. suum* the use of intestinal material to prepare extracts enhances the amount of available tubulin but with smaller nematode species e.g. *O.circumcincta* and *H.contortus* whole parasites are used because of the difficulty of extracting intestinal material. For the tubulin extracts approximately 3% of the protein in the crude extract is tubulin and this could predispose to variations in results of binding assays (see later).

Other methods to prepare highly purified tubulin extracts from parasites are available (Gull *et al*, 1987) involving separation on a DEAE Sephadex column followed by temperature dependent cycles of assembly and disassembly of tubulin into microtubules combined with separation using ultracentrifugation. This method requires very large numbers of parasites 50g wet weight of *A.suum* yields aproximately 0.75mg purified tubulin. This method therefore is impractical for studies on tubulin binding where low numbers of parasite larvae are available for testing resistance.

Lacey and Snowdon (in preparation) have described a method for purifying tubulin from mammalian brain using amino-activated gel chromatography. This method has been evaluated for purification of tubulin from nematodes, cestodes and trematodes (Lacey and Gill, personal communication) but again this method is limited because large numbers of parasites are required. Also purification of tubulin from nematodes using this method is less successful than with other groups of helminths because of the presence of contaminating proteins which cannot be separated in the extract. Tubulin extracted using

temperature dependent assembly of microtubules isolates the tubulin which polymerises and depolymerises readily whereas gel chromatography isolates a greater proportion of the tubulin. The importance of the different types of tubulin and the possible effect they have on the binding characteristics of the extract has not been established.

For the tubulin binding assays to compare the binding of benzimidazoles to tubulin extracts from resistant and susceptible parasites it is impractical to use highly purified tubulin extracts. The extraction method used to obtain tubulin from *A.suum* which was shown to be successful using SDS-PAGE was modified for use with *O. circumcincta* and *H.contortus* larvae and eggs. The preparation of extracts from homogenised nematode parasites for use in tubulin binding assays using ultracentrifugation or centrifugation in a bench top Eppendorf centrifuge has been described (Lacey and Prichard, 1986; Lacey and Snowdon, 1988). Both methods yielded crude extracts with suitable protein concentrations when sufficient larvae were used (>50,000) and the volume of the homogenate was maintained at a low level (<1ml). Using bench-top centrifugation was quicker and ensured that the tubulin extract was collected rapidly reducing the overall time taken to perform the binding assay and therefore may reduce the denaturation and loss of protein from the sample.

TUBULIN BINDING ASSAYS

Displacement of Colchicine Binding Assay using Crude Tubulin Extracts Prepared from *Ascaris suum*

This assay utilises the principle that colchicine binds to tubulin in a similar way to the benzimidazoles and therefore after incubation with benzimidazoles there is a reduction in the amount of tritiated colchicine which can subsequently bind to the tubulin extract. The reduction in binding of tritiated colchicine in the presence of benzimidazoles can be measured by comparing the final counts bound with those in a control sample, i.e. tubulin extract with tritiated colchicine alone. This method was modified from Sherline *et al.*,

(1974) and Barrowman *et al.*,(1984).

Materials and Methods

Reagents.

Buffer - 10mM KH_2PO_4/K_2HPO_4 containing 1mM $MgSO_4$, 0.1mM GTP and 1uM PMSF, pH 7.

Radiolabelled colchicine - (ringA-4-^{3}H) colchicine obtained from Amersham International. Stock solution 10uM in ethanol/buffer 1:10, 5-15Ci/mmol (185-550GBq/mmol).

Benzimidazoles - Stock solutions of MBZ and ABZ 0.4mM(100ug/ml) in ethanol were diluted 1 in 10 with buffer for use.

Charcoal suspension - 2mg/ml activated charcoal (Norit-A activated charcoal - Sigma Chemical Co. Ltd) in 1%(w/v) BSA

Method.

1. One ml of crude tubulin extract from *A.suum* (see previously) was incubated with 10ul MBZ or ABZ (0.4uM) for 3h at 37°C in a glass tube (control was prepared by incubating 1ml tubulin extract with 10ul buffer and blank by incubating 1ml buffer with 10ul buffer).

2. To each tube was added 100ul tritiated colchicine and tubes were incubated for 1.5h at 37°C.

3. Separation of tubulin bound tritiated colchicine and unbound tritiated colchicine was carried out using a gel filtration column (method A) or activated charcoal extraction (method B).

Method A

4A. The gel filtration column (see below) was run to almost dryness and the sample or control was loaded onto the column.

5A. The sample was eluted from the column using buffer and 1ml fractions were collected

from the base of the column. A 100ul aliquot of each fraction was added to 10ml Optiphase X and counted in a Packard counter for 1min.

6A. The tritiated colchicine bound to tubulin is eluted in the void volume. The counts from the fractions 6-10 contained the tubulin bound fractions. The total counts bound to tubulin were calculated in each of the samples and the inhibition of binding of tritiated colchicine to tubulin for MBZ or ABZ was calculated by subtracting the values of binding in these samples from the control.

Method B

4B. To each sample, control or blank was added 10ml charcoal suspension, mixed and allowed to stand for 10min.

5B. Charcoal mixtures were centrifuged at 2,200g for 10min. A 100ul aliquot of the supernatant was placed in 10ml Optiphase X and counted in a Packard counter for 1min.

6B. The percentage inhibition of binding of tritiated colchicine to tubulin by MBZ or ABZ was calculated using the following equation; $\frac{\text{cpm control-cpm blank}}{\text{cpm sample-cpm blank}} \times 100$

Preparation and calibration of gel filtration column

A gel filtration column was prepared for use in tubulin binding assays to separate drug bound to tubulin from unbound drug. The gel filtration column works on the principle that as a solution passes down the column the molecules within the solution have different abilities to enter the pores formed in the column which contain the stationary phase of the solution. Smaller molecules which can enter the gel pores move more slowly through the column because they spend a proportion of their time in the stationary phase whereas larger molecules cannot enter the pores and pass down the column in the mobile phase. Therefore molecules are eluted from the column in order of decreasing molecular size.

The material used in the gel filtration column was Sephadex G-100 which has a

fractionation range of 4,000-150,000 molecular weight for proteins.

Reagents.

Sephadex G-100 - bead size 40-120microns (Sigma Chemical Co.Ltd.)

Eluting buffer - 10mM KH_2PO_4/K_2HPO_4 containing 1mM $MgSO_4$, 0.1 mM GTP and 1uM PMSF, pH 7.

Method.

1. Sephadex G-100 (2g) was placed in excess buffer for 48-72 h at room temperature to swell and was degassed thoroughly.

2. A 20 cm gel filtration column was prepared in a sintered glass column 30cm X 1.4 cm.

3. The column was allowed to settle and flushed several times with eluting buffer.

4. The column was calibrated using dextran blue (0.5ml dextran blue in 50%(v/v) glycerol) and potassium dichromate (10% (w/v)). The void volume was 9-10 ml.

Results

Crude tubulin extract prepared from intestinal material of *A.suum* with protein concentration of 15.3mg/ml was used in the assay. The assay was carried out initially using method A to separate colchicine bound to tubulin and unbound drug. Binding of tritiated colchicine to the tubulin extract was inhibited by MBZ and ABZ and inhibition of colchicine binding is given in Table 3.3. The assay was repeated on three occasions using method B to separated bound and unbound tritiated colchicine after incubation with an inhibitor MBZ or ABZ. Results of the three assays were combined and are shown in Table 3.3.

Discussion

Colchicine an antimitotic compound has been shown to bind irreversibly to tubulin from mammalian brain, fungi and helminths (Dustin,1984). The benzimidazoles are thought to bind to the same site on the tubulin molecule as colchicine (Friedman and Platzer, 1978) and therefore the binding of benzimidazoles to tubulin can be measured indirectly by

Table 3.3

Inhibition of binding of tritiated colchicine to tubulin extracts from *A.suum* in the presence of mebendazole or albendazole

Inhibitor	% Inhibition of Colchicine Binding
*0.40uM MBZ	92.8
*0.40uM ABZ	48.4
**0.40uM MBZ	32.4±7.4
**0.40uM ABZ	46.8±11.3

* Tritiated colchicine bound to tubulin extract separated from unbound drug using Sephadex column (Method A)

**Tritiated colchicine bound to tubulin extract separated from unbound drug by charcoal extraction (Method B)

measuring the inhibition of binding of tritiated colchicine by the benzimidazole compound. Separation of bound from free tritiated colchicine in the assay can be achieved using a gel filtration column (Borisy and Taylor, 1967) or charcoal extraction (Sherline *et al.*, 1974) and both these methods were investigated. An alternative method of separation using DEAE cellulose filter paper (Borisy, 1972) was not investigated because values for binding are on average 15-20% lower than with the gel filtration column method. The gel filtration column method of separation is time consuming and is not suitable for multiple determinations which prompted the development of the charcoal extraction method (Sherline *et al*, 1974).

Results for inhibition of binding of tritiated colchicine with column separation (Table 3.3) are similar to those obtained by Barrowman *et al.* ,(1984) who found that ABZ inhibited binding by 42.6% and MBZ by 88.2%. These results indicate that MBZ has a greater affinity for nematode tubulin than ABZ. Repeats of the inhibition of colchicine binding assay using charcoal extraction showed similar results for ABZ (Table 3.3) but inhibition of binding was much less with MBZ. Several problems were encountered with the charcoal extraction method. The standard deviation of the mean is quite high indicating a problem with reproducibilty in the assay. The type of charcoal used in the extraction has an effect on the assay (Sherline *et al.*, 1974) and Sigma activated charcoal was used to try to minimise variation caused by differences in stability of the tubulin-colchicine complex to types of charcoal.

The indirect measurement of benzimidazole binding by competition with tritiated colchicine is affected by the affinity of both the benzimidazole and colchicine for the binding sites on tubulin. Where the benzimidazole has a high affinity for the tubulin binding site the specific activity of the tritiated colchicine can be a limiting factor in the assay. With MBZ where inhibition of colchicine binding is high a low concentration of colchicine and therefore a low level of radioactive counts binds to the tubulin

subsequently. This low level of radioactivity can cause variability in the assay because any variation in the background counts can have a significant effect on the results. To overcome this problem binding of benzimidazoles to tubulin should be measured directly using radiolabelled benzimidazole compounds. The significance of the inhibition of binding of tritiated colchicine by a benzimidazole as a measure of the activity of the benzimidazole *in vivo* is questionable. Barrowman *et al*, (1984) found that inhibition of colchicine binding was similar with ABZ, ABSX and ABSO even although ABSO has little anthelmintic activity *in vivo* and for the other benzimidazoles tested degree of inhibition of colchicine binding did not reflect known efficacy of the compounds *in vivo*. The direct measurement of binding of benzimidazoles to nematode tubulin therefore may be of greater significance for measuring efficacy of the individual benzimidazole compounds (see later).

Another limiting factor in measuring binding of benzimidazoles to nematode tubulin extracts by this indirect method with tritiated colchicine is the low soluble protein content in the extracts and the fact that tubulin constitutes a small percentage (<5%) of the soluble protein component. In order to investigate binding of benzimidazoles to tubulin extracts of *O.circumcincta* or *H.contortus*, where the protein concentration is ten fold less than extracts from *A.suum* intestine, a more sensitive method is required.

TRITIATION OF BENZIMIDAZOLES

Labelling of some of the benzimidazole drugs with tritium was investigated in order that binding of the benzimidazoles to tubulin could be measured directly by combining the drugs with tubulin extracts, removing unbound drug residues and then counting the labelled material bound to the tubulin fraction (see later). Two methods with various modifications were investigated.

Materials and Methods

Method 1 for the Preparation of Tritiated Benzimidazoles

Reagents./

Reagents.

Albendazole, Fenbendazole and Thiabendazole - donated by the appropriate drug company

Sodium boro(^{3}H) hydride - Amersham International 5-20 Ci/mmol (185-740GBq/mmol)

Other compounds were obtained from Sigma Chemical Co Ltd or BDH Chemicals Ltd.

Method.

Method 1 was modified from the method of Lacey (1985), involving bromination of the benzimidazole followed by debromination in the presence of tritium labelled sodium borohydride and a palladium chloride catalyst.

1. The pure benzimidazole compound, ABZ, FBZ or TBZ, 0.5g (2.5×10^{-3} moles), was dissolved in 10ml glacial acetic acid. To this was added 1.5 molar equivalents (600mg) of bromine and the reaction was stirred for 1h.

2. The precipitate which formed was collected and air dried overnight. This brominated product was then recrystallised from methoxyethanol over a 24-48h period.

3. A quantity of the recrystallised material was dissolved in methanol (100ug/ml) and purity assessed using high performance liquid chromatography (HPLC).

4. Ten umoles (0.003g) of the brominated benzimidazole was dissolved in 400ul 20% DMSO in methanol. To this was added 30umoles of palladium chloride as a 0.4% solution in methanol (1.33ml of a 0.4% solution). Then added 50 umoles of sodium boro(^{3}H)hydride dissolved in 30ul of water and the reaction was stirred for 30 min.

5. The reaction mixture was diluted to 2ml with methanol and filtered through a sintered glass funnel. The funnel was washed 5 times with 2ml aliquots of methanol.

6. The washings were collected, combined and evaporated to dryness under a stream of air for approx. 36h.

7. The product was then purified using method A or B

Purification Method A

7A. The residue was dissolved in 10ml chloroform and applied to a silica Sep-Pak (Waters) pre-washed with chloroform. The material passing through the column during loading was retained, evaporated to dryness under a stream of air and the residue was redissolved in 0.5ml methanol.

8A. The loaded Sep-Pak was washed with 20ml chloroform and washings were collected, evaporated to dryness under air and the residue was redissolved in 0.5ml methanol.

9A. The product was eluted from the column using 2 x 10ml 10% methanol in chloroform and the eluate collected, evaporated to dryness and the residue redissolved in 0.5ml methanol.

10A. All the fractions eluted from the Sep-Pak column were tested for purity by HPLC.

Purification Method B

7B. The residue was dissolved in 3ml dichloromethane (DCM) and loaded onto a silica Bond Elut (Analytichem International) pre-conditioned with 2ml DCM and allowed to run by gravity flow. The material passing through the column during loading was collected, evaporated to dryness under air and redissolved in 0.5ml methanol.

8B. The flask containing the benzimidazole sample was rinsed with 2 x 3ml DCM and material passing through the column was collected, evaporated to dryness under air and redissolved in 0.5ml methanol.

9B. The column was washed with 5ml DCM and washings collected , evaporated to dryness under air and redissolved in 0.5ml methanol.

10B. The product was eluted from the column using 5ml 25%methanol/ DCM and eluate collected, evaporated to dryness in air and redissolved in 0.5ml methanol.

11B. All the fractions collected from the Bond Elut column were assessed for drug purity using HPLC.

Quantitative and Qualitative Analysis of Products of Bromination and Tritiation of Benzimidazoles

High-performance liquid chromatography (HPLC) was used to test the purity of the products of the bromination and tritiation of the benzimidazoles using the method of Marriner and Bogan,(1980).

Materials.

Pump- Gilson model 302

Detector- Cecil CE2012 variable wavelength ultra violet spectrophotometer

Column- 100mm x 5mm (Shandon Southern) packed with ODS-Hypersil 5 micron.

Wavelength- 292nm

Absorbance- 0.05 A.u.f.s

Solvent A-Methanol: Ammonium carbonate (0.05M) 75:25 - used to detect ABZ, ^{3}HABZ, brominated ABZ, FBZ, ^{3}HFBZ, brominated FBZ, TBZ and brominated TBZ.

Solvent B - Methanol: water 65:35 + 0.5ul perchloric acid (1.1%v/v) per ml of solvent - used to detect oxidised metabolites of the benzimidazoles - ABSX, ABSO, OFZ and 5hydroxyTBZ.

Flow rate- 1ml/min

Standard concentrations of the individual pure benzimidazole compounds and their metabolites were reconstituted in methanol over a range of concentrations 10ug/ml, 20ug/ml, 50ug/ml and 100ug/ml to allow quantitation of the tritiated benzimidazole compounds.

During HPLC analysis fractions were collected from the base of the column corresponding to each peak obtained spectrophotometrically. A 10ul aliquot of each of these samples was placed in 10ml Optiphase X scintillation fluid and counted for 1min in a Packard counter

Thin Layer Chromatography of Tritiated Albendazole

(Method modified from Gyurik *et al*, 1981)

Reagents.

Solvent - chloroform: methanol: concentrated ammonium hydroxide 90:10:1

Silica gel - silica gel 250 microns containing inorganic phosphor (GF Analtech)

Method.

1. Glass plates were coated with silica gel. The plates were dried and heated overnight at 50°C to activate the gel

2. Test compounds- ABZ (100ug/ml) and ^{3}HABZ (315ug/ml) 100ul were spotted in duplicate onto the plate and allowed to dry.

3. Plate was placed in solvent for 1h then allowed to dry.

4 Plate was examined under UV light (254nm)

5. The silica gel at the area of the spots was scraped from the plate and either placed in 10ml Optiphase X and counted in a Packard counter or shaken in methanol, centrifuged to remove the silica and injected onto an HPLC system to detect ABZ.

Method II for the Preparation of Tritiated Benzimidazoles

Reagents.

Tritium labelled MBZ and ABZ were obtained from Dr.E.Lacey (CSIRO Division of Animal Health, McMaster Laboratory, Sydney) Labelling of the drugs was carried out by catalytic exchange using palladium on calcium carbonate in pure dioxane at 60°C under tritium gas (Lacey *et al*, to be published). Using HPLC and mass spectrometry the ^{3}HMBZ and ^{3}HABZ were analysed and quantified into aliquots of 29.5ug which were stored in 2ml methanol in glass vials

Buffer - 0.025 MES buffer pH 6.5 containing 1mM EGTA and 0.5mM $MgSO_4$

Method./

Method.

1. To prepare the tritiated benzimidazoles for use in the tubulin binding assays vials containing 29.5ug of the compounds in methanol were evaporated to dryness under a stream of nitrogen.

2. The residue was redissolved in 2ml DMSO and made up to 10ml with MES buffer producing a 10uM solution.

4. Calculation of specific activity of tritiated benzimidazole was carried out. Six 10ul aliquots of 10uM ^{3}HMBZ or ^{3}HABZ in DMSO/MES buffer mixture were placed in 5ml Biofluor and counted for 1min. The specific activity (dpm/pmole) for each compound was calculated.

Results and Discussion

Tritiation of Albendazole

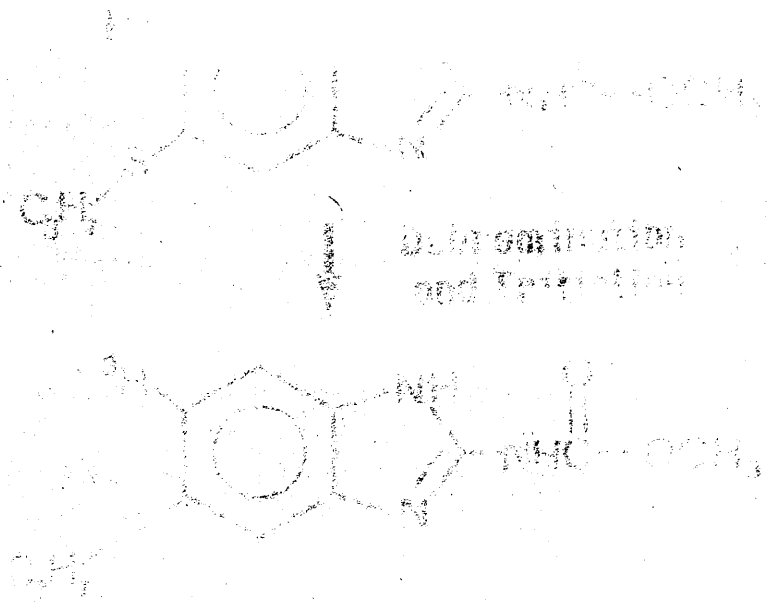
Using method I, ABZ was brominated then reacted with tritiated sodium borohydride in the presence of a palladium chloride catalyst to form tritiated ABZ. The reaction is shown in Fig. 3.3. with bromination at the C-5 position followed by debromination at this position and substitution by tritium.

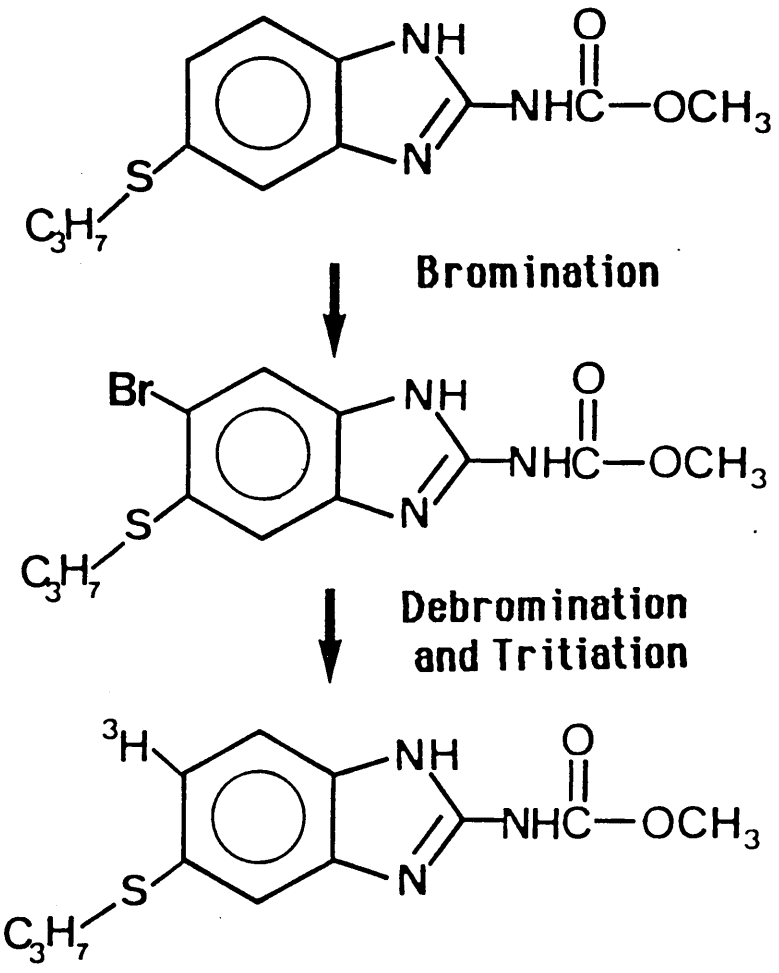
Results of HPLC Analysis of Products of the Bromination and Tritiation of Albendazole

The product of the bromination reaction was recrystallised and a 100ug/ml solution in methanol was injected onto an HPLC column and comparisons made with injections of pure ABZ. A small peak corresponding to that of ABZ was found and the concentration of ABZ in the sample was calculated as 1ug/ml and the remaining peak, showing a longer retention time than pure ABZ was assumed to correspond to the brominated compound (Table 3.4). A pure brominated ABZ standard was not available and therefore the exact concentration of this compound could not be calculated by HPLC. Measurement of ABZ in the product of the bromination reaction showed that approximately 1% of the ABZ had failed to brominate.

Figure 3.3

Mechanism of bromination and tritiation of albendazole.




NH
O
NHC—OCH3
N
S
C3H7
Bromination
Br
Debromination
and Tritiation
3H

After reaction of the brominated ABZ with tritiated sodium borohydride the product was purified initially using a Sep-Pak (Method A) and fractions collected from the base of the column during loading, washing and elution. An aliquot of each of these fractions was tested by HPLC. Injection of the loading sample produced a high peak with retention time identical to ABZ, tritiated ABZ, and a smaller peak corresponding to the brominated compound. The sample contained contaminants which could have been free sodium borohydride. The major part of the loaded material failed to adhere to the silica in the Sep-Pak column and passed out in the void volume. Washings collected from the Sep-Pak also contained tritiated ABZ and a small amount of unconverted brominated ABZ. The eluted fraction contained 2.5ug/ml tritiated ABZ. No brominated ABZ was detected in this sample.

The poor loading and separation achieved with the Sep-Pak column prompted further investigation into the use of an alternative system. Method B was developed using a Bond elut with dichloromethane as the solvent. The products collected from the Sep-Pak separation (loading, washing and elution samples) were combined and evaporated to dryness in a stream of air then the residue was redissolved in dichloromethane and loaded onto a Bond elut column. HPLC analysis was carried out on an aliquot of each fraction collected from the base of the Bond elut column during loading, rinsing, washing and elution. Samples collected during loading, rinsing and washing of the column contained no detectable levels of ABZ. The eluate produced a large peak with retention time identical to pure ABZ and this was assumed to be tritiated ABZ. There was also a peak with a greater retention time which corresponded to brominated ABZ. A 1 in 20 dilution of the eluate contained 31.54 ug/ml tritiated ABZ (119uM). Injection of this tritiated ABZ sample on a methanol/perchloric acid column to detect ABSX and ABSO confirmed the presence of these oxidised products but at low concentrations, less than 1% of the overall tritiated ABZ concentration.

During HPLC analysis fractions were collected from the base of the column and an aliquot

was counted to determine the presence of the tritiated compound. The fractions taken before the ABZ peak gave a mean of 533cpm, those taken at the time of the ABZ peak 30,360cpm and fractions after the peak 307cpm.

Results of Thin Layer Chromatography of Albendazole and Tritiated Albendazole

Thin layer chromatography with ABZ and the tritiated ABZ produced detectable spots under UV light and the distance travelled by each of the compounds was identical (Fig. 3.4). The spot for tritiated ABZ was more prominent than the ABZ spot reflecting the higher concentration of tritiated ABZ added to the plate. The silica was scraped from the plate at the area of the spots and the presence of pure ABZ confirmed by HPLC. For the duplicate samples the silica scraped from the area of the spot was placed in Optiphase X and counted. The spot from the tritiated ABZ sample gave 8,689,832cpm.

Tritiation of Fenbendazole

Fenbendazole was brominated then tritiated using method I. The chemical reaction is similar to that given for ABZ (Fig. 3.3).

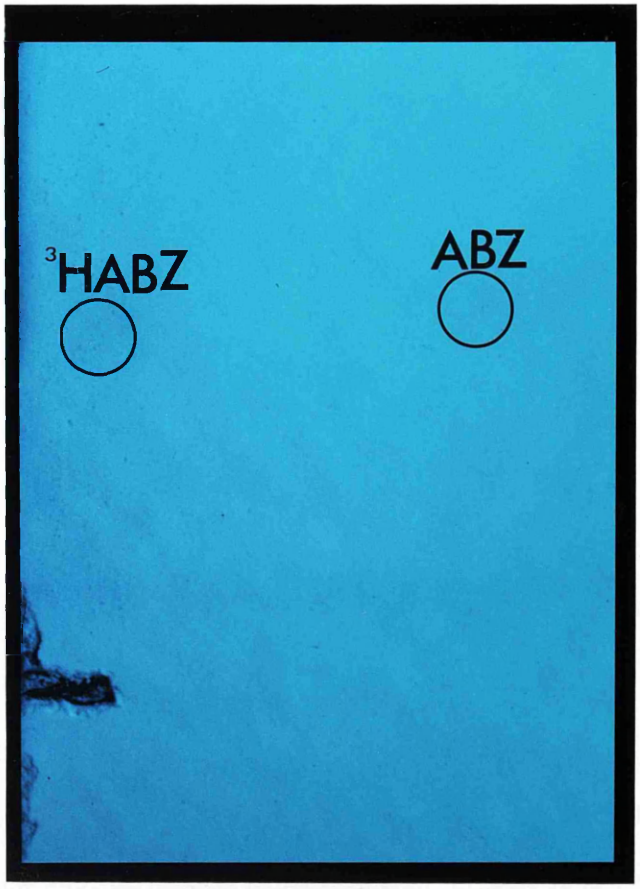
Results of HPLC Analysis of the Products of Bromination and Tritiation of Fenbendazole

The recrystallised product of the bromination reaction was dissolved in methanol (100ug/ml) and injected on HPLC and compared with a standard solution of pure FBZ. The bromination product did not show a peak corresponding to FBZ but there was a large peak indicating the presence of a compound with a longer retention time than FBZ which probably corresponds to the brominated FBZ (Table 3.4).

After reaction of the brominated FBZ with tritiated sodium borohydride the product was purified using a Bond-elut column and fractions collected from the base of the column were analysed. The load sample contained a high peak corresponding to FBZ , tritiated FBZ, and a smaller peak for the brominated FBZ. The rinse sample contained both tritiated FBZ

Figure 3.4

Thin layer chromatogram of albendazole standard and tritiated albendazole.


^{3}HABZ
ABZ

and brominated FBZ. There was no tritiated FBZ or brominated FBZ in the wash sample or eluate. The load and rinse samples were combined and evaporated to dryness, redissolved in dichloromethane and loaded onto another Bond-elut column to repeat the purification step. Again fractions were collected from the column and analysed by HPLC. The load sample gave a high peak corresponding to tritiated FBZ and a peak for brominated FBZ. A 1 in 10 dilution of this load sample contained tritiated FBZ at a concentration of 25 ug/ml (83.6uM). None of the other fractions collected from the Bond-elut contained tritiated FBZ or brominated FBZ. The load sample was injected onto a column with methanol/ perchloric acid as the solvent. Small peaks corresponding to OFZ and FBZSO were found but it was calculated that less than 1% of the tritiated FBZ in this fraction was in these oxidised forms.

During HPLC analysis fractions were collected from the base of the column and an aliquot was counted to determine the presence of the tritiated compound. The fractions collected prior to the FBZ peak showed a mean of 7,555cpm, those collected at the FBZ peak 4,502,380cpm, those collected during the brominated FBZ peak 8,578cpm and fractions after the peaks 1,066cpm.

Tritiation of Thiabendazole

Thiabendazole was brominated then reacted with tritiated sodium borohydride in the presence of a palladium chloride catalyst using method I.

Results of HPLC Analysis of Products of the Bromination and Tritiation of Thiabendazole.

The recrystallised product from the bromination reaction (100ug/ml) was injected onto an HPLC column and comparisons were made using a pure TBZ standard. The sample produced a small peak equivalent to 5ug/ml TBZ and a larger peak for a compound with a longer retention time than TBZ which was assumed to correspond to the brominated TBZ (Table 3.4).From this result it was calculated that 5% of the original TBZ had failed to

brominate during the reaction.

The brominated TBZ was reacted with tritiated sodium borohydride and the product purified using method B through a Bond-elut column with fractions collected from the base of the column analysed by HPLC. The load and rinse samples produced a small peak corresponding to TBZ and a large peak for brominated TBZ. The wash sample did not contain any detectable levels of TBZ or brominated TBZ. The eluate showed a small peak for TBZ and a larger peak corresponding to brominated TBZ. Fractions collected from the base of the column were counted to detect tritiated compounds. An aliquot of the fractions collected during the TBZ peak showed 3,823cpm and during the brominated TBZ peak 476cpm.

There was poor conversion of brominated TBZ to tritiated TBZ and the fractions collected from the Bond-elut were combined, evaporated to dryness under a stream of air and the residue redissolved in 20% DMSO in methanol and the tritiation reaction repeated. The product was purified using method B and fractions collected from the column analysed by HPLC. None of the fractions contained TBZ. All the fractions collected from the Bond-elut column had high counts for radioactivity but the counts were not associated with TBZ.

This experiment to tritiate TBZ by bromination followed by tritiation using tritiated sodium borohydride and a palladium chloride catalyst was repeated twice with similar results to above. The brominated compound failed to debrominate and negligible amounts of tritiated TBZ were formed.

Specific Activity of Tritiated Benzimidazoles

The specific activity of the tritiated ABZ and FBZ produced by method I described above and tritiated MBZ and ABZ produced by method II is given in Table 3.5.

Table 3.4

Retention times of benzimidazole compounds and brominated benzimidazole compounds during HPLC analysis using methanol: ammonium carbonate (65:35) as the solvent.

Compound	Approximate Retention Time *
Albendazole	2.6min
Brominated Albendazole	3.4min
Thiabendazole	1.8min
Brominated Thiabendazole	3.2min
Fenbendazole	4.4min
Brominated Fenbendazole	6.2min

* Retention times can be variable and values are approximate.

Table 3.5

Specific activity of radiolabelled benzimidazoles

Compound	Specific Activity (dpm/pmole)
*Albendazole (Batch 1)	265
*Albendazole (Batch 2)	360
*Fenbendazole	110
**Albendazole	9754
**Mebendazole	2685

* Tritiated using Method I

** Tritiated using Method II

Tritiation of Benzimidazoles - Discussion

Radiolabelling of benzimidazoles with tritium was investigated to try to produce compounds which could be used to investigate the binding of this class of drugs to the cytoskeletal protein, tubulin, in nematodes. In the past labelling of benzimidazoles with ^{14}C produced compounds of sufficient specific activity to investigate their metabolism, pharmacokinetics and biochemistry (DiCuollo *et al*, 1974; Prichard *et al*, 1981; Davidse and Flach, 1977). Tritiation of the benzimidazoles produces compounds with higher specific activity and therefore better suited for biochemical tests involving the binding of benzimidazoles to small amounts of nematode tubulin.

Using the method of Lacey (1985) tritiation of TBZ, ABZ and FBZ was attempted (method 1). Bromination of all 3 compounds was successful with 95%, 99% and 100% of the original compound becoming brominated for TBZ, ABZ and FBZ respectively. The differing solubilities of the brominated compounds compared with the parent benzimidazole is reflected by the increased retention times of all the brominated compounds compared with the parent compounds on HPLC analysis (Table 3.4).

The purification of the products of the tritiation reaction was carried out using a Sep-Pak or Bond-elut column to try to remove tritium not associated with the benzimidazole. Purification of tritiated ABZ using a Sep-Pak was not achieved because the benzimidazole compound was not retained by the column. The original method described by Lacey (1985) used a Sep-Pak for separation, however, in the present studies a Bond-elut column and different solvent system achieved better results for ABZ. During the experiment to tritiate FBZ purification on a Bond-elut column was not achieved because the sample failed to load onto the column. During subsequent HPLC analysis and radioactivity estimation of the tritiated FBZ there was a high level of radioactivity in the material collected prior to the FBZ peak. This was due to non-volatile tritium not bound to FBZ which had not been removed during purification using the Bond-elut. The analysis

of tritiated ABZ showed significantly lower counts for radioactivity in the sample collected from the HPLC column before the peak of ABZ suggesting that the unbound tritium had been removed successfully during the purification step through a Bond-elut for ABZ.

For both FBZ and ABZ the counts obtained in fractions collected from the HPLC column during the peak of the benzimidazole showed significantly higher levels than samples before and after the peak. This indicated that the tritium was associated with the benzimidazole. Thin layer chromatography of the tritiated ABZ confirmed that the tritium was bound to the ABZ. The calculated specific activity for tritiated ABZ therefore gives a good indication of the amount of tritium bound to the benzimidazole. The value for specific activity of FBZ was lower which suggested that there was poorer conversion of the brominated compound to the tritiated product and possibly replacement of the bromine by an untritiated hydrogen during the reaction. The value for specific activity also may be less accurate than for ABZ because some of the counts were not associated with the FBZ but were due to unbound tritium. This could affect the accuracy of calculations made during binding experiments and possibly lead to over estimation of the amount of drug binding to tubulin in binding assays (see later).

The use of bromination followed by reaction with tritiated sodium borohydride in the presence of a palladium chloride catalyst as a method to tritiate TBZ gave poor results. The yield of brominated compound was lower than with ABZ and FBZ but at approximately 95% should have been adequate for debromination and tritiation. Analysis of the products of tritiation followed by purification showed a poor yield of tritiated TBZ in all experiments. Further attempts to react the partially tritiated compound with tritiated sodium borohydride resulted in total loss of the TBZ. The products of the reaction had high radioactivity counts but this tritium was not associated with TBZ. Tritiation of TBZ by this method failed because in the presence of the palladium chloride catalyst the brominated TBZ was broken down rather than debrominating with replacement of the bromine by

tritium. The chemical structure and properties of the TBZ molecule therefore prevent tritiation of the molecule using this method.

To improve the specific activity of the tritiated benzimidazoles they were labelled by a different method (Lacey *et al*, in preparation) involving catalytic exchange using palladium on calcium carbonate in pure dioxane at 60°C in the presence of tritium gas. The tritium has been shown to be incorporated at position 4 of the benzimidazole nucleus in ABZ and is assumed to occur at this position in the other benzimidazoles. The compounds were purified and analysed using HPLC and thin layer chromatography. The specific activity found for the tritiated ABZ prepared by this method was greater than tritiated ABZ synthesised previously using a brominated intermediate (Table 3.5). A high specific activity was also obtained for MBZ produced by method II.

The production of tritiated compounds with a high specific activity facilitates the studies on the binding of benzimidazoles to nematode tubulin. The differences in the binding of benzimidazoles to tubulin extracts from anthelmintic resistant and susceptible parasites can be quantified more accurately using tritiated drugs with high specific activity. For some benzimidazole resistant strains of parasites the quantity of benzimidazole binding to the tubulin extracts is very low and therefore to measure binding the use of a compound with high specific activity is necessary (see later).

Tritium has a radioactive half life of 12.36 years (Strominger *et al*.,1958) and therefore the specific activity of the synthesised tritiated benzimidazoles remained constant over the period of use. There was evidence of oxidation of the benzimidazoles on storage and during preparation. During the synthesis of tritiated ABZ and FBZ by method I the solvents were evaporated under a stream of air rather than nitrogen as described by Lacey (1985). HPLC analysis of the products showed less than 1% of the final product was in the oxidised form suggesting that evaporation under air had little effect on the oxidation of the benzimidazoles. The tritiated compounds synthesised by method I were stored in

methanol and there was negligible oxidation over the period of storage (up to 12 months). Tritiated ABZ and MBZ prepared by method II were stored in methanol and then prepared in DMSO and buffer for use in binding assays. Oxidation of the benzimidazoles was rapid in the presence of DMSO and compounds in DMSO and buffer were used for 3-4 weeks then replaced because the increasing levels of oxidised products influence the results of binding assays (see later).

Tubulin Binding Assays using Tritiated Benzimidazoles

Materials and Methods

Modified Tubulin Binding Assay using Tubulin Extracts from *A. suum*, *O. circumcincta* (infective larvae and eggs) and *H. contortus* (infective larvae) with Tritiated ABZ and FBZ

Reagents.

Buffer A - 10uM KH_2PO_4/K_2HPO_4 containing 1mM $MgSO_4$, 0.1mM GTP, 25% (v/v) glycerol and 1uM PMSF, pH 7.

Buffer B - Buffer A without glycerol

Tritiated ABZ - Prepared by method I (see previously) and diluted to 11.9uM, 23.8uM or 11.9 uM in methanol. Specific activity 360dpm/pmole.

Tritiated FBZ - Prepared by method I (see previously) and diluted to 83.6uM or 8.36uM in methanol. Specific activity 110dpm/pmole

Charcoal suspension - 2mg/ml activated charcoal (Norit-A activated charcoal, Sigma Chemical Co. Ltd.) in 1%(w/v) BSA.

Method.

1. 90 ul tubulin extract prepared by method A or B (see previously) was placed in a 5ml plastic tube and 10ul tritiated ABZ or FBZ added (blank 90ul buffer A plus 10 ul tritiated drug) mixed and the tubes incubated at 37°C for 15min in a waterbath.

2. Unbound tritiated drug was separated from tubulin bound drug using either gel filtration (method A) or charcoal extraction (method B).

Method A

3A. The gel filtration column (see previously) was allowed to run to almost dryness. The sample or blank was loaded onto the top of the column and eluted from the column using buffer B.

4A. Fractions, each of 1ml, were collected from the base of the column and a 100ul aliquot of each fraction was placed in 10ml Optiphases X and counted in a Packard counter for 1min.

5A. The total counts of tritiated drug bound to the tubulin which was eluted in the void volume was calculated and corrected using the blank. Using the specific activity of the tritiated drug and the protein content of the tubulin extract the binding in pmoles/mg protein was calculated.

Method B

3B. Charcoal extraction of the unbound tritiated drug was carried out. 0.5ml of the charcoal suspension was added to each tube, vortexed and incubated at 37°C for 5 min in a shaking waterbath.

4B. The tubes were centrifuged at 3,500g for 10min.

5B. An aliquot of the supernatant (0.4ml) was placed in 5ml Ecosint in a plastic scintillation vial and counted in a β counter(Packard Model 2650 Tricarb) The blanks and samples were counted for 1min.

6B. Adjustments were made for quenching and the results corrected by subtraction of the blanks then multiplied by 1.5 to give the total counts in the sample. To calculate the binding of ABZ or FBZ to the tubulin extracts in pmoles/assay the total counts in each sample were divided by the specific activity of ABZ or FBZ (dpm/pmole) and then to calculate the binding in pmoles/mg protein this value (pmoles/assay) was divided by the

protein concentration in the sample.

Binding Assays using Tubulin Extracted from *A.suum* and Tritiated Albendazole

Results

A modified tubulin binding assay using tubulin extracted from *A.suum* intestinal material was investigated. Varying concentrations of tritiated drug were used in the assay to try to determine the optimum concentration for use in tubulin binding assays. The tubulin extract had a protein concentration of 16mg/ml measured by the Lowry protein assay. The tritiated drug was ABZ from batch 1 with specific activity of 265 dpm/pmole. The tubulin bound ^{3}HABZ was separated from unbound drug using charcoal extraction (method B). The assay was repeated on three occasions and all results are shown in Table 3.6. For Assay 2 tubulin samples were incubated with tritiated drug for 15min and in Assay 1 and 3 incubation was for 3h prior to separation of bound and unbound drug.

Discussion

Binding of tritiated ABZ to tubulin extracts from *A.suum* was related to the concentration of tritiated drug incubated with the extract (Table 3.6). The duration of incubation was not critical. Assays 2 and 3 were performed on the same day using identical samples and varying the incubation time from 15min in the former to 3h in the latter. There was no significant difference, calculated using a Students t-test, between samples incubated for 15min or 3h at the same concentration of tritiated drug. Saturation of benzimidazole binding sites on tubulin must occur rapidly and prolonging the incubation time did not increase the binding.

Reproducibility of the assay was good with no differences between assays 1, 2 and 3 except for the sample incubated with 5.95 uM ^{3}HABZ in Assay 1 where the value for drug binding was low possibly due to experimental error.

Table 3.6

Binding of tritiated albendazole to tubulin extracts from *A.suum*

Concentration ^{3}HABZ	Binding of ^{3}HABZ (pmoles/mg protein)		
(uM)	Assay 1	Assay 2	Assay 3
23.80	11.56	12.25	12.92
11.90	8.85	7.36	8.09
5.95	1.97	5.89	6.92
3.97	5.26	5.51	4.31
1.98	1.92	2.74	1.81

The protein concentration with tubulin extracts from *A.suum* was significantly higher than extracts from *O.circumcincta* and *H.contortus* larvae. In these assays (Assay 1, 2 and 3) individual samples contained a constant amount of protein (1.44mg/tube). Binding of tritiated benzimidazoles has been shown to vary with protein concentration (Lacey and Prichard, 1986) and therefore the binding of tritiated drug to tubulin is calculated taking protein concentration into account as pmoles per mg of protein in the tubulin extract. The results for this assay were consistent and higher concentrations of tritiated ABZ gave increased binding. It was decided that for future tubulin binding assays a concentration of 23.8 uM or 11.9uM ^{3}HABZ would be used to ensure a high level of binding to the tubulin molecules. Increasing the concentration of ^{3}HABZ above this level could increase the error in the assay because the efficiency of charcoal in removing unbound tritiated drug could become a limiting factor (see later)

Binding of Tritiated Albendazole to Tubulin Extracted from *O. circumcincta* (Bearsden Strain)

Results

Using a range of numbers of infective larvae or eggs of *O.circumcincta* , Bearsden benzimidazole susceptible strain, tubulin was extracted using Method B. Protein in the resultant extracts was determined by the Lowry method and results are shown in Table 3.7. The extracts were used in tubulin binding assays using tritiated ABZ from batch 2 (23.8 uM) with specific activity of 360dpm/pmole. Bound and unbound drug were separated using charcoal extraction or Sephadex column separation. The binding of ^{3}HABZ calculated as pmoles per mg protein in the tubulin extract is shown in Table 3.7.

Discussion

The tubulin extracts from more than 2,000 infective larvae of *O.circumcincta* contained similar protein concentrations. Increasing the number of larvae used to make

Table 3.7

Binding of tritiated albendazole to tubulin extracts from infective larvae or eggs of the Bearsden benzimidazole susceptible strain of *O. circumcincta*

Tubulin Extract	Protein (mg/ml)	Binding ^{3}HABZ (pmoles/mg protein)	
		Charcoal separation	Column separation
3,000 eggs	0.23	1270.48	-
1,000 larvae	0.11	-	1263.30
2,000 larvae	0.22	4.57	-
3,000 larvae	0.20	41.90	1246.11
4,000 larvae	0.24	160.19	-
5,000 larvae	0.24	119.91	-
10,000 larvae	0.26	-	577.35

the extract did not produce a proportional increase in the protein concentration in the tubulin extract (Table 3.7). The homogenisation of the larvae could have been at fault causing variability in the amount of protein extracted from the larvae.

The concentration of tritiated ABZ bound to the extracts which was stable to charcoal extraction was much higher in the tubulin extract from eggs than larvae of *O. circumcincta*. Also increasing numbers of larvae from 2,000-4,000 showed increased binding of tritiated ABZ although the extract made using 5,000 larvae bound less than the extract from 4,000 larvae. The binding of drug to the tubulin extracts was calculated taking protein concentration into account and therefore there should be no difference in the binding of benzimidazoles to extracts using different numbers of larvae. For the extracts using 4,000 and 5,000 larvae the protein concentrations were identical and yet the binding of tritiated ABZ differed. It is possible that the protein concentration is not a direct measure of the tubulin content of the extract from the parasites and the amount of tubulin as a proportion of the total protein in the extract could vary from sample to sample.

There could be variability in the stability of the tubulin drug complex to charcoal extraction leading to within-assay variation in the measurement of tubulin bound drug. For each sample duplicates or triplicates were tested and a mean result was taken for calculation of the amount of drug bound to the extract. Occasionally there were obvious outliers where the charcoal had failed to extract the total amount of unbound tritiated drug and these values were ignored when calculating the tubulin binding of benzimidazoles. However, if this incomplete extraction occurs to a lesser extent it could lead to variation in the results for the assay. Separation of bound and unbound tritiated drug using a Sephadex column was examined and the results were compared with results of binding assays using charcoal extraction. There is no apparent reason for the higher values for binding of tritiated ABZ in the assays using column separation. The charcoal may extract some of the

tubulin bound drug as well as the unbound drug but this would not account for the large variation in the results between the two methods. A comparison of separation of tubulin bound and free tritiated colchicine using charcoal or a Sephadex column (Sherline *et al*, 1974) showed no significant differences between the results in assays using the two methods. Because benzimidazoles are thought to bind to the same site on tubulin as colchicine it is likely that the benzimidazole tubulin complex will behave in a similar way to the tubulin colchicine complex in both charcoal and column separation.

The high binding of tritiated ABZ to the tubulin extracted from eggs may be due to a higher percentage of tubulin in this extract compared with the extracts from larvae. Alternatively, there may be other macromolecules in the extract capable of binding tritiated ABZ and therefore increasing the amount of tritiated ABZ bound through non-specific binding. Eggs were collected from faecal material with thorough washing of the sample, however, contaminating faecal material which is closely associated with the eggs may influence the binding of tritiated benzimidazoles in the assay. Clean larval samples of *O.circumcincta* are readily available as a suitable starting material for tubulin extraction and assays of binding of benzimidazoles. Using higher numbers of larvae and thorough homogenisation during the preparation of tubulin extracts from *O.circumcincta* larvae may aid in achieving higher concentrations of protein in the tubulin extract.

Comparison of Binding of Tritiated Albendazole to Tubulin Extracts from Benzimidazole Susceptible and Resistant Strains of *O.circumcincta* (Bearsden and HFRO Strains)

Results

Tubulin extracts were prepared using infective larvae or eggs of *O.circumcincta* by Method B. The two strains of *O.circumcincta* used were the Bearsden benzimidazole

susceptible strain and the HFRO benzimidazole resistant strain. The protein concentration in the tubulin extracts was calculated using a Lowry protein assay and results are shown in Tables 3.8 and 3.9. The tubulin extracts were used in binding assays using tritiated ABZ batch 2 (23.8uM) with specific activity of 360 dpm/pmole. The bound and unbound drug were separated using a Sephadex column or charcoal extraction. Results of binding of tritiated ABZ were expressed as pmoles/mg protein in the tubulin extract and are shown in Tables 3.8 and 3.9.

Discussion

Lacey and Prichard (1986) found that binding of tritiated benzimidazoles to benzimidazole resistant strains of *Haemonchus contortus* was significantly lower than binding to benzimidazole susceptible strains. Similar differences in binding of benzimidazoles were found between benzimidazole resistant and susceptible strains of *O.circumcincta* and *Trichostrongylus colubriformis* (Lacey and Snowdon,1988) and the binding of benzimidazoles to tubulin extracts from nematode parasites was suggested as a possible assay to detect benzimidazole resistance.

Results in Table 3.8 show reduced binding of tritiated ABZ to tubulin extracted from eggs of the benzimidazole resistant stain of *O.circumcincta* compared with the susceptible strain. However, binding of tritiated ABZ was much higher to tubulin extracts from larvae of the resistant strain than tubulin extracts using larvae of the susceptible strain. Similar results were obtained in a repeat of the binding assay (Table 3.9). The binding of tritiated ABZ to the tubulin extracts was inconsistent between assays. In Table 3.9 the two extracts using 10,000 resistant larvae bound similar amounts of tritiated ABZ but this differed from results of binding to an extract made with 10,000 resistant larvae in the previous assay (Table 3.8). Binding of tritiated ABZ to tubulin extracts from eggs of the two strains of *O.circumcincta* (Table 3.8) was much lower than binding calculated in a previous assay (Table 3.7). This could be due to increased care taken to ensure the

Table 3.8

Binding of tritiated albendazole to tubulin extracts from eggs and infective larvae of benzimidazole susceptible and resisitant strains of *O.circumcincta* (Bearsden and HFRO strains)

Tubulin Extract	Protein (mg/ml)	Binding ^{3}HABZ (pmoles/mg protein)	
		Charcoal separation	Column separation
6,000 eggs - S	0.10	346.70	-
6,000 eggs - R	0.10	112.03	-
15,000 larvae - S	0.28	318.43	131.45
10,000 larvae - R	0.24	4004.76	665.95
4,000 larvae - R	0.11	-	841.32

S - Bearsden susceptible strain of *O.circumcincta*

R - HFRO resisitant strain of *O.circumcincta*

Table 3.9

Binding of tritiated albendazole to tubulin extracts from infective larvae of benzimidazole susceptible and resistant strains of *O.circumcincta* (Bearsden and HFRO strains)

Tubulin Extract	Protein (mg/ml)	Binding ^{3}HABZ (pmoles/mg protein)	
		Charcoal separation	Column separation
10,000 larvae - S	0.39	1233.93	322.79
10,000 larvae - R	0.18	5967.49	11771.43
10,000 larvae - R	0.21	5556.87	-
5,000 larvae - R	0.09	389.03	-

S - Bearsden benzimidazole susceptible strain of *O.circumcincta*

R - HFRO benzimidazole resistant strain of *O.circumcincta*

samples were clean and free from faecal material prior to homogenisation in preparation of the tubulin extracts or due to inconsistency in the assay. There was a marked variation in the binding measured using a column or charcoal for separation of the bound and unbound drug. For some tubulin extracts binding measured after charcoal extraction was higher than with column separation but with other extracts this trend was reversed.

Overall the results of these binding assays were extremely variable and comparisons of binding of benzimidazoles to resistant or susceptible strains of *O.circumcincta* could not be made because of the within assay and between assay variability in binding. The reproducibility of the results of binding assays was a major problem and the reasons for the extreme variability in the results were unknown. The protein concentration within the tubulin extract could have been at fault. A low level of protein and therefore tubulin in the extract could result in poor specific binding of benzimidazole and the contribution to the total binding of benzimidazole made by non-specific binding could distort the results. The efficiency of the charcoal at removing unbound tritiated drug also may be of great importance especially where low levels of drug were binding to tubulin. The contribution to the overall counts in the sample made by a small amount of unbound drug could be significant if the tubulin extract was binding a small amount of tritiated drug. According to Sherline *et al.*, (1974) activated charcoal removed 96% of tritiated colchicine from samples. Calculation of the efficiency of the charcoal at removing unbound tritiated drug in the binding assays using tritiated ABZ described above showed results of 94.3% and 94.9% which is probably as good as can be attained using this method. Efficiency of removal of unbound tritiated drug was greater with a column but this method has the disadvantage of being more time-consuming and therefore of little use for multiple sample assays.

The nature of the benzimidazole used in the binding assay may have an effect on the binding profile because of the differing affinities of the individual benzimidazoles for

tubulin (Lacey and Pritchard, 1986). Lacey and Snowdon (1988) in their description of benzimidazole binding to tubulin extracts as a method of detecting resistance to benzimidazoles used tritiated mebendazole as their test benzimidazole. The use of different benzimidazole compounds in the binding assays to tubulin extracted from benzimidazole resistant and susceptible strains of *O.circumcincta* was investigated using tritiated FBZ in addition to tritiated ABZ to try to establish if the nature of the benzimidazole was a reason for the variable results obtained in the assays.

Binding of Tritiated Fenbendazole to Tubulin Extracts from Benzimidazole Susceptible and Resistant Strains of *O.circumcincta* (Bearsden and HFRO Strains)

Results

Tubulin extracts were prepared using Method B from 5,000 infective larvae of the Bearsden benzimidazole susceptible strain and the HFRO benzimidazole resistant strain of *O.circumcincta*. The protein concentrations in the tubulin extracts which were calculated using a Lowry protein assay are shown in Table 3.10. These extracts were used in a binding assay with tritiated FBZ (83.9uM, 110dpm/pmole). The bound and unbound drug were separated using a Sephadex column and results of binding of tritated FBZ to the tubulin extracts in pmoles/mg protein are given in Table 3.10. A graph was drawn of the counts in fractions eluted from the Sephadex column during separation of tubulin bound and unbound tritiated FBZ (Fig. 3.5)

Discussion

There was no significant difference in the binding of tritiated FBZ to tubulin extracts from the benzimidazole resistant and susceptible strains of *O.circumcincta* (Table 3.10). Lacey and Prichard, (1986) noted a higher level of binding of benzimidazoles to tubulin extracted from benzimidazole susceptible parasites compared with binding to

Table 3.10

Binding of tritiated fenbendazole to tubulin extracts from benzimidazole susceptible and resistant strains of *O.circumcincta* (Bearsden and HFRO strains)

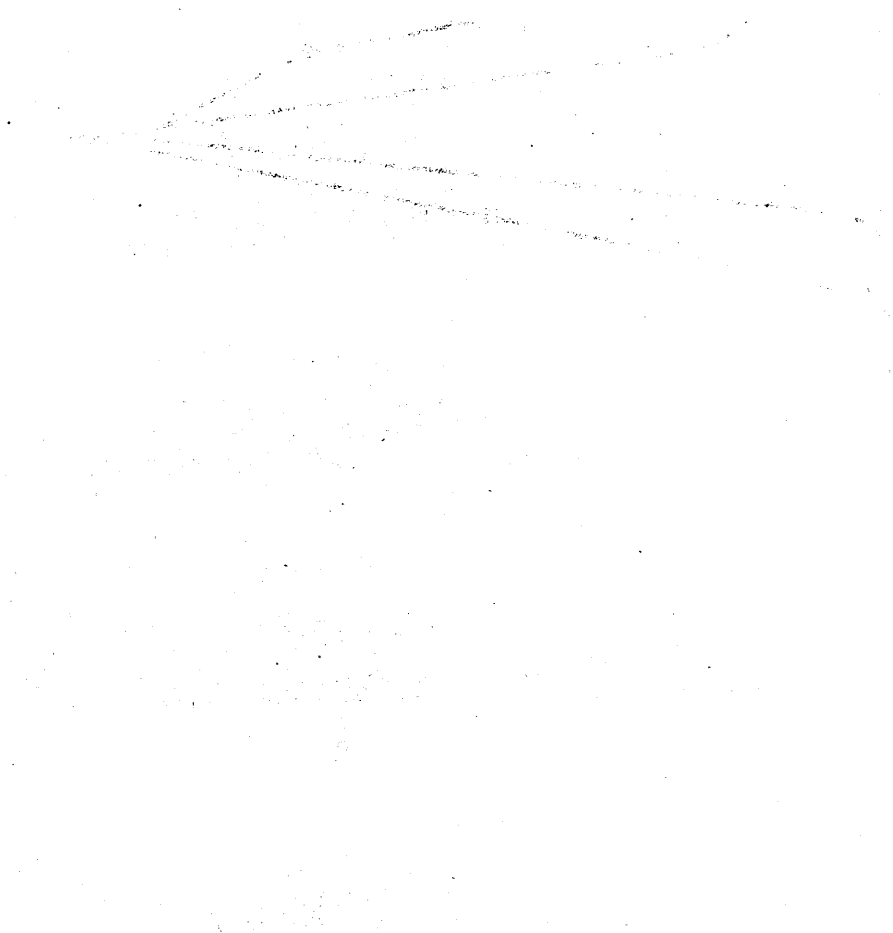
Extract	Protein (mg/ml)	Binding ^{3}HFBZ (pmoles/mg protein)
5,000 larvae - **S**	0.13	2870.94
5,000 larvae - **R**	0.15	2776.44

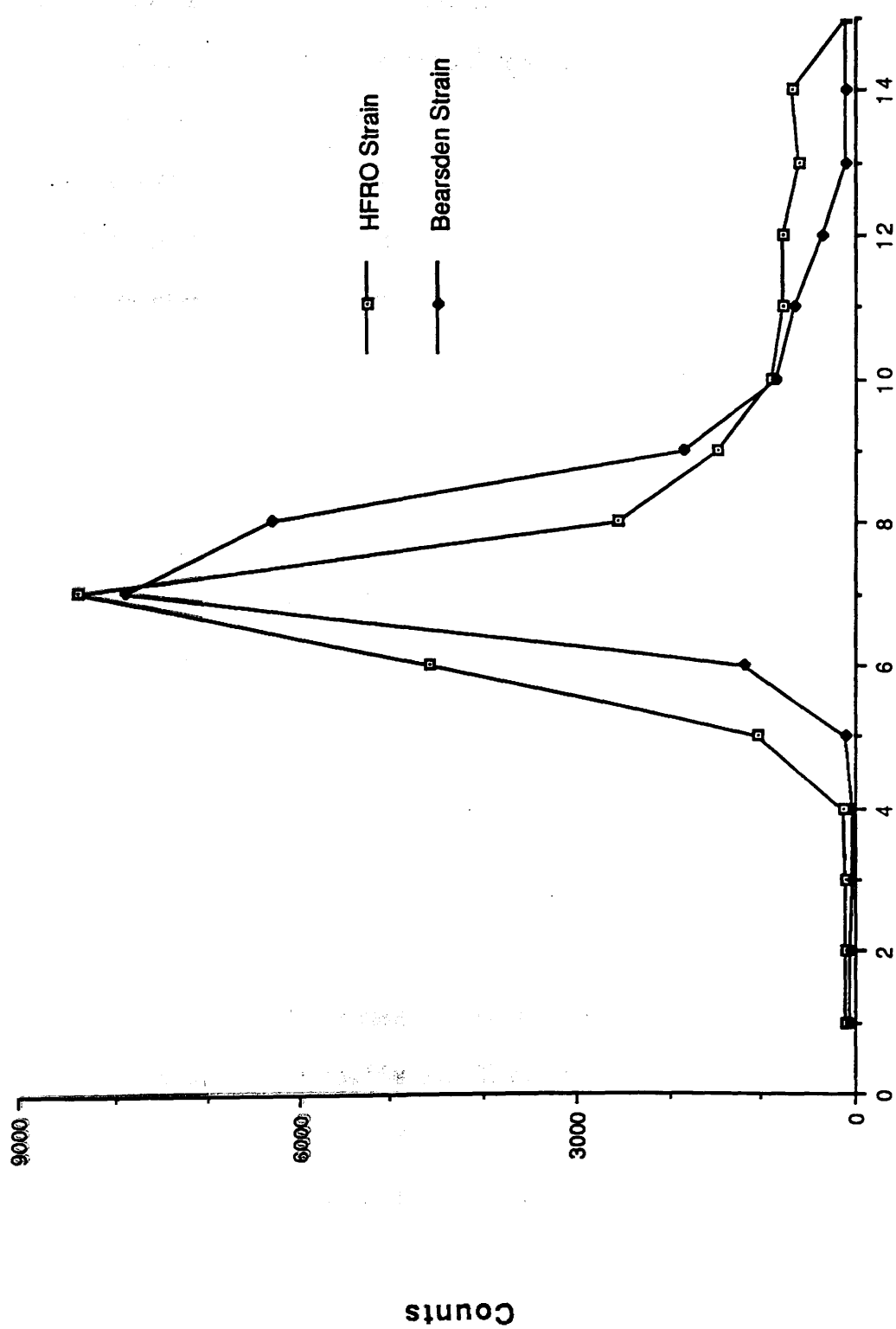
S - Bearsden benzimidazole susceptible strain of *O.circumcincta*

R - HFRO benzimidazole resistant strain of *O.circumcincta*

Figure 3.5

Counts in eluate from Sephadex column used to separate tubulin bound and unbound tritiated fenbendazole using tubulin extracts from *O.circumcincta* HFRO and Bearsden strains.




HFRO Strain
Bearsden Strain
Counts
9000
6000
3000
0
0
2
4
6
8
10
12
14
Fraction no.

benzimidazole resistant strains. In previous assays (Tables 3.8 and 3.9) using tritiated ABZ binding to tubulin extracts from infective larvae of a benzimidazole resistant strain of *O.circumcincta* was higher than to extracts from larvae of a benzimidazole susceptible strain. Using tritiated FBZ there was no difference in the level of binding to extracts from these same resistant and susceptible strains of *O.circumcincta* (Table 3.10). The high level of binding measured in these assays to extracts from a benzimidazole resistant strain of *O.circumcincta* conflicts with results described by Lacey and Snowdon (1988). In the assay described above using tritiated FBZ the unbound and tubulin bound drug were separated using a Sephadex column in case instability to charcoal extraction was causing variability in the results of the binding assays (see previously). Using column separation and collection of fractions from the column for counting, a graph can be drawn showing the elution of the tritiated drug (Fig. 3.5). The tritiated drug bound to high molecular weight material is eluted in the void volume (fractions 5-12) and the unbound tritiated FBZ which has a lower molecular weight is eluted later, in fraction 20 onwards approximately. The graph of counts eluted from the column during separation of the bound and unbound tritiated FBZ showed high counts at the period of elution of high molecular weight compounds (Fig 3.5) then a fall to basal levels. There was a rise in counts from fractions 20-30 . From these results it can be concluded that the tritiated FBZ is bound to material with a high molecular weight which is assumed to be tubulin. The tubulin extracts from benzimidazole resistant and susceptible strains of *O.circumcincta* had similar protein concentrations (Table 3.10) and a similar pattern of binding of tritiated FBZ and therefore the binding assay in this form cannot be used as a method to detect resistance to benzimidazoles in strains of *O.circumcincta*.

The Effect of Host Treatment with Fenbendazole on the Binding of Benzimidazoles to Tubulin Extracted from Eggs of *O.circumcincta* (HFRO Strain)

Results

Eggs of *O.circumcinta* were collected from the faeces of a lamb (DB16) which had been infected experimentally with the HFRO benzimidazole resistant strain of *O.circumcincta* for passage 5 (see previously). Eggs were collected prior to oral treatment of the lamb with FBZ at a dose rate of 5mg/kg and after treatment on 3 occasions at 24h, 48h and 8 days post treatment. Using 20,000 eggs collected at each occasion tubulin was extracted and the protein concentration in the extracts determined using a Lowry protein assay. The protein concentration in the extracts was adjusted to give a concentration of 0.11mg/ml in each extract. The extracts were used in tubulin binding assays with tritiated FBZ (83.6uM, 110dpm/pmole) and tritiated ABZ (119uM, 360dpm/pmole). The tubulin bound and unbound drug were separated using a Sephadex column.

Results of binding of tritiated ABZ and FBZ to the tubulin extracts in pmoles /mg protein are given in Table 3.11. The counts obtained during elution of the tubulin bound fraction of the samples during Sephadex column separation for each of the tubulin extracts with tritiated ABZ or tritiated FBZ are shown in Figs. 3.6 and 3.7 respectively.

Discussion

Binding of benzimidazoles to tubulin extracts from *O.circumcincta* eggs passed in the faeces of a lamb treated with orally administered FBZ varied (Table 3.11). There was increased binding of tritiated ABZ to tubulin extracts from eggs collected 24h and 48h after FBZ treatment. However, in binding assays using tritiated FBZ, increased binding was observed at 24h and binding was reduced at 48h compared with pre-treatment levels. The reason for the increase in binding to tubulin extracts from eggs collected immediately post-treatment is unknown. It is possible that treatment of the host with FBZ reduces the

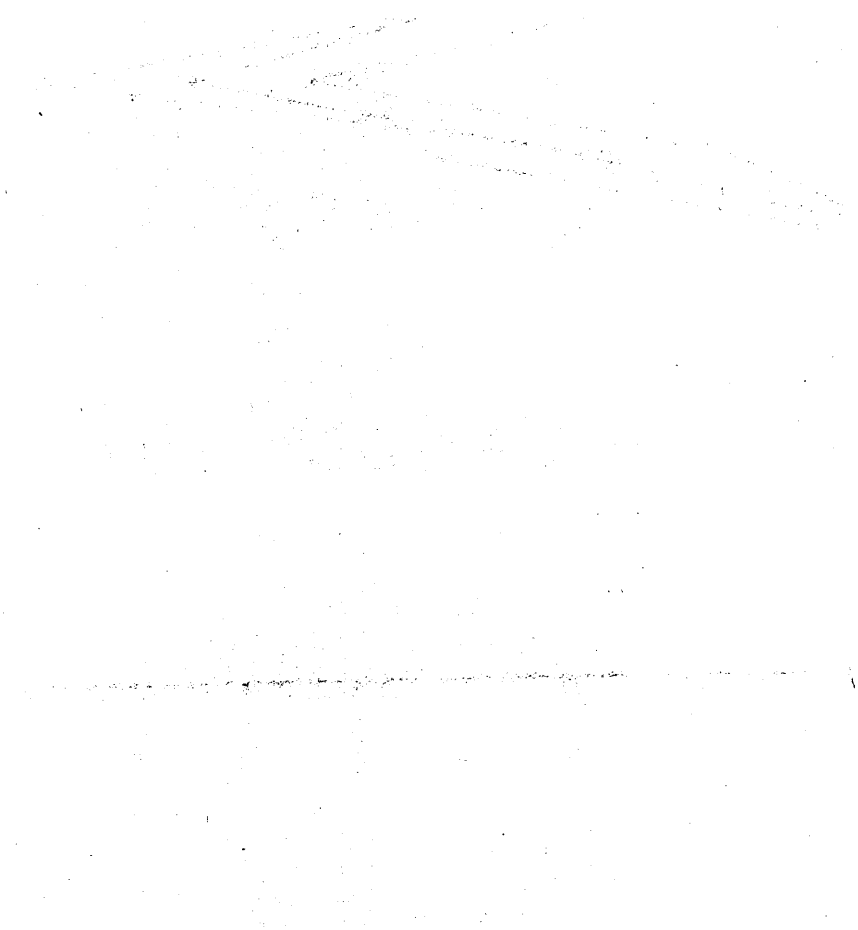
Table 3.11

Binding of tritiated benzimidazoles to tubulin extracted from eggs of *O.circumcincta* (HFRO strain) collected before and after oral treatment of the host with fenbendazole

Extract	Binding of Tritiated Benzimidazole (pmoles/mg protein)	
	Albendazole	Fenbendazole
Pre-treatment	131.21	452.53
24h Post-treatment	181.53	538.02
48h Post-treatment	203.51	404.52
8days Post-treatment	166.95	464.67

Figure 3.6

Counts obtained during elution of tubulin bound tritiated albendazole from a Sephadex column with tubulin extracts from eggs of *O.circumcincta* HFRO strain collected prior to and at 24h, 48h and 8 days after treatment of the host with fenbendazole.



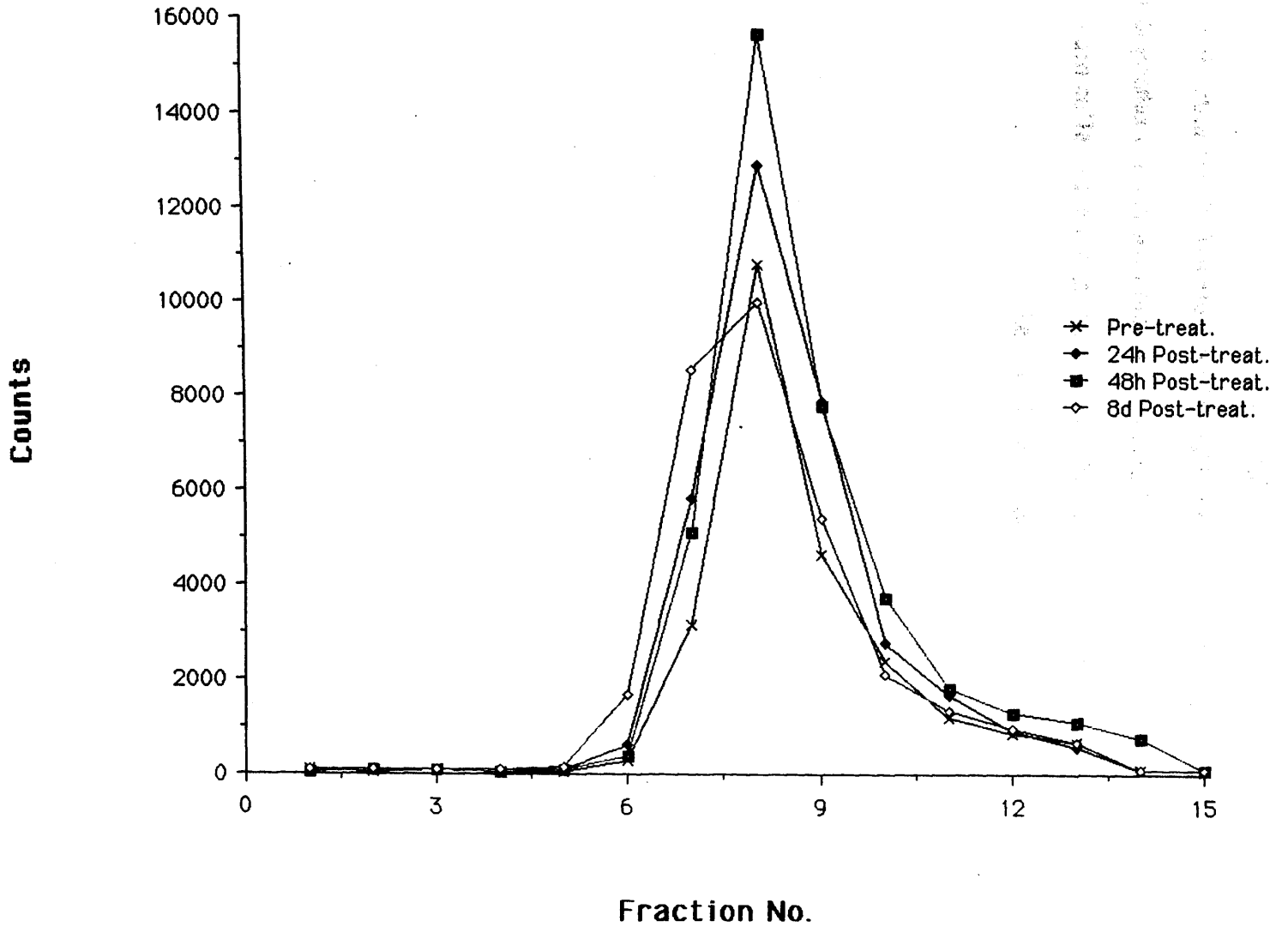
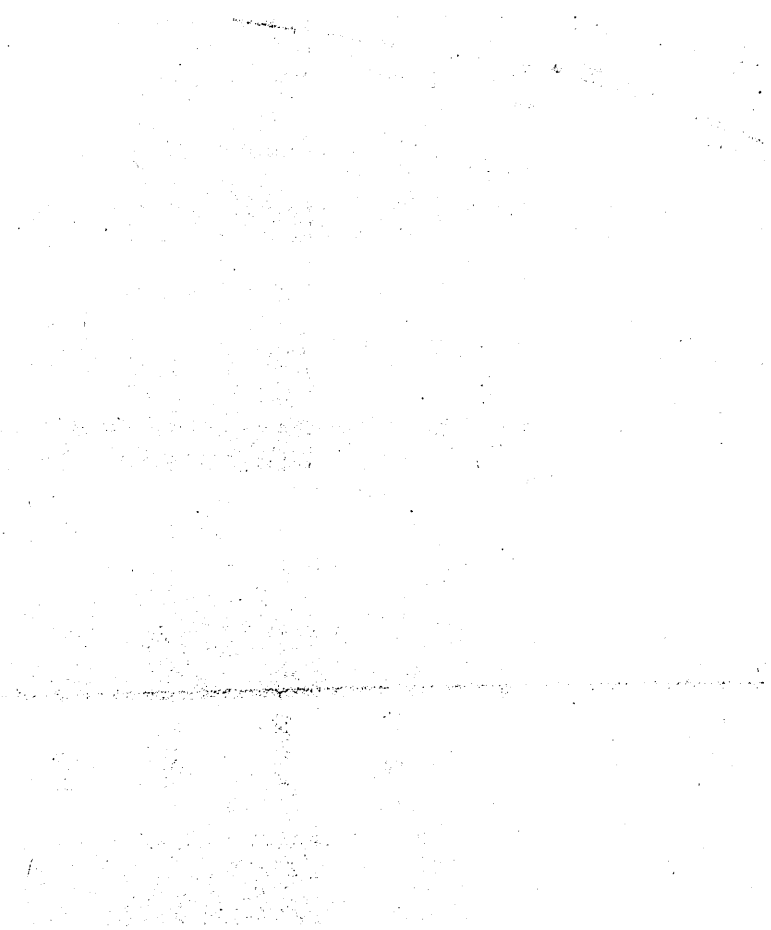
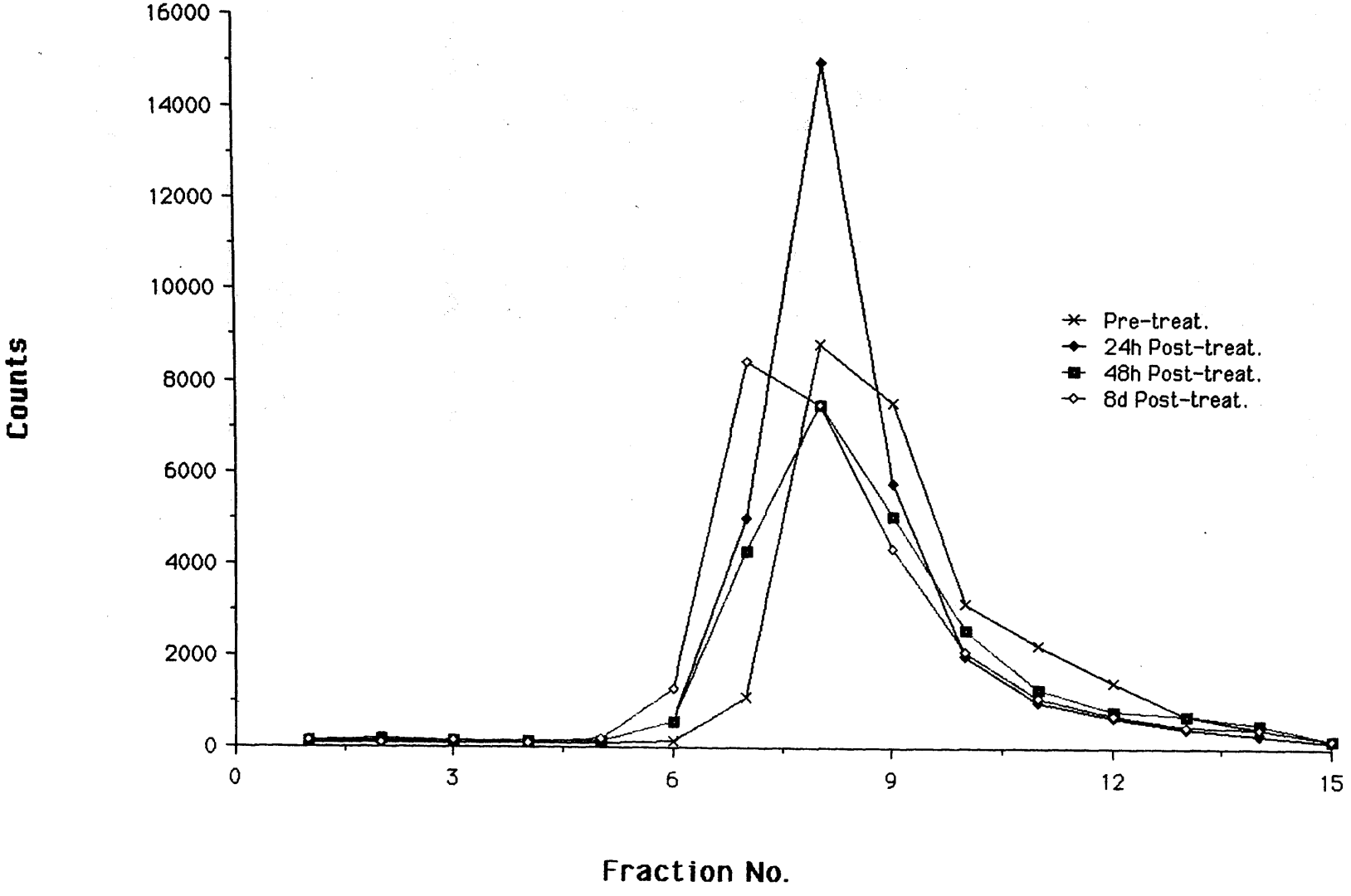

16000
14000
12000
10000
8000
6000
4000
2000
0
Counts
0
3
6
9
12
15
Fraction No.
Pre-treat.
24h Post-treat.
48h Post-treat.
8d Post-treat.

Figure 3.7

Counts obtained during elution of tubulin bound tritiated fenbendazole from a Sephadex column with tubulin extracts from eggs of *O.circumcincta* HFRO strain collected prior to and at 24h, 48h and 8 days after treatment of the host with fenbendazole.




16000
14000
12000
10000
8000
6000
4000
2000
0
Counts
0
3
6
9
12
15
Fraction No.
Pre-treat.
24h Post-treat.
48h Post-treat.
8d Post-treat.

formation of microtubules in adult parasites (Borgers *et al*, 1975b) and their eggs. This may produce a change in the equilibrium between tubulin subunits and the microtubule structures with possible exposure of an increased number of benzimidazole binding sites. From Figs. 3.6 and 3.7 the distribution of counts bound to high molecular weight compounds eluted from the Sephadex column was similar for all the samples suggesting that any increase in binding occurred to macromolecules of similar distribution and high molecular weights. The tubulin extracts were prepared to contain the same amount of protein but the proportion of tubulin in extracts from eggs collected immediately post-treatment could be higher.

Alternatively, there could be an alteration in the adult population of the parasites leading to changes in the eggs produced by the parasites. After treatment of the host with FBZ there was a drop in the faecal egg count. Either fewer adults are laying eggs or the rate of egg laying in the same population is reduced. If the latter was the case then the eggs were possibly held within the female parasites for a longer period with possible increased accumulation of tubulin. Egg hatch assays carried out on eggs passed in faeces in the 24-72h period after oral treatment of the host with FBZ showed elevated ED50s particularly when ABZ was used in the assay (see previously). The reason for these observed differences in the results of egg hatch assays may have a link with reasons for the observed changes in the binding of benzimidazoles to tubulin extracts from eggs collected during the same period post-treatment.

The results of this group of binding assays were fairly consistent compared with previous assays. The tubulin extracts were prepared using higher numbers of eggs than previously which may have resulted in the improved reproducibility (see later).

Binding of Tritiated Albendazole and Tritiated Fenbendazole to Tubulin Extracts Prepared using High Numbers of Infective Larvae of a Benzimidazole Susceptible and Resistant Strain of *O.circumcincta* (Bearsden and HFRO Strains)

Results

Tubulin extracts were prepared by Method A using infective larvae of HFRO and Bearsden strains of *O.circumcincta*. The protein concentration in the extracts was assessed using a Lowry protein assay and the results are shown in Table 3.12. The extracts were used in duplicate tubulin binding assays using tritiated FBZ (8.36uM, 110dpm/pmole) and tritiated ABZ (11.9uM, 360dpm/pmole) with results shown in Table 3.13. Tubulin bound and unbound tritiated drug were separated using charcoal. The susceptibility factor, the concentration of drug bound to the susceptible strain divided by the concentration of drug bound to the resistant strain, was calculated for each assay along with the resistance factor, the concentration of drug bound to the resistant strain divided by the concentration of drug bound to the susceptible strain, and are shown in Table 3.13.

Discussion

The tubulin extracts contained a higher level of protein than any extracts used previously (Table 3.12) with >70ug of protein in each sample of extract used in the binding assays. Binding of tritiated FBZ in pmoles/mg protein was higher than binding of tritiated ABZ which was similar to results in the previous binding assay using tubulin extracts from eggs of *O.circumcincta* HFRO strain (Table 3.11). The binding of tritiated ABZ to tubulin Extracts A and B was lower than binding to Extracts C and D but levels of binding of tritiated FBZ did not show such a large fluctuation. In all the assays the concentration of tritiated ABZ or FBZ bound was greater to the tubulin extracts from the susceptible strain of *O.circumcincta* than the resistant strain. This is contrary to results obtained in previous assays but similar to results of Lacey and Snowdon (1988).

Table 3.12

Protein concentration in tubulin extracts prepared from infective larvae of Bearsden and HFRO strains of *O.circumcincta*

Extract (no. of Larvae)	Protein Concentration (mg/ml)
A - 200,000 - **S**	2.16
B - 150,000 - **R**	4.55
C - 150,000 - **S**	0.82
D - 150,000 - **R**	1.32

S - Bearsden benzimidazole susceptible strain of *O.circumcincta*

R - HFRO benzimidazole resistant strain of *O.circumcincta*

Table 3.13

Binding of Tritiated Benzimidazoles to Tubulin Extracts from Large Numbers of Infective Larvae of Benzimidazole Resistant and Susceptible Strains of *O.circumcincta*.

Tubulin Extract	Binding (pmoles/mg protein)			
	^{3}HABZ		^{3}HFBZ	
	Assay 1	Assay 2	Assay 1	Assay 2
A - 200,000 larvae - **S**	22.20	26.39	110.50	89.74
B - 150,000 larvae - **R**	2.32	4.30	25.90	17.33
Resistance Factor **S/R**	9.57	6.14	4.27	5.18
Susceptibility Factor **R/S**	0.11	0.16	0.23	0.19
C - 150,000 larvae - **S**	117.66	50.14	149.32	64.81
D - 150,000 larvae - **R**	51.61	43.66	16.85	22.00
Resistance Factor **S/R**	2.28	1.15	8.86	2.95
Susceptibility Factor **R/S**	0.44	0.87	0.11	0.34

S - Bearsden benzimidazole susceptible strain of *O.circumcincta*

R - HFRO benzimidazole resistant strain of *O.circumcincta*

However, the reproducibility of results from one assay to another is poor (Table 3.13). When measuring resistance a susceptibility factor or resistance factor is calculated to draw comparisons between various resistant and susceptible strains of nematodes. From the results in Table 3.13 these factors show great between assay variation. For tritiated ABZ the resistance factors range from 1.15 to 9.57 and for tritiated FBZ from 2.95 to 8.86 for the same resistant and susceptible strains. The quantification of resistance from the results of this assay is therefore very unreliable. From egg hatch assay results (see previously) a ratio for resistant/ susceptible of about 6 is expected with these strains. Further discussion on the reproducibility of results in binding assays is included later.

Binding of Tritiated Albendazole or Fenbendazole to Tubulin Extracts from *Haemonchus contortus* (McMaster and VRSG Strains)

Results

Tubulin extracts were prepared by Method A using various numbers of infective larvae of the two strains. The protein concentration in the extracts was assessed using a Lowry protein assay and results are shown in Table 3.14. The extracts were used in binding assays with tritiated FBZ (8.36uM, 110dpm/pmole) and tritiated ABZ (10.8uM, 360 dpm/pmole). Separation of the tubulin bound and unbound benzimidazole was carried out using charcoal extraction. A Sephadex column was used to separate bound and unbound tritiated ABZ in repeat assays. Results of these binding assays are shown in Table 3.15.

Discussion

Differences in the binding of tritiated benzimidazoles to tubulin extracts from benzimidazole susceptible and resistant strains of *H.contortus* were found (Table 3.15). With tubulin Extracts A and B and tritiated ABZ there was an approximate three fold increase in binding to the extract from the susceptible strain compared with the extract from the resistrant strain. However, with tritiated FBZ the overall binding of drug was

Table 3.14

Protein concentration in tubulin extracts prepared from infective larvae of *Haemonchus contortus* (McMaster and VRSG strains)

Tubulin Extract (no. of Larvae)	Protein Concentration (mg/ml)
A - 100,000 - McMaster - **S**	0.62
B - 100,000 - VRSG - **R**	0.55
C - 120,000 - McMaster - **S**	0.98
D - 55,000 - VRSG - **R**	0.27

S - Benzimidazole susceptible strain

R - Benzimidazole resistant strain

Table 3.15

Binding of tritiated ABZ or tritiated FBZ to tubulin extracts from *H.contortus* (McMaster and VRSG strains)

Tubulin Extract[1]	Binding of Benzimidazole (pmoles/mg protein)		
	^{3}HABZ *	^{3}HABZ**	^{3}HFBZ*
McMaster - **A**	65.55	58.23	117.13
VRSG - **B**	20.49	21.36	99.07
Resistance Factor	3.20	2.73	1.18
Susceptibility Factor	0.31	0.37	0.85
McMaster - **C**	56.06	40.85	165.65
VRSG - **D**	64.80	76.64	308.35
Resistance Factor	0.87	0.53	0.54
Susceptibility Factor	1.16	1.88	1.86

1 Number of larvae used to prepare tubulin extracts and protein concentration of the extracts given in Table 3.14.

*Bound and unbound drug separated using charcoal extraction

**Bound and unbound drug separated using a Sephadex column

greater but the difference between binding to the resistant strain and binding to the susceptible strain was much lower. For tritiated ABZ separation of bound and unbound drug using a Sephadex column gave similar results to assays where charcoal extraction was used. With tubulin Extracts C and D comparison of binding of tritiated ABZ or FBZ to the susceptible strain with binding to the resistant strain gave the opposite results. The extract derived from the resistant strain bound a higher level of drug than the extract from the susceptible strain. Extract D was prepared using 55,000 infective larvae compared with 120,000 larvae used for Extract C. The lower resulting protein concentration in Extract D could be important in influencing the results of the tubulin binding assays. The effect of protein concentration in the tubulin extract on the binding of tritiated benzimidazoles is discussed in more detail later.

Lacey and Prichard (1986) used these strains of *H.contortus* in their work on identification of benzimidazole resistant strains of nematodes using the tubulin binding assay. These authors calculated the ratios of binding of drug to the susceptible strain (McMaster) as compared with binding to the resistant strain (VRSG) for individual benzimidazole compounds. For ABZ the ratio was 60.8±18.9 and for FBZ 10.2±1.4. These ratios do not correspond with results (resistance factors) shown in Table 3.15 from binding assays using identical strains of *H.contortus*. These gross differences in results using the tubulin binding assay required further investigation to try to elucidate if consistent, reliable results could be obtained with this method.

Tubulin Binding Assay using Tubulin Extracts from Benzimidazole Resistant or Susceptible *H.contortus* and *O. circumcincta* Infective Larvae and Tritiated ABZ or MBZ. (Method from Lacey and Prichard ,1986)

Materials and Method

Reagents./

Reagents.

Tritiated ABZ and MBZ - Tritiated drugs prepared by method II (see previously) as 10 uM solution in DMSO / MES buffer. Specific activity ABZ - 9754 dpm/pmole and MBZ - 2685 dpm/pmole

Buffer- 0.025M MES pH6.5 containing 0.1mM EGTA, 1.5mM $MgSO_4$ and 0.1M GTP.

Charcoal suspension - 2mg/ml charcoal (Norit-A activated charcoal - Sigma Chemical Co. Ltd.) in 1% (w/v)BSA.

Method

1. Duplicates of 90ul of tubulin extracts prepared by method A (see previously) and dilutions of the extracts giving a range of protein concentrations from approx. 10-120ug/ml were placed in 1.5ml Eppendorf tubes (blanks 90ul buffer).

2. To each tube was added 10ul tritiated ABZ or MBZ vortexed and the tubes were incubated for 30 min at 37°C in a shaking waterbath .

3. 0.5ml of the charcoal suspension was added to each tube, vortexed and incubated at 37°C for 5 min in a shaking waterbath.

4 The tubes were centrifuged at 10,000 r.p.m. in an Eppendorf bench-top centrifuge for 10min.

5. An aliquot of the supernatant (0.4ml) was placed in 5ml Biofluor in a glass scintillation vial and counted in a β counter (Packard Model 2650 Tricarb). The blanks were counted for 10min and the samples for 1min.

6. Adjustments were made for quenching and the results corrected by subtraction of the blanks then multiplied by 1.5 to give the total counts in the sample. To calculate the binding of ABZ or MBZ to the tubulin extracts in pmoles/assay the total counts in each sample was divided by the specific activity of ABZ or MBZ (dpm/pmole) and then to calculate the binding in pmoles/mg protein this value (pmoles/assay) was divided by the protein concentration in the sample.

7. Graphs of binding, pmoles/assay or pmoles/mg protein, against protein concentration in the tubulin extract were drawn and the mean value for binding of ^{3}HABZ or ^{3}HMBZ to each individual strain calculated using values from the linear portion of the graphs.

Tubulin Binding Assays using Tubulin Extracts from *H.contortus* McMaster and VRSG Strains with Tritiated Mebendazole and Tritiated Albendazole

Results

Tubulin extracts were prepared from infective larvae of *H.contortus*, McMaster and VRSG strains. The protein concentrations in the extracts were determined using the BioRad assay and results are shown in Table 3.16. These extracts were used in tubulin binding assays with tritiated MBZ or tritiated ABZ. A range of protein concentrations was used for each extract in the assays (Table 3.16). Graphs of protein concentration against binding of tritiated drug for Assays 4 and 5 with MBZ are shown in Figs. 3.8 and 3.9 and for Assays 3 and 4 with ABZ in Figs. 3.10 and 3.11 The mean and standard deviation for binding of tritiated MBZ or ABZ for each assay are shown in Tables 3.17 and 3.18. For tritiated MBZ curves of binding to tubulin extracted from the McMaster strain (Fig. 3.12) or VRSG strain (Fig. 3.13) for each assay against protein concentration were drawn. Similar curves were drawn for assays using tritiated ABZ (Figs. 3.14 and 3.15).

Discussion

The tubulin extracts were prepared using high numbers of *H.contortus* infective larvae (>100,000 larvae) producing protein concentrations in the extracts over the range 0.69-2.96 mg/ml. Using the BioRad protein assay was less time consuming than the Lowry assay and gave comparable results for protein concentrations in the extracts. Lacey and Snowdon (1988) found a linear relationship betweeen protein concentration in the extract and number of larvae with a yield of 6.5 ug protein per 1,000 larvae. The range

Table 3.16

Tubulin extracts prepared from *H.contortus* McMaster and VRSG strains

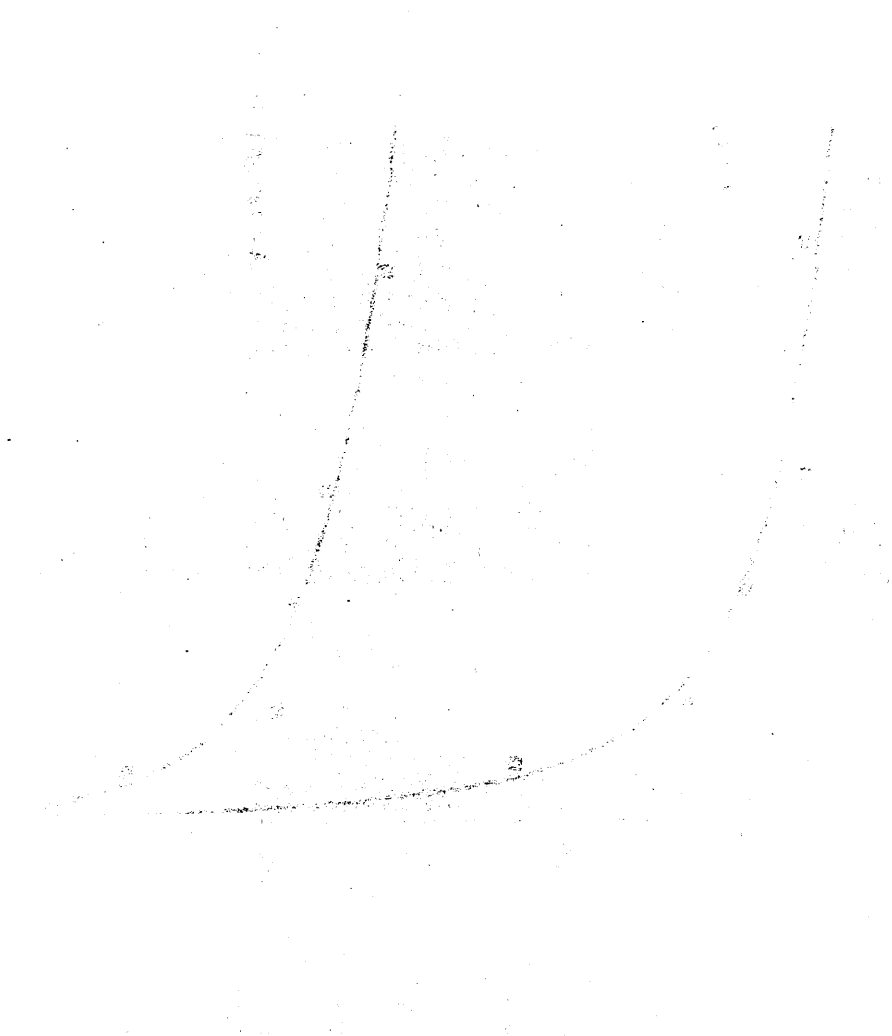
Assay Number	Number of Larvae	Protein concentration (mg/ml)	Protein in Assay (ug)
1	400,000 - S	2.88	22 - 98
	400,000 - R	2.96	26 - 119
2	100,000 - S	0.87	35 - 61
	100,000 - R	0.89	44 - 80
3	450,000 - S	1.88	9 - 169
	450,000 - R	2.01	5 - 181
4	400,000 - S	1.61	8 - 144
	400,000 - R	1.69	8 - 153
5	300,000 - S	1.46	4 - 131
	300,000 - R	1.56	4 - 141
6	200,000 - S	1.82	9 - 163
	200,000 - S	0.69	3 - 62

S - McMaster benzimidazole susceptible strain of *H.contortus*

R - VRSG benzimidazole resistant strain of *H.contortus*

Figure 3.8

Binding of tritiated mebendazole (pmoles/mg protein) to tubulin extracted from *H.contortus* VRSG and McMaster strains in Assay 4.



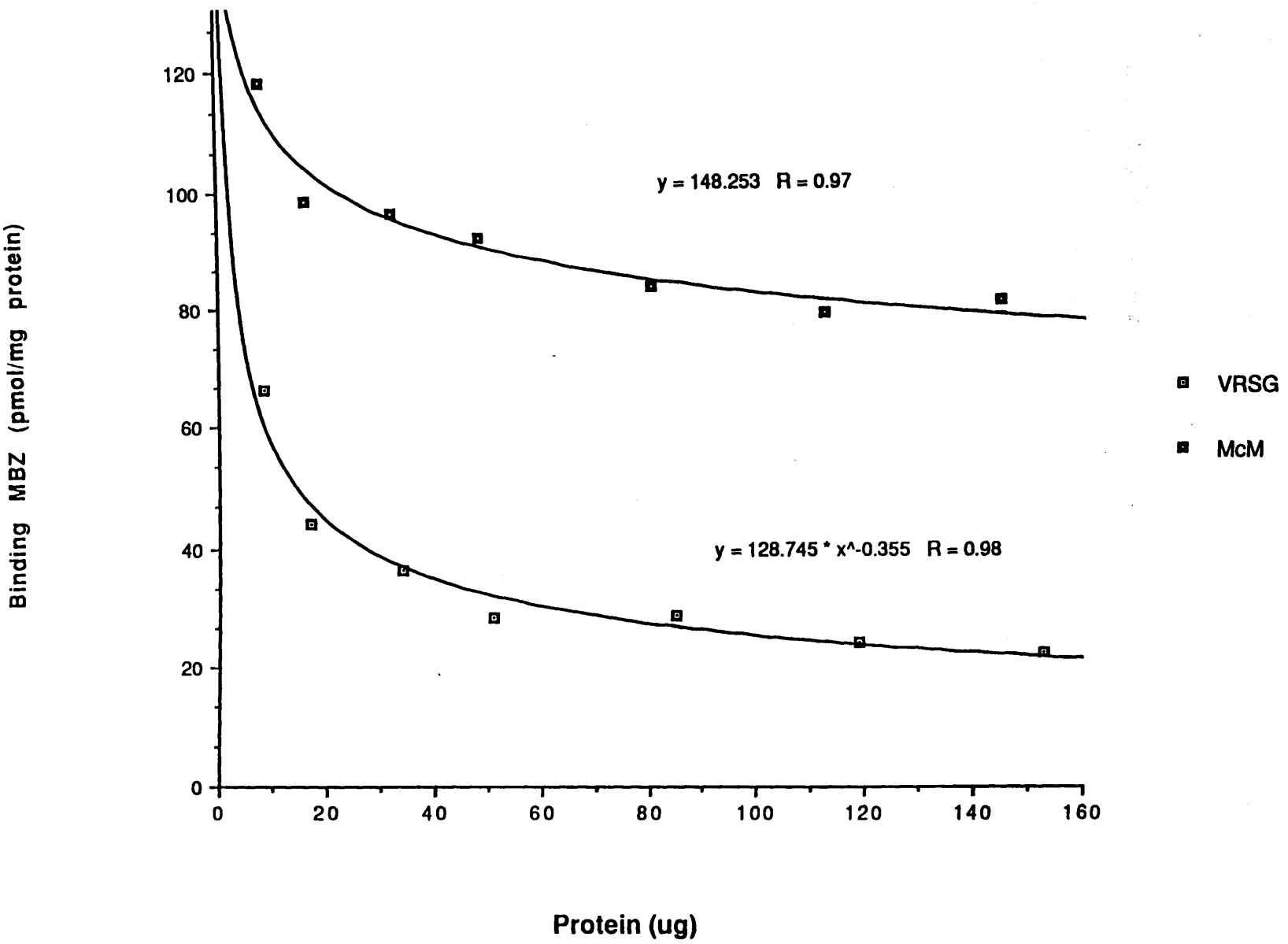
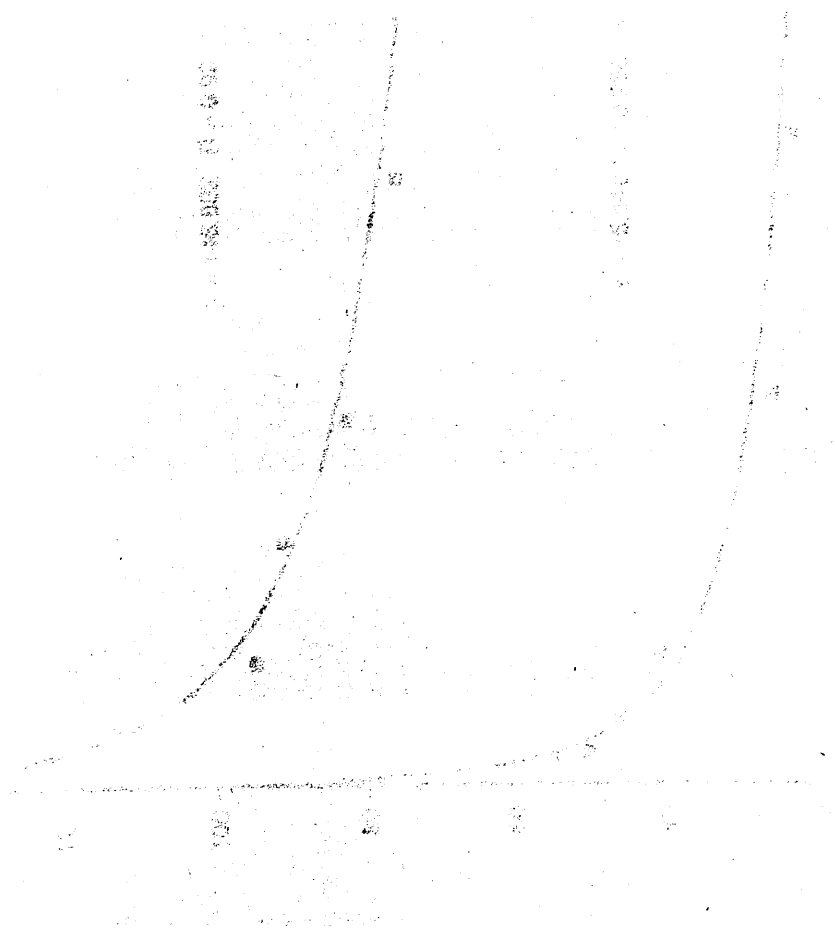

Binding MBZ (pmol/mg protein)
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
Protein (ug)
y = 148.253 R = 0.97
y = 128.745 * x^-0.355 R = 0.98
VRSG
McM

Figure 3.9

Binding of tritiated mebendazole (pmoles/mg protein) to tubulin extracted from *H.contortus* VRSG and McMaster strains in Assay 5.



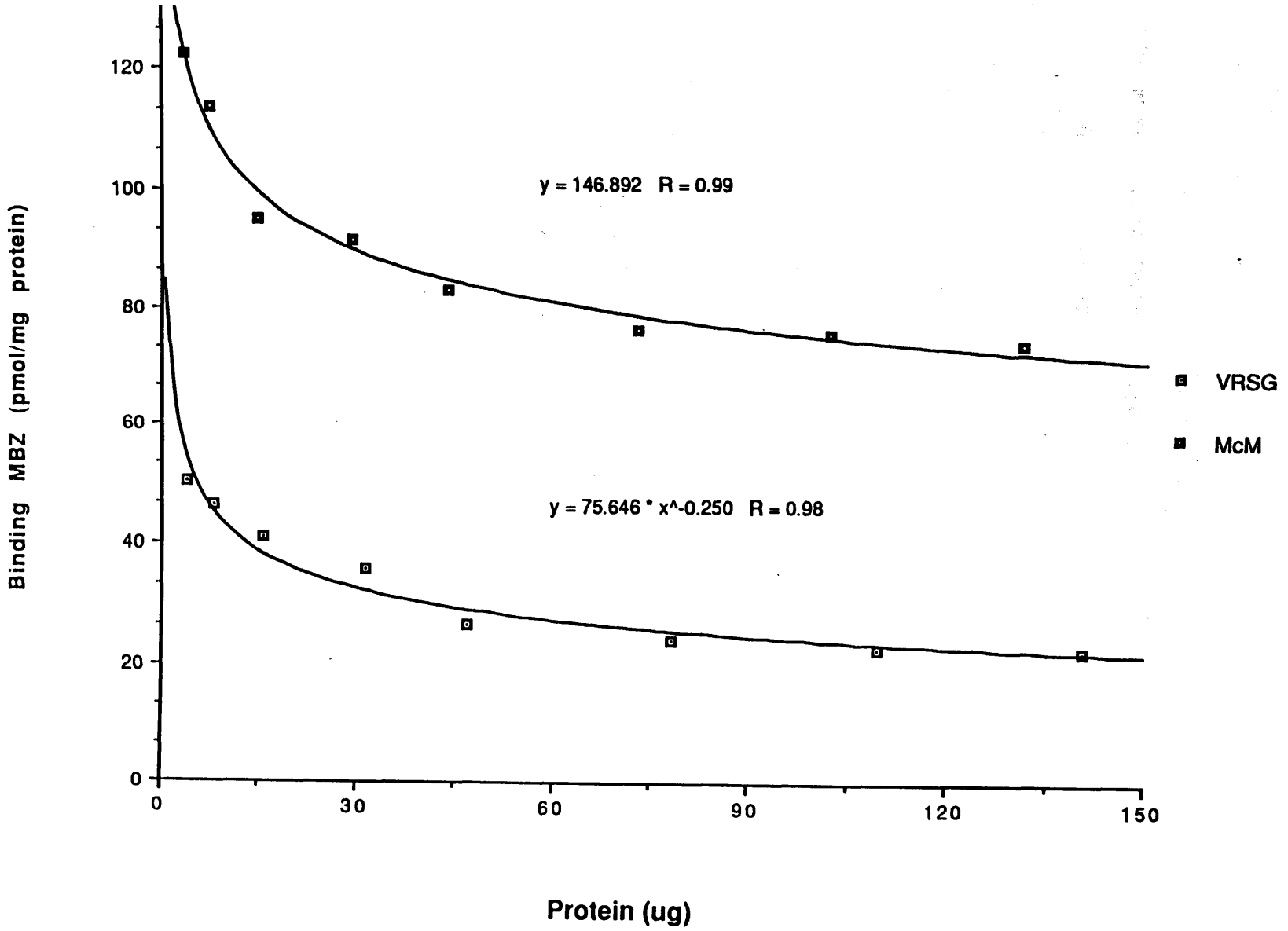
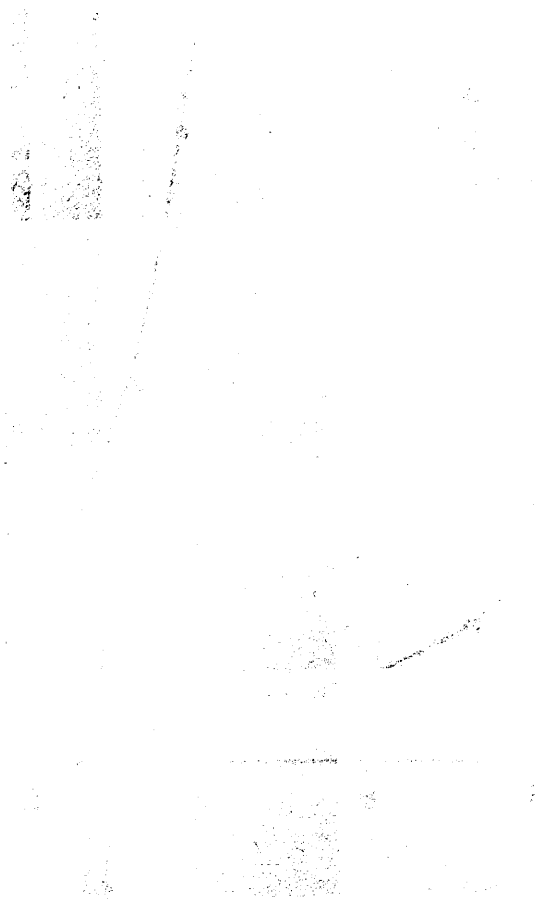

Binding MBZ (pmol/mg protein)
120
100
80
60
40
20
0
0
30
60
90
120
150
Protein (ug)
y = 146.892 R = 0.99
y = 75.646 * x^-0.250 R = 0.98
VRSG
McM

Figure 3.10

Binding of tritiated albendazole (pmoles/mg protein) to tubulin extracted from *H.contortus* VRSG and McMaster strains in Assay 3.



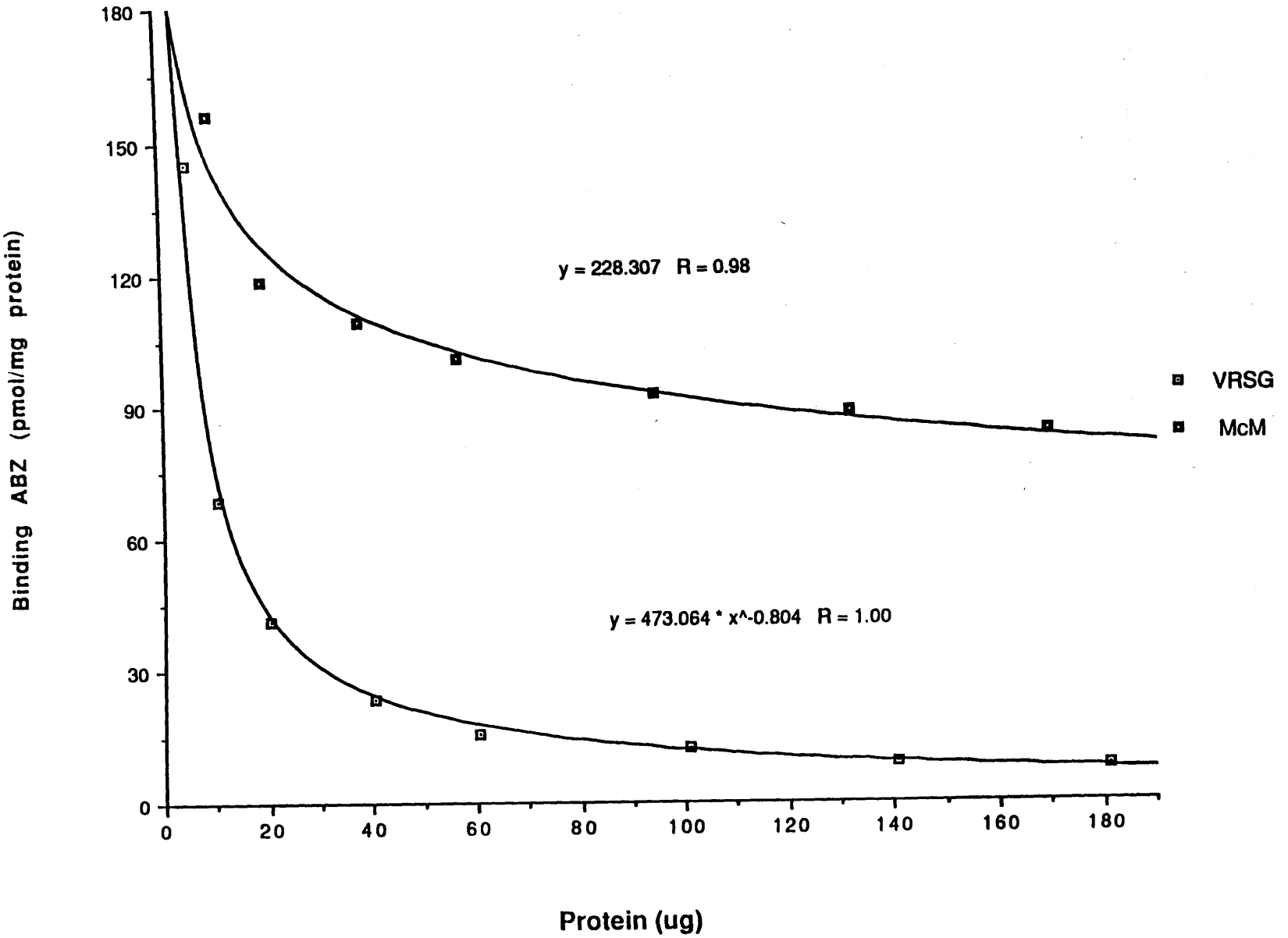
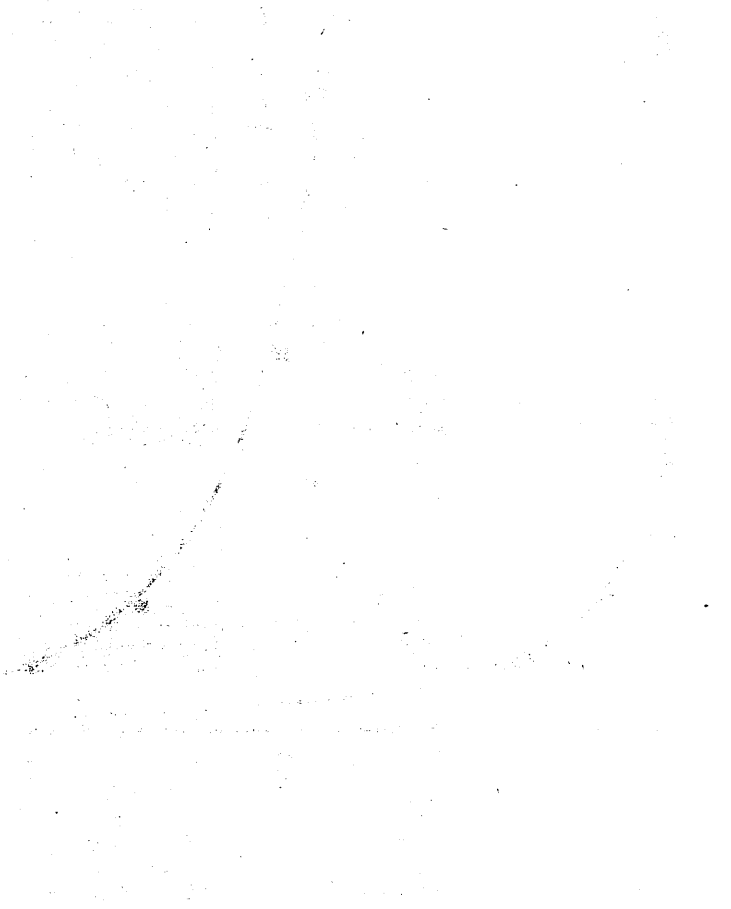

Binding ABZ (pmol/mg protein)
180
150
120
90
60
30
0
0
20
40
60
80
100
120
140
160
180
Protein (ug)
y = 228.307 R = 0.98
y = 473.064 * x^-0.804 R = 1.00
VRSG
McM

Figure 3.11

Binding of tritiated albendazole (pmoles/mg protein) to tubulin extracted from *H.contortus* VRSG and McMaster strains in Assay 4.



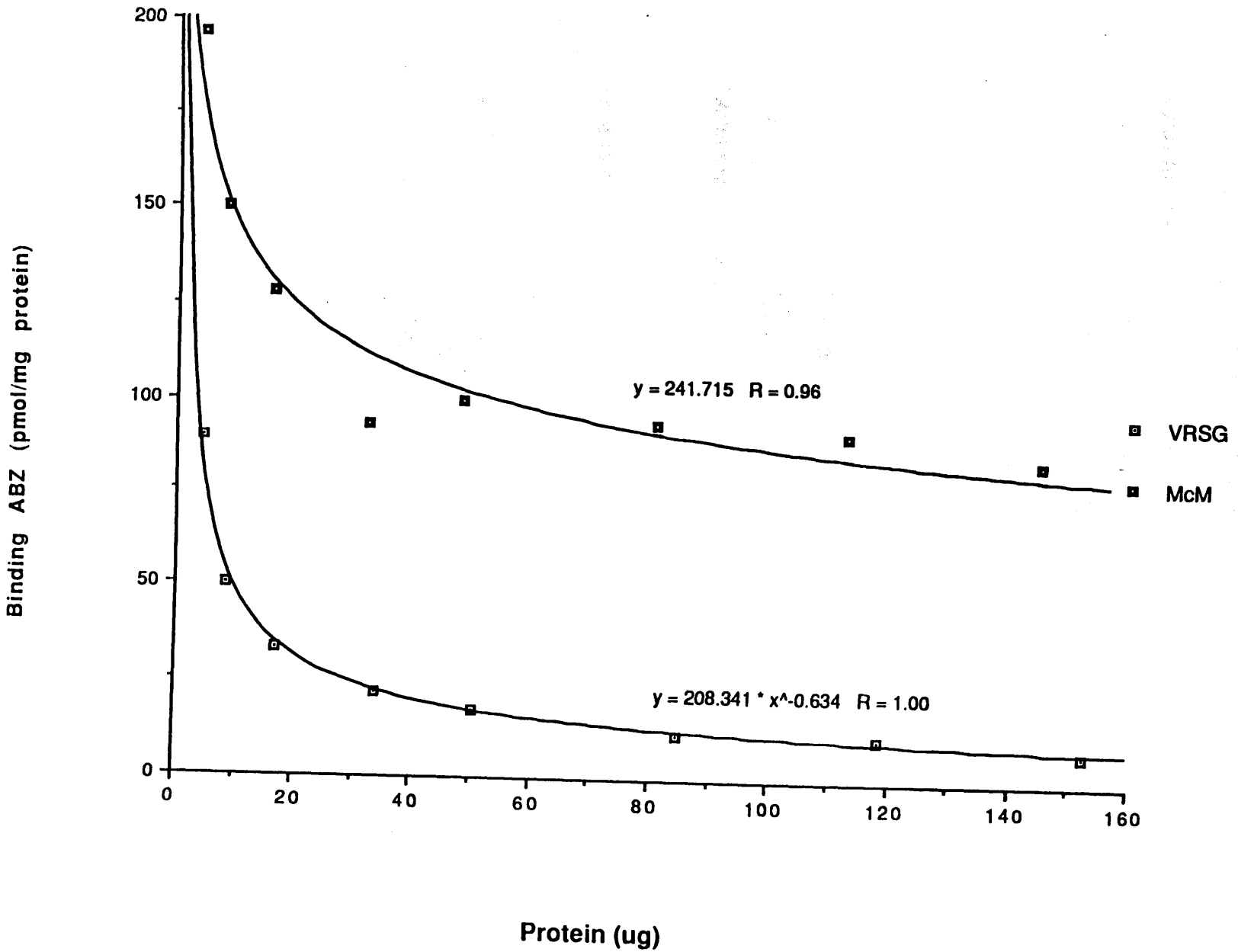

Binding ABZ (pmol/mg protein)
200
150
100
50
0
0
20
40
60
80
100
120
140
160
Protein (ug)
y = 241.715 R = 0.96
y = 208.341 * x^-0.634 R = 1.00
VRSG
McM

Table 3.17

Results of tubulin binding assays using tubulin extracts from *H.contortus* McMaster and VRSG strains with tritiated mebendazole

Assay Number	Binding of ^{3}HMBZ (pmoles/mg protein)		Resistance Factor	Susceptibility Factor
	McMaster	VRSG		
1	83.90±3.65	22.28±2.21	3.77	0.27
2	96.10±5.32	24.05±1.97	4.00	0.25
4	84.33±5.79	26.00±3.51	3.24	0.31
5	77.34±4.15	24.06±1.91	3.21	0.31
6	94.35±4.58	-	-	-
6	97.13±6.84	-	-	-

(Results are mean values ± standard deviation of the mean)

Table 3.18

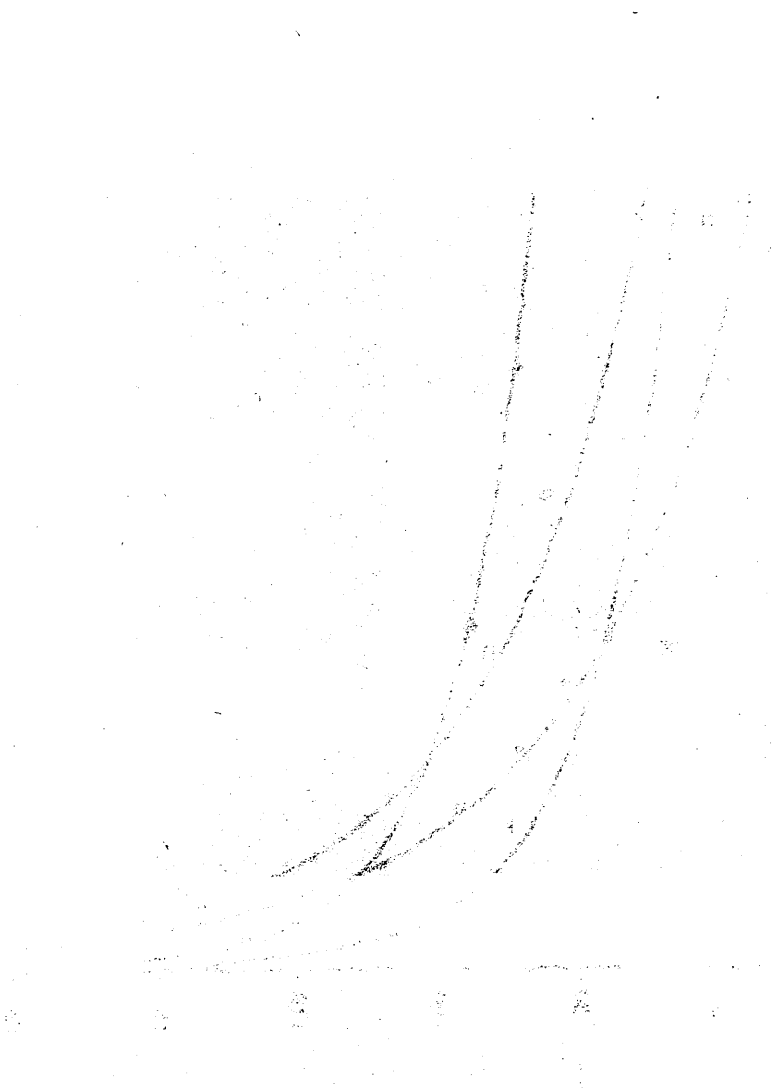
Results of tubulin binding assays using tubulin extracts from *H.contortus* McMaster and VRSG strains with tritiated albendazole

Assay Number	Binding of ^{3}HABZ (pmoles/mg protein)		Resistance Factor	Susceptibility Factor
	McMaster	VRSG		
2	108.73±9.78	16.28±6.00	6.68	0.15
3	94.73±9.97	13.49±5.88	7.02	0.14
4	92.34±5.90	14.16±5.44	6.52	0.15
5	88.40±7.0	-	-	-

(Results are mean values ± standard deviation of the mean)

Figure 3.12

Binding of tritiated mebendazole (pmoles/mg protein) to tubulin extracted from *H.contortus* McMaster strain in Assays 1, 2, 4 and 5.



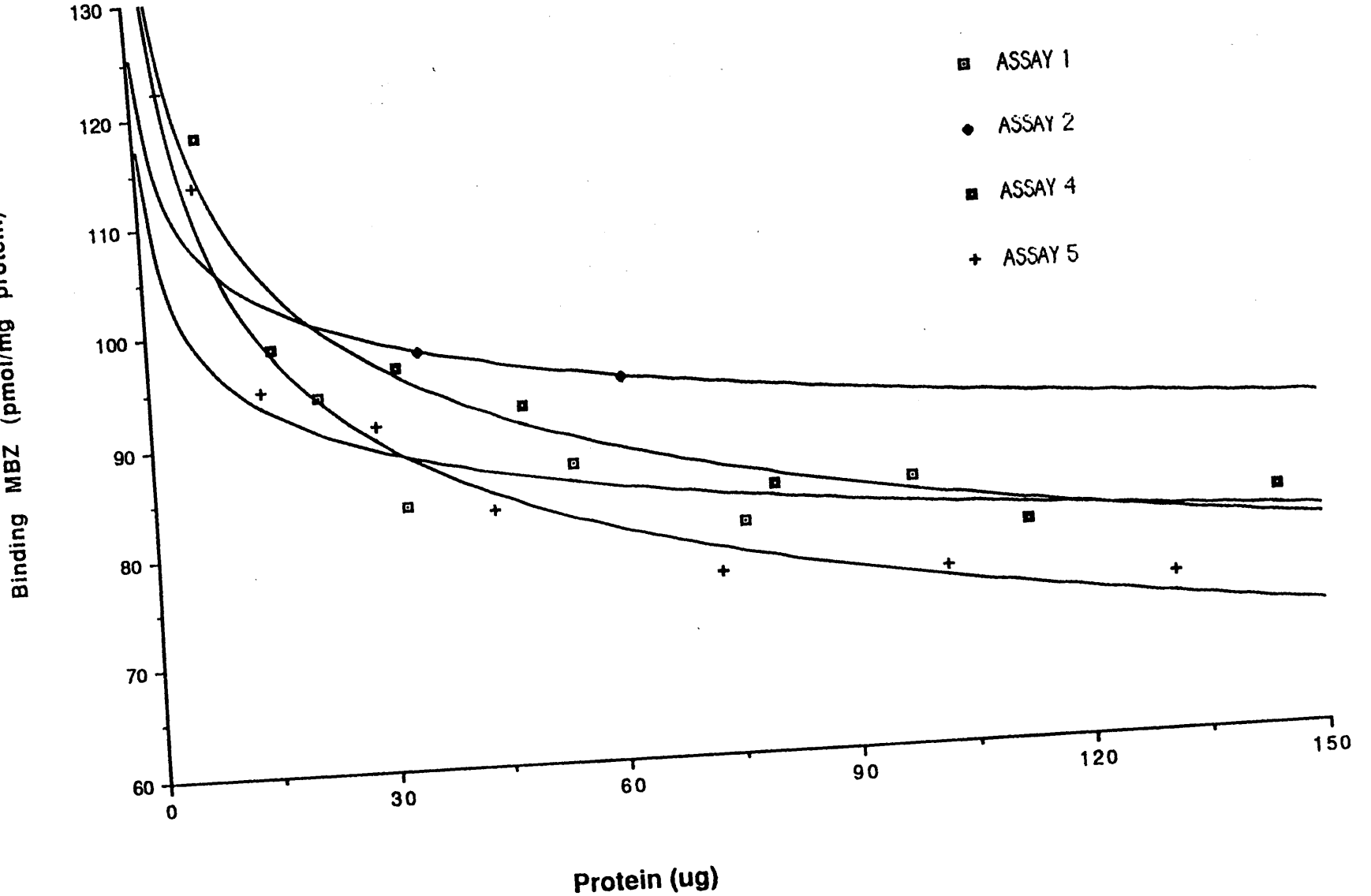

130
120
110
100
90
80
70
60
0
30
60
90
120
150
Binding MBZ (pmol/mg protein)
Protein (ug)
ASSAY 1
ASSAY 2
ASSAY 4
ASSAY 5

Figure 3.13

Binding of tritiated mebendazole (pmoles/mg protein) to tubulin extracted from *H.contortus* VRSG strain in Assays 1, 2, 4 and 5.

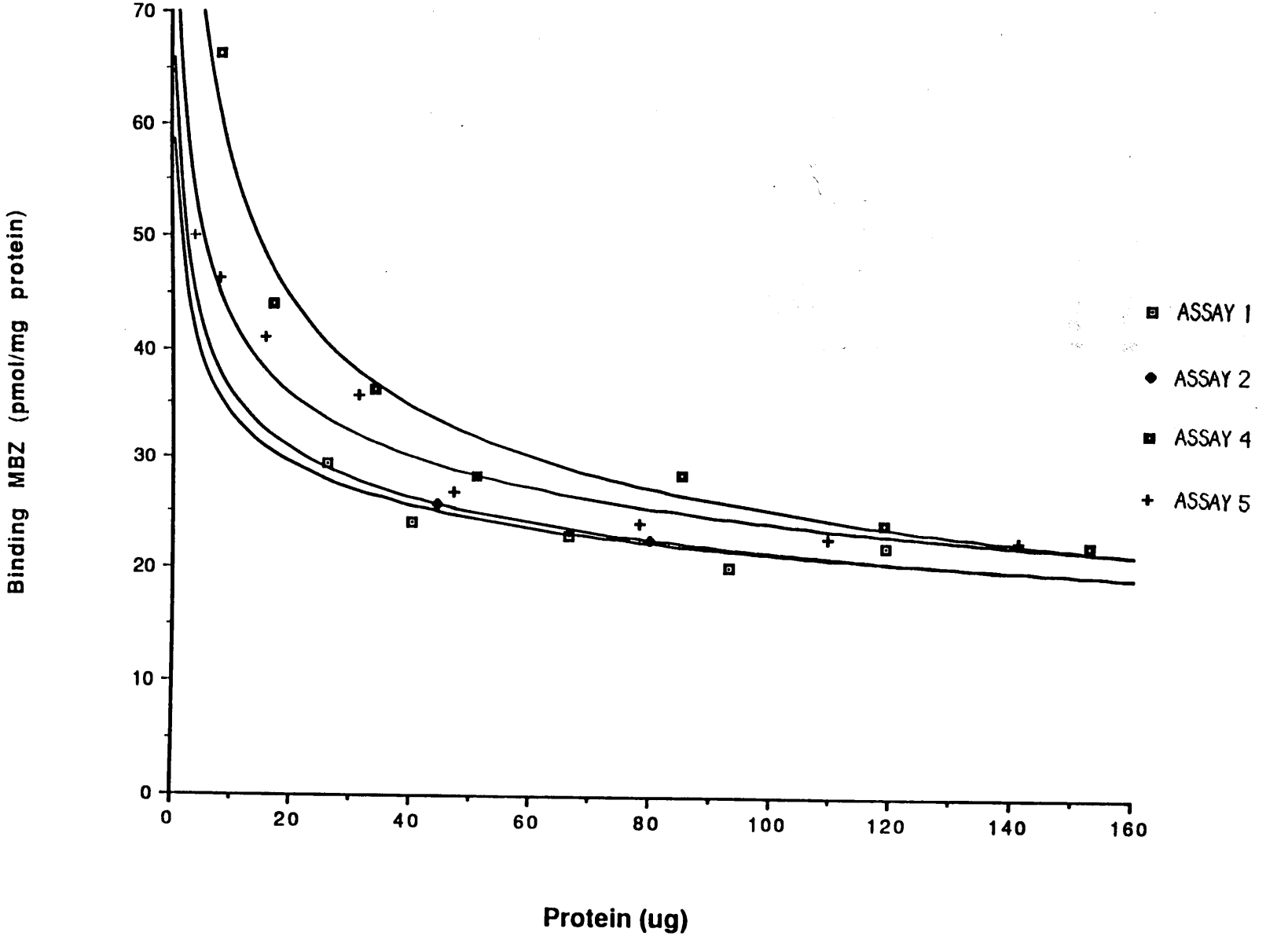

Binding MBZ (pmol/mg protein)
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
160
Protein (ug)
ASSAY 1
ASSAY 2
ASSAY 4
ASSAY 5

Figure 3.14

Binding of tritiated albendazole (pmoles/mg protein) to tubulin extracted from *H.contortus* McMaster strain in Assays 2, 3, 4 and 5.

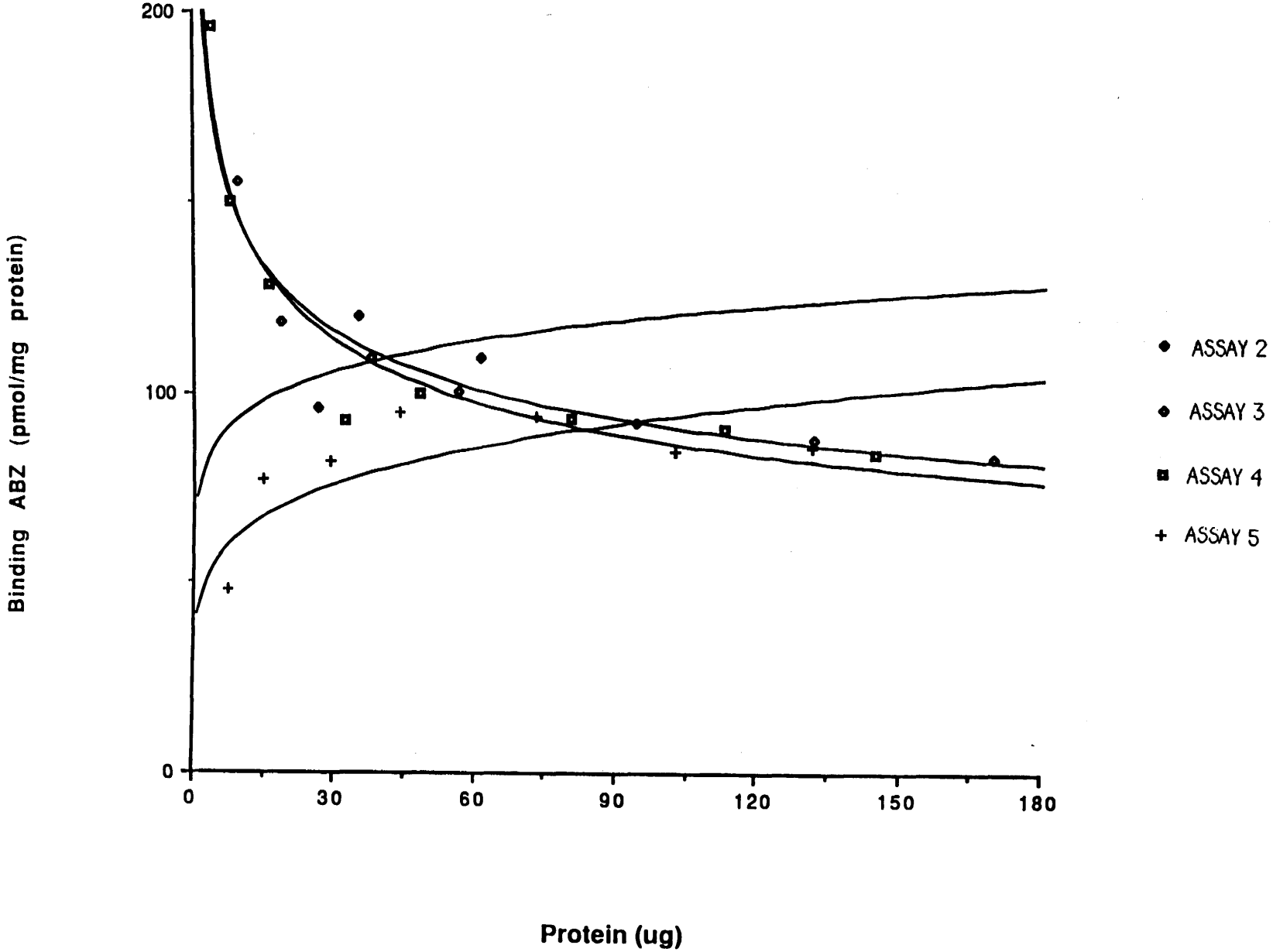
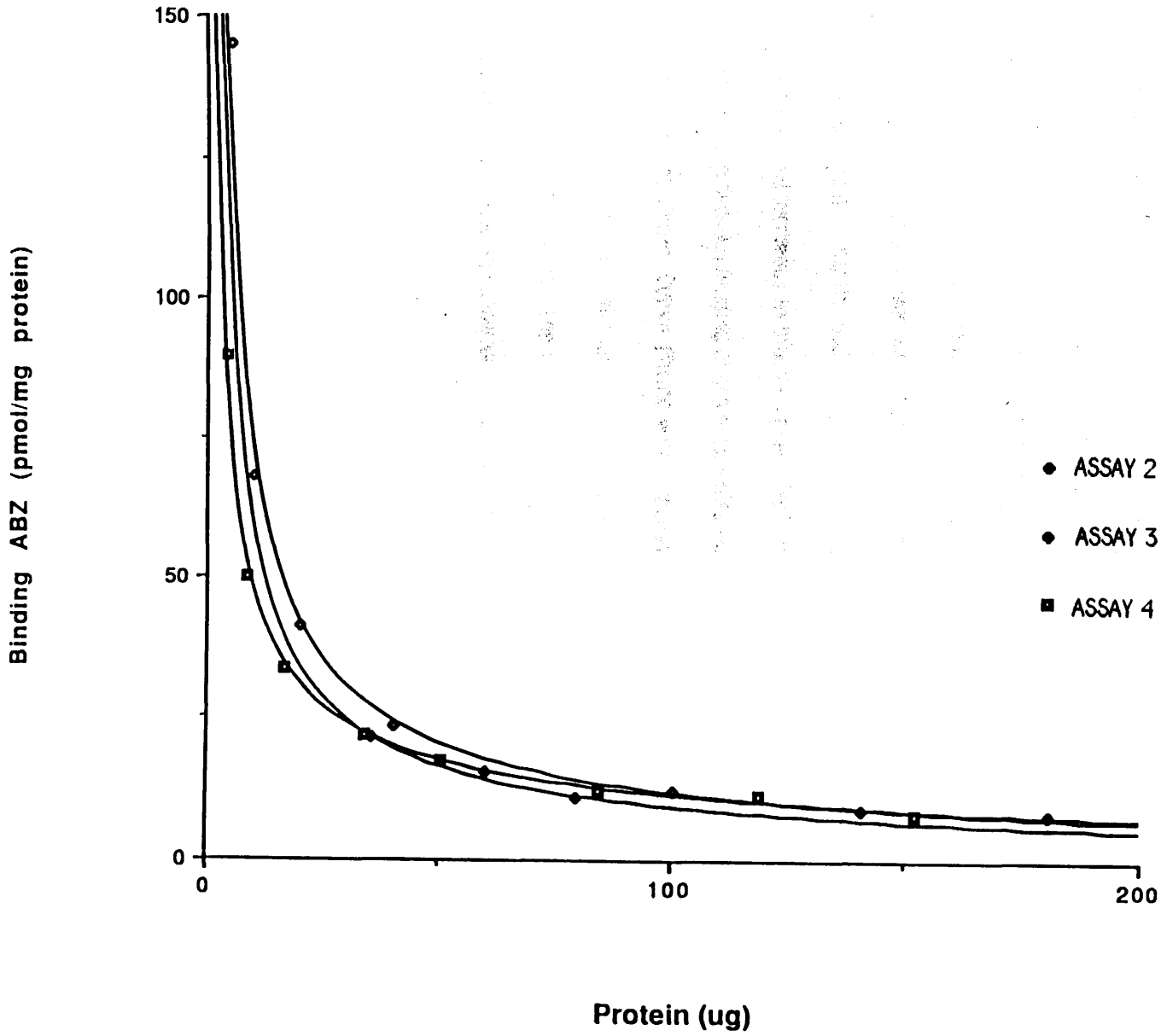

200
100
0
0
30
60
90
120
150
180
Binding ABZ (pmol/mg protein)
Protein (ug)
ASSAY 2
ASSAY 3
ASSAY 4
ASSAY 5

Figure 3.15

Binding of tritiated albendazole (pmoles/mg protein) to tubulin extracted from *H.contortus* VRSG strain in Assays 2, 3 and 4 .

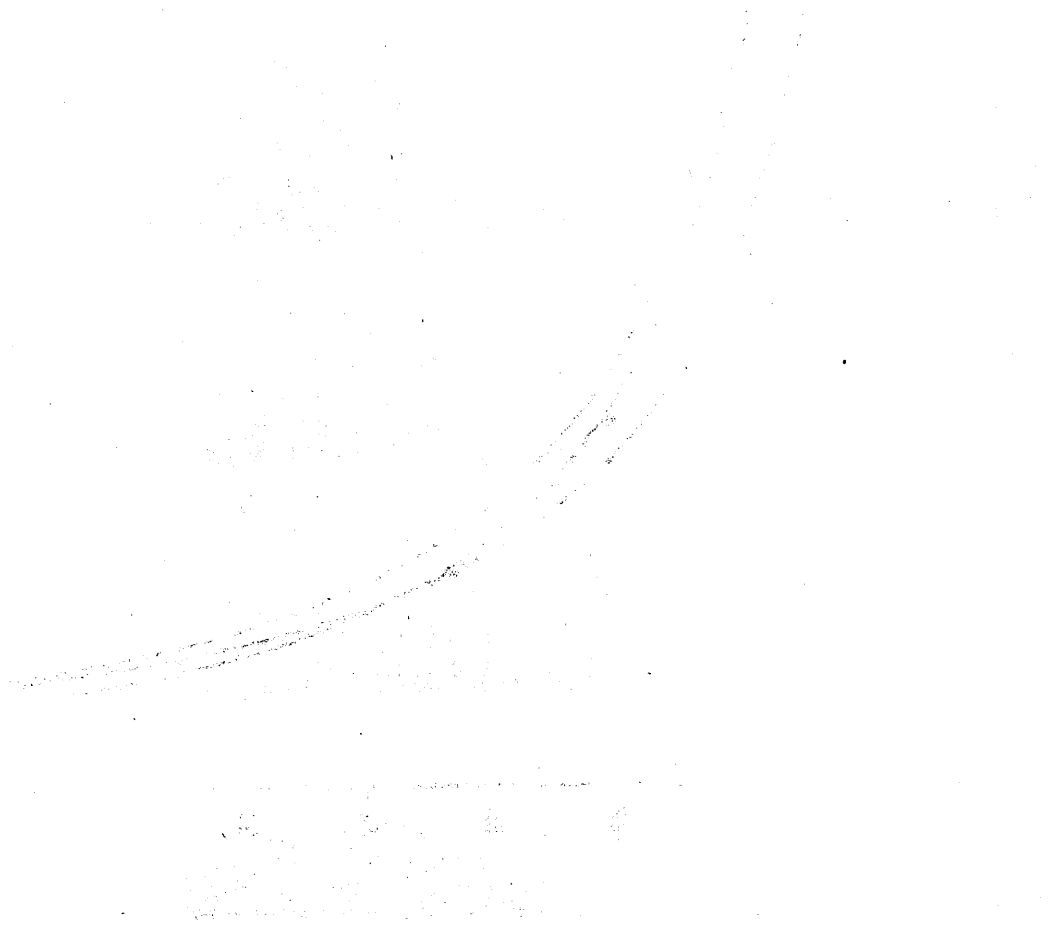

Binding ABZ (pmol/mg protein)
150
100
50
0
0
100
200
Protein (ug)
ASSAY 2
ASSAY 3
ASSAY 4

for protein extracts in the current experiments was approximately 7-9ug per 1,000 larvae.

The effect of protein concentration in the extracts on the binding of benzimidazoles was studied by using a wide range of protein concentrations for each tubulin extract in the binding assays. The binding of benzimidazoles to tubulin extracts is expressed in pmoles/mg protein in the extract to take account of the variation in binding with increasing protein concentrations. However from Figs. 3.12-3.15 where binding is plotted against protein concentration there is increasing binding of benzimidazoles with increasing protein concentration. Lacey and Snowdon (1988) found binding of benzimidazoles to be linear with respect to protein concentration down to 5ug/assay with deviation from linearity occurring below 10ug/assay. These workers observed both increases and decreases in binding below this level of protein. From Figs. 3.12-3.15 the relationship between binding of benzimidazoles and protein concentration for both ABZ and MBZ was not linear. Curve fitting with exponential curves produced good correlations R>0.97. When linear calculations were calculated they were poorer with a range of 0.62-0.96 for R values. The limit of detection of the assay, i.e. the protein concentration below which Lacey and Snowdon (1988) expected deviation from linearity was defined as 20ug protein per assay. From Figs. 3.12-3.15 there was a sharp rise or fall (Fig. 3.14) in the level of benzimidazoles around a protein concentration of 30ug with deviation at higher protein concentration in assays using the resistant strain of *H.contortus*. Taking 35ug of protein per assay as the limit of detection and discarding figures for binding using protein values below this level curves of binding against protein concentration were redrawn for binding of ABZ to extracts from the VRSG strain (Fig. 3.16) and MBZ to extracts from the VRSG strain (Fig. 3.17). Again the best fit curves were drawn and were shown to be exponential curves rather than the linear relationship shown by Lacey and Snowdon (1988).

Figure 3.16

Binding of tritiated albendazole (pmoles/mg protein) to tubulin extracted from *H.contortus* VRSG strain in Assays 2, 3 and 4 using data for samples containing >35 ug protein.



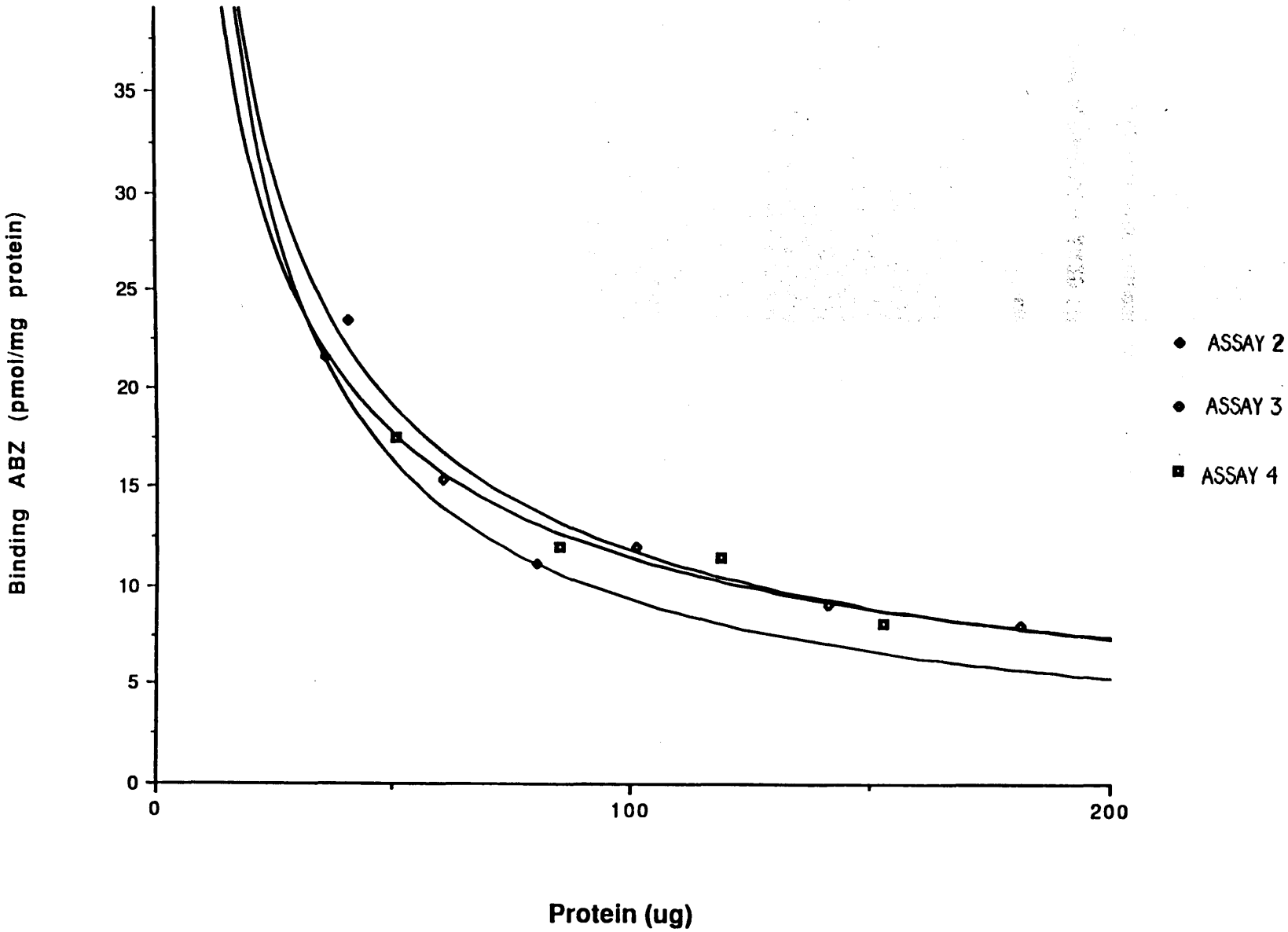
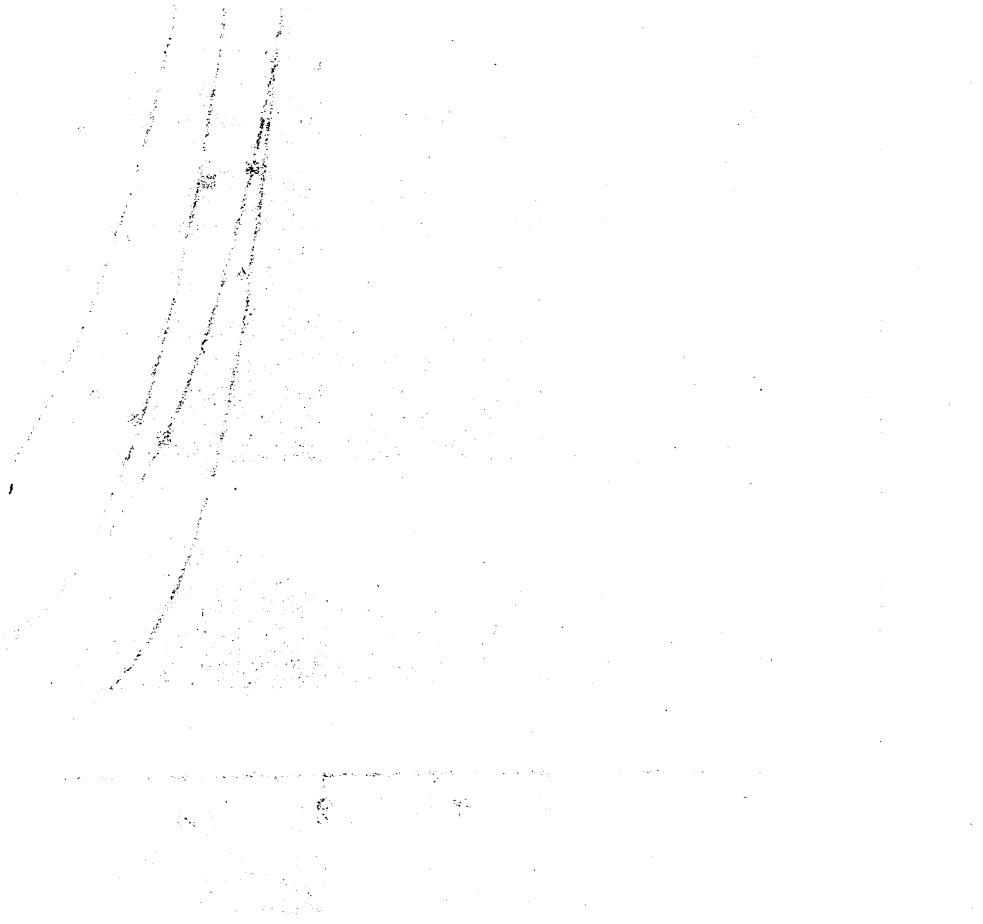
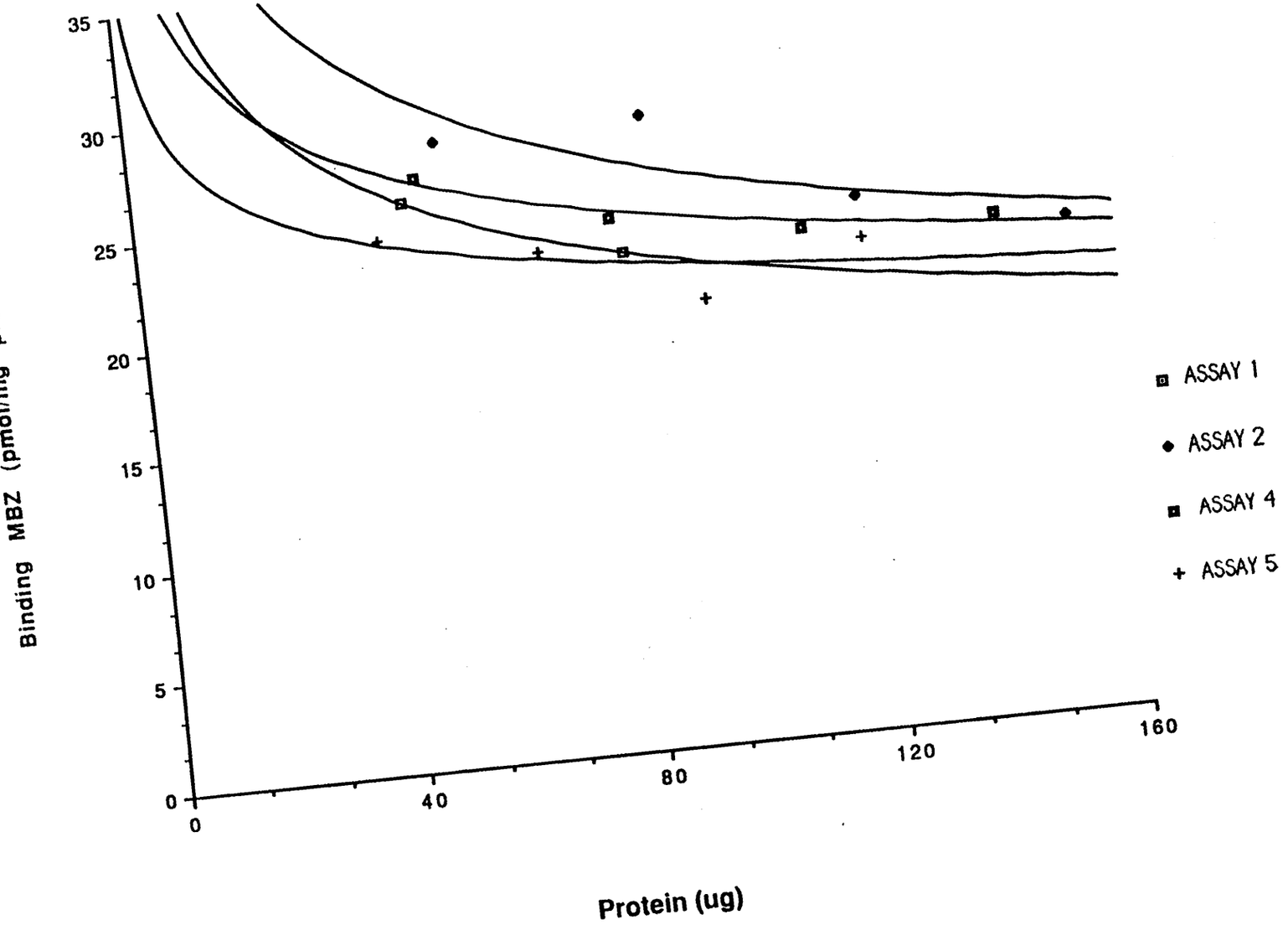

Binding ABZ (pmol/mg protein)
35
30
25
20
15
10
5
0
0
100
200
Protein (ug)
ASSAY 2
ASSAY 3
ASSAY 4

Figure 3.17

Binding of tritiated mebendazole (pmoles/mg protein) to tubulin extracted from *H.contortus* VRSG strain in Assays 2, 3 and 4 using data for samples containing >35 ug protein.




Binding MBZ (pmol/mg protein)
35
30
25
20
15
10
5
0
0
40
80
120
160
Protein (ug)
ASSAY 1
ASSAY 2
ASSAY 4
ASSAY 5

The curves for individual assays were similar showing consistent results among the assays. Using values for binding to tubulin extracts containing >35ug protein per assay a mean value and standard deviation for the binding of MBZ or ABZ for each assay was calculated (Tables 3.17 and 3.18). In the binding assays using ABZ the standard deviation of the mean was greater than when MBZ was used in the assays. Between assay variation was less in assays using MBZ compared with assays using ABZ. However, with both ABZ and MBZ results of binding assays showed reduced binding to benzimidazole resistant strains of *H.contortus* However, the difference in binding of benzimidazole to tubulin extracts from benzimidazole resistant and susceptible strains is greater when ABZ was used as the ligand rather than MBZ. Lacey and Prichard (1986) noted that resistance factors were higher when benzimidazoles which are alkyl derivatives e.g. ABZ were used in the assays compared with phenyl derivatives e.g. MBZ. The differences in the resistance factors were less marked in the current study than results shown by Lacey and Prichard (1986). In order to define the level of resistance of a particular stain of *H.contortus* a range of different strains would need to be used and their binding compared with a single susceptible strain using one of the benzimidazoles. Lacey and Prichard (1986) recommended MBZ as the ligand of first choice because of the increased consistency of results with this benzimidazole compared with the others.

Binding of Tritiated Mebendazole or Albendazole to Tubulin Extracts from Various Strains of *O.circumcincta*

Results

Tubulin extracts were prepared by Method A using >100,000 infective larvae of each of the strains of *O.circumcincta* shown in Table 3.19. Binding assays were carried out using tritiated ABZ and MBZ on a range of protein concentrations of each tubulin extract. The mean binding for each of the benzimidazoles was calculated for the individual assays using

Table 3.19

Strains of *Ostertagia circumcincta* used in tubulin binding assays

Source	Strain	Resistance Status
Lamb R94 (Glasgow)	Bearsden	Susceptible
Lamb DB34 (Glasgow)	HFRO	Resistant
Lamb FR61 (Glasgow)	HFRO transplant-F3 Generation	Resistant (partially)
C.S.I.R.O. (Sydney)	McMaster	Susceptible
C.S.I.R.O. (Melbourne)	KS	Susceptible
C.S.I.R.O. (Melbourne)	KR	Resistant
C.S.I.R.O. (Melbourne)	KS X KR (40:60)	Resistant (partially)

results for the binding to tubulin extracts with >35 ug protein in the assay. The mean binding of tritiated MBZ and tritiated ABZ to tubulin extracts are shown in Tables 3.20 and 3.21 respectively. For some of the strains 2 or 3 assays were performed using the same batch of larvae but different tubulin extracts were prepared for each individual assay.

Discussion

The sources of the strains of *O.circumcincta* used in the binding assays are shown in Table 3.19. The resistant strain obtained from C.S.I.R.O., Melbourne (KR strain) had been maintained in a laboratory situation with pressurisation using benzimidazoles to produce a highly resistant strain. The KS X KR strain was produced by combining KS and KR infective larvae in the ratio of 40:60 and using this mixture to infect lambs. The progeny of the cross were collected by culturing faeces of the infected lambs. The binding assays were performed on strains of *O.circumcincta* with varying benzimidazole resistance status in order to try to validate the use of the tubulin binding assay as a test for the detection of benzimidazole resistance and to draw comparisons of the resistance level among the different strains of *O.circumcincta* .

As in previous binding assays the protein concentration of the tubulin extract used in the assay had an effect on the binding of benzimidazoles. The results for binding (Tables 3.20 and 3.21) were calculated over a narrow range of protein concentrations, 35-100 ug protein per assay, in order to minimise any variation in binding due to changes in protein concentration.

For some of the strains binding assays were carried out on several occasions and results showed marked between assay variation e.g. for the KR strain binding varied from 46.23 pmoles MBZ/mg protein on occasion one to 18.57pmoles MBZ/mg protein on occasion 2 which is a 60% drop in binding. Similarly on occasion 3 the binding for each strain was significantly different from results on the previous 2 occasions both in assays using MBZ

Table 3.20

Binding of tritiated mebendazole to tubulin extracts from *O.circumcincta*

Strain	Binding of ^{3}HMBZ (pmoles/mg protein)		
	1	2	3
Bearsden	102.50±4.09	73.30±4.10	-
HFRO	34.13±4.10	25.40±1.37	-
HFRO transplant	82.14±2.35	-	-
McMaster	92.09±2.41	-	-
KS	77.83±3.58	-	-
KR	46.23±3.35	18.57±2.06	32.18±3.17
KS X KR	71.53±4.31	46.51±3.57	62.76±4.05

(Results are mean values ± standard deviation of the mean)

Table 3.21

Binding of tritiated albendazole to tubulin extracts from *O.circumcincta*

Strain	Binding ^{3}HABZ (pmoles/mg protein)		
	1	2	3
Bearsden	105.88±5.71	-	-
HFRO	18.56±3.06	-	-
HFRO transplant	74.79±4.52	-	-
McMaster	140.84±6.14	-	-
KS	112.50±4.38	-	-
KR	39.38±4.25	18.23±2.20	31.46±4.77
KS X KR	101.17±6.11	57.43±4.20	84.99±5.90

(Results are mean values ± standard deviation of the mean)

and in assays with ABZ. Lacey and Snowdon (1988) found between experiment variation to be from 5-25% over a 12 month period using strains of *T.colubriformis*, *H.contortus* and *O.circumcincta* In previous assays (Tables 3.17 and 3.18) with *H.contortus* day to day variation in binding of benzimidazoles to tubulin extracts was of this order. However variation in binding with strains of *O.circumcincta* in the present experiments was much greater.

A possible reason for variability in the results of binding assays was changes in the protein content of the tubulin extracts or changes in the percentage or availability of tubulin in the extracts. Using the same number of larvae of the same strain of *O.circumcincta* homogenisation was carried out using separate homogenisers because this step is the most likely to produce variability in the tubulin extraction (Lacey, personal communication, 1988). However, there was no significant difference in binding to tubulin extracts prepared using different homogenisers. Another problem with the assay is that total protein is measured in the extracts rather than the tubulin content. Tubulin constitutes <3% of the total protein in the tubulin extract and therefore it is unlikely that any change in the tubulin content in the extract would be reflected by a change in the overall protein concentration. Assay of the tubulin content would be more satisfactory using e.g. an ELISA for tubulin (see later). Standardisation of the ELISA assay is difficult because slight differences exist between the binding of anti-tubulin antibody to tubulin from resistant and susceptible strains. There could be day to day variation in the metabolism of infective larvae causing alteration in the amount of extractable protein and tubulin. However it would be surprising if these changes could account for differences in binding to *O.circumcincta* when they do not occur to such an extent in *H.contortus* where binding is less variable.

These changes in binding observed for the Bearsden, HFRO, KR and KS X KR strains assayed on different occasions confirm that in order to make any comparisons among

different strains of *O.circumcincta* the assays need to be carried out on the same day using exactly the same method and equipment. Comparison of the ratio of binding of MBZ to KR with KS X KR gave values of 0.64, 0.40 and 0.51 on the 3 occasions and therefore the validity of the assay as a method of quantitating benzimidazole resistance accurately in strains of *O.circumcincta* is questionable.

There were also differences in the level of resistance measured in the strains when ABZ was used as the ligand in the assays rather than MBZ. The strains were ranked in order of susceptibility (most susceptible=1) according to results of binding assays using MBZ and ABZ (Table 3.22). The only strains which retain the same order with both benzimidazoles are the highly resistant strains KR and HFRO. The HFRO transplant strain which has been shown to be partially resistant was ranked above the susceptible strain KS using results for binding assays with MBZ. The HFRO transplant strain from lamb FR61 was tested for resistance using egg hatch assays and results were compared with those from egg hatch assays on the HFRO and Bearsden strains (see previously). The HFRO transplant strain was resistant with results from egg hatch assays showing resistance slighltly less than the HFRO strain. However, results from binding assays (Tables 3.20 and 3.21) suggest that the HFRO transplant strain was fairly susceptible. Similarly the resistance of the KS X KR strain should lie about midway between that of the KS and KR strains according to results of egg hatch assays (Martin, personal communication,1988) and again results of binding assays show the KS X KR strain to be fairly susceptible. Differences between results of egg hatch assays and binding assays could be due to the influence of the stage of development of the parasite being tested. For tubulin binding assays tubulin is extracted from infective larvae whereas egg hatch assays are carried out using undeveloped parasite eggs. There could be differences in the level of resistance which can be measured at different stages of the life cycle of the parasites, which could in turn be linked to dependence on tubulin during that particular phase of the life cycle.

Table 3.22

Susceptibility to benzimidazole anthelmintics of strains of *O.circumcincta* ranked using result from tubulin binding assays with mebendazole or albendazole

Strain	Order of Susceptibility	
	Assays with MBZ	Assays with ABZ
Bearsden	1	3
McMaster	2	1
HFRO transplant	3	5
KS	4	2
KS X KR	5	4
KR	6	6
HFRO	7	7

In order to detect resistance using egg hatch assays the population of parasites has to have a high level of resistance (Martin, personal communication,1988). The tubulin binding assay appears to have similar drawbacks with highly resistant strains detectable but identification and quantitation of benzimidazole resistance in less resistant strains appears to give much less reliable results.

Competitive Binding Assay with Tritiated Albendazole using Tubulin Extracted from *O.circumcincta* with Albendazole, Albendazole sulphoxide, Albendazole sulphone and Colchicine

Materials and Method

Reagents.

Tritiated ABZ - prepared by method I (see previously) and diluted to 10.8uM in methanol. Specific activity 360dpm/pmole

Buffer - 0.025M MES buffer containing 0.1mM EGTA, 0.5mM $MgSO_4$ and 0.1M GTP, pH 6.5.

Charcoal suspension - 2mg/ml activated charcoal (Norit-A activated charcoal - Sigma Chemical Co. Ltd.) in 1%(w/v) BSA.

Stock solutions of inhibitors - solutions of ABSX and ABSO and colchicine were prepared (10.8uM) in methanol

Method.

1. Tubulin extracts prepared by method A (see previously) from *O.circumcincta* infective larvae of Bearsden strain, benzimidazole susceptible, adjusted to a protein concentration of 2-3mg/ml.

2. 90ul tubulin extract (or 90ul buffer-blank) was placed in 5ml plastic tubes. 10ul of one of the inhibitors (or 10ul methanol in the blank and standard) was added. The tubes were vortexed and incubated at 37°C for 20min in a waterbath. The samples were carried out in triplicate.

3. 10ul of ^{3}HABZ was added to each tube (10ul methanol to blank), vortexed and incubated for 15min at 37°C in a waterbath.

4. 0.5ml charcoal suspension was added to all the tubes, mixed and incubated for 10min at 37°C in a waterbath.

5. All tubes were centrifuged at 3,500g for 10min.

6. A 0.4ml aliquot of the supernatant in each tube was added to 10ml Ecoscint in a scintillation vial and counted for 1min in a Packard counter.

7. The counts for the standards and samples were corrected by subtracting the average counts obtained for the blanks. These results were divided by the specific activity of ^{3}HABZ and the protein concentration of the tubulin extract to give the binding of ^{3}HABZ in pmoles/mgprotein. The effect of the inhibitor was measured by comparing the binding of ^{3}HABZ in the standard with binding of ^{3}HABZ in the presence of an inhibitor. The results were expressed as a percentage inhibition of binding.

Results

The amount of tritiated ABZ bound to tubulin extracts after pre-incubation with the inhibitors is shown in Table 3.23.

Discussion

Colchicine and the benzimidazoles are thought to bind to the tubulin molecule at the same binding site (Friedman and Platzer, 1980b). The benzimidazoles and colchicine therefore compete for the binding site. Prior incubation with one of these molecules should reduce subsequent binding of tritiated ABZ to the tubulin molecule. This competition for binding sites was exploited in the original binding assays (see previously) when binding of benzimidazoles was measured indirectly by displacement of colchicine (Sherline *et al*, 1974 ; Barrowman *et al*, 1984). The low counts for radioactivity of the tritiated colchicine were a limiting factor in the use of this assay. With tritiated benzimidazoles inhibition of binding of the drug can be measured using a modification of

Table 3.23

Binding of tritiated ABZ (pmoles/mg protein) to tubulin extracted from *O.circumcincta* after incubation with a range of inhibitors (ABZ, ABSX, ABSO and Colchicine)

Assay Number	Inhibitor				
	Blank	ABZ	ABSX	ABSO	Colchicine
1	49.77	-	50.20	58.30	-
2	22.87	19.96	63.17	36.49	-
3	27.29	22.52	24.67	20.43	42.19

the tubulin binding assay by adding a step, pre-incubation of the tubulin extract with an inhibitor, before addition of the tritiated ABZ.

Barrowman *et al.*,(1984) found that ABSX and ABSO acted as inhibitors of colchicine binding. From the results given in Table 3.23 they did not inhibit binding of tritiated ABZ in Assays 1 and 2 and inhibited binding to a slight extent in Assay 3. Pre-incubation with ABZ caused a low level of inhibition in Assays 2 and 3 but the values for binding of tritiated ABZ were not significantly different from the blanks. In Assay 3 pre-incubation with colchicine caused an increase in binding of tritiated ABZ to the extract which is contrary to the expected result.

The reason for the increase in binding of tritiated ABZ in Assay 3 after pre-incubation with ABSX or ABSO and in Assay 3 after pre-incubation with colchicine was unknown. There may be a high level of non-specific binding which masks the inhibitory effect of the pre-incubated substances but this would not explain the enhancement of binding of tritiated ABZ which was observed in some cases. From previous tubulin binding assays using a Sephadex column to separate the bound and unbound tritiated drug the high counts corresponded to drug bound to a high molecular weight component which was assumed to be tubulin. However, this may not be the case and binding to some other component of the crude extract could be influencing the results of the assays.

Previous tubulin binding assays using tubulin extracted from *O.circumcincta* showed variability in results. This may account for some of the discrepancies in results between assays in the present competitive binding assay. A similar type of assay using *H. contortus* tubulin was investigated using a range of concentrations of the inhibitor substances ABZ, ABSX and ABSO.

Displacement Assay using Tubulin Extracts from *H.contortus* with Tritiated Albendazole and the Inhibitors Albendazole, Albendazole Sulphoxide and Albendazole Sulphone.

Materials and Method

Reagents.

Stock solutions of inhibitors - Stock solutions of ABZ, ABSX and ABSO at a concentration of 5,000uM were made up in DMSO. Serial dilutions 1:2 in DMSO were made and a range of solutions was used in the assay to give final concentrations of 0.025-3.125uM for ABZ and 0.39-50uM for ABSX and ABSO.

Buffer - 0.025 MES buffer, pH 6.5 containing 0.1mM EGTA, 0.5mM $MgSO_4$ and 0.1M GTP

Tritiated ABZ prepared by method II with specific activity of 9754 dpm/pmole.

Charcoal suspension - 2mg/ml activated charcoal (Norit-A activated charcoal- Sigma Chemical Co. Ltd) in 1%(w/v) BSA.

Method.

1. Crude tubulin extract prepared by method A (see previously) from *H.contortus* McMaster strain (Benzimidazole susceptible) was adjusted to a protein concentration of 80-100ug/100ul using buffer. 90ul of tubulin extract was placed in each 1.5ml Eppendorf tube (Blank 90ul buffer).

2. To each tube 2ul of inhibitor was added - duplicates of each concentration of the inhibitor solutions were used (blank and standard 2ul DMSO added) vortexed and incubated for 30 min at 37°C in a shaking water bath.

3. 10ul of ^{3}HABZ was added to each tube, vortexed and incubated for 30min at 37°C in a shaking water bath.

4 Added 0.5ml charcoal suspension to each tube, vortexed and incubated for 5min at 37°C in a shaking water bath then the tubes were centrifuged at 10,000 r.p.m. in an Eppendorf bench-top centrifuge for 10min.

5. A 0.4ml aliquot of the supernatant from each tube was placed in 5ml Biofluor in a glass scintillation vial and counted in a β counter (Packard Model 2650 Tricarb).

6. The blank was counted for 10 min and the average dpm subtracted from the counts of all the other samples and standards.

7. The standards and samples with inhibitor substances were counted for 1min. After correction for quenching, the final concentration of ^{3}HABZ binding in each sample and standard was calculated in pmoles/mg protein by dividing the counts in each by the specific activity of the ^{3}HABZ and the protein concentration of the tubulin extracts.

8. The reduction in binding caused by each of the inhibitors was calculated by comparing the H^{3}ABZ binding in the standards with that found in the samples. This reduction was expressed as percentage inhibition.

Results

Binding of tritiated ABZ to the tubulin extracts after prior incubation with buffer or increasing concentrations of ABZ, ABSX or ABSO was calculated. The percentage inhibition of binding of tritiated ABZ was calculated for each concentration of the inhibitors and a graph of percentage inhibition against log concentration of inhibitor was drawn (Fig. 3.18). The best-fit straight line plot was drawn for each inhibitor and from this the LD50, the concentration of inhibitor at which binding of tritiated ABZ was reduced by 50% was calculated. The LD50 for each of the inhibitors is shown in Table 3.24.

Discussion

Pre-incubation of tubulin extracts with ABZ, ABSX or ABSO reduced the subsequent binding of tritiated ABZ. The potency of the compounds as inhibitors differed with ABZ being more potent than ABSX and ABSO. Barrowman *et al.*, (1984) using inhibition of colchicine binding assays found ABSX and ABSO bound almost as strongly as ABZ to tubulin extracted from *A.suum*. However, in the present study the inhibition of binding of tritiated ABZ is less with the metabolites ABSX and ABSO than with the parent compound

Table 3.24

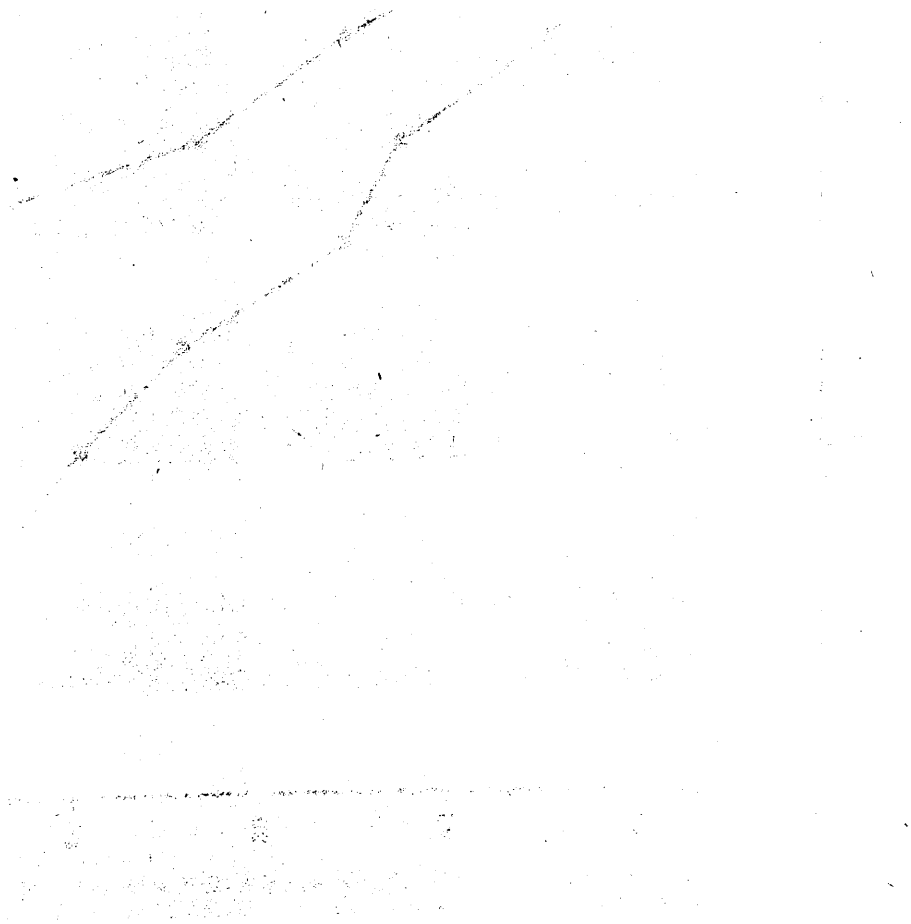
Inhibition of binding of tritiated ABZ to tubulin extracted from *H.contortus* in the presence of ABZ, ABSX and ABSO

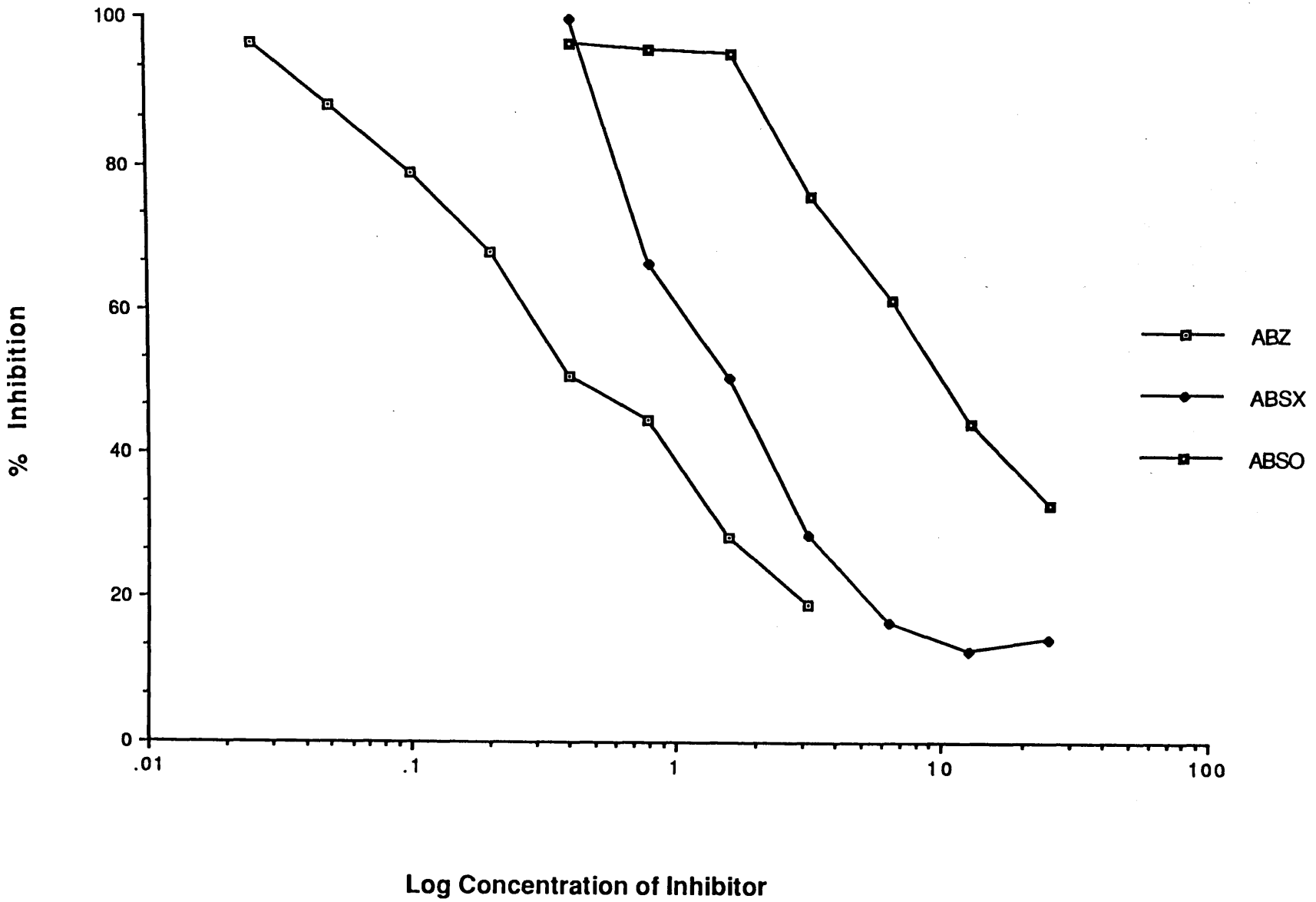
Inhibitor	* LD 50 (uM)
Albendazole	0.48
Albendazole sulphoxide	2.35
Albendazole sulphone	11.13

*Concentration of inhibitor which reduced binding of tritiated albendazole by 50%

Figure 3.18

Percentage inhibition of binding of tritiated albendazole to tubulin extracted from *H.contortus* VRSG strain in the presence of albendazole, albendazole sulphoxide and albendazole sulphone.




100
80
60
40
20
0
% Inhibition
.01
.1
1
10
100
Log Concentration of Inhibitor
ABZ
ABSX
ABSO

ABZ.

The relationship between strength of binding of a benzimidazole to tubulin and efficacy as an anthelmintic is probably not simple. The benzimidazoles have several possible modes of action (see introduction) and some of these may not be linked with tubulin binding. Therefore conclusions drawn from data on the ability to displace tritiated ABZ from the tubulin binding site as a measure of the strength of tubulin binding may be misleading. Lacey and Prichard (1986) found that binding to tubulin differed from one benzimidazole compound to another with greater binding found with MBZ and OFZ than with ABZ, FBZ, PBZ and OBZ. However, this does not reflect the known *in vivo* efficacy of these drugs. The efficacy of ABSO as an anthelmintic is negligible but from studies by Barrowman *et al.*, (1984) and the present studies ABSO binds to tubulin.

Another important factor is the concentration of drug available in the host animal. After oral administration of ABZ to ruminant animals high levels of ABSX are found and negligible levels of ABZ. Even if ABSX were poorer at binding to tubulin than ABZ and therefore had poorer activity than the parent compound the higher available concentrations of ABSX would more than compensate for the poorer binding of this metabolite to tubulin.

In the previous competitive binding assay using *O.circumcincta* tubulin ABZ at a concentration of 1.08uM inhibited binding of tritiated ABZ to a slight extent and ABSX and ABSO at the same concentration had a variable effect on binding. The use of *H.contortus* tubulin and tritiated ABZ with a higher specific activity in the present assay produced greater differences in binding in the presence of lower concentrations of the inhibitors. This may be due to the reduction in the importance of non-specific binding because of the higher counts associated with the tritiated ABZ or the possible influence of the parasite species used to prepare tubulin extracts for the binding assays (see previously).

General Discussion and Conclusions

The mechanism of action of the benzimidazole anthelmintics is thought to include inhibition of formation of microtubules in cells (Borgers and De Nollin, 1975). Benzimidazoles have been shown to bind to tubulin and inhibit the assembly of tubulin into microtubules *in vitro* (Friedman and Platzer, 1978; Ireland *et al*.,1979). The binding of benzimidazoles to tubulin is thought to occur at the same site as colchicine binds (Hoebeke *et al*, 1976). Studies with tubulin extracted from *A.suum* confirmed that benzimidazoles compete with colchicine for binding sites in tubulin extracts and this competitive binding could be used as a method to measure the binding of benzimidazoles to tubulin. Binding of benzimidazoles was also measured directly using tritiated benzimidazoles but the results from a range of assays using nematode tubulin were variable.

The protein content in tubulin extracts exerted a strong influence on the binding of benzimidazoles. The initial studies using the tubulin binding assay involved tubulin extracts with low protein concentrations and results were very variable. Latterly with higher protein concentrations in the tubulin extracts the assay appeared more robust but reproducibility of results especially with extracts from *O.circumcincta* was poor. Various factors could be implicated including tubulin content in the extract, specific activity of the tritiated benzimidazole used and method of separation of the tubulin bound and unbound drug.

The differences in binding of benzimidazoles to tubulin extracts from benzimidazole susceptible and resistant strains was inconsistent. Lacey and Snowdon (1988) have advocated tubulin binding of benzimidazoles as a routine diagnostic assay for detection of benzimidazole resistance in nematode parasites. From the results presented above it was obvious that the test has various disadvantages. Early work showed that the test was of no value to differentiate between strains of parasites with tubulin extracts using low

numbers of infective larvae. Results were of some value when extracts were prepared using >100,000 infective larvae but this places limitations on the test for diagnosis of resistance because availability of infective larvae may be a problem.

Alteration in the binding of benzimidazoles may not be the entire mechanism by which parasites have evolved resistance. In studies on the mechanism of action of benzimidazoles and their effect on microtubule assembly, (Gull *et al*, 1987) differences in the supramolecular structure of microtubules between mammals and helminth species were described. These authors found differences between groups of cells within nematode parasites. This is shown as differences in the arrangement and number of protofilaments in microtubules in cells. The selective action of benzimidazoles on helminth microtubule formation is likely to involve differences in tubulin, changes in the molecular structure of microtubules and possibly different mechanisms of microtubule assembly. Resistance to benzimidazoles may be acquired by an alteration in any one of these constituents.

Tubulin has been highly conserved throughout evolutionary development of species and there is little variation in the component α and β tubulin molecules. However, there is species variation in the molecular structure of helminth microtubules (Davis and Gull,1983; Gull *et al*, 1987). In the tubulin binding assays the binding of benzimidazoles to a crude extract of tubulin was measured. Differences in binding could reflect differences in the binding site for benzimidazoles. However, at low protein concentrations binding of benzimidazoles was extremely variable. This suggested that changes were occurring in the binding site when a low concentration of tubulin was present in the extract. At low levels of tubulin the assembly of microtubules or their component parts may be affected with changes in the tertiary structure of the tubulin molecules causing variation in benzimidazole binding. The differences in benzimidazole binding to benzimidazole susceptible and resistant strains may reflect differences in tertiary structure of tubulin or possibly other macromolecules in the extracts. Reliability of the

tubulin binding assay with tubulin extracts from *H.contortus* was greater than with extracts from *O.circumcincta* This may be due to species differences in the structure of microtubules and their method of assembly. A similar mechanism could account for differences in binding between extracts from infective larvae or eggs of the same species of nematode.

When parasites develop resistance to benzimidazoles there is side-resistance to other members of the group of anthelmintics. In the tubulin binding assays there is benzimidazole dependent variation in results for binding. Lacey and Prichard (1986) recommended MBZ as the benzimidazole of choice in tubulin binding assays because results using this compound are more consistent than with other benzimidazole compounds. From Table 3.22 the level of resistance measured in strains of *O.circumcincta* differed with the benzimidazole compound used in the assay. To use the tubulin binding assay as a method to diagnose resistance accurately, results require to be consistent and from the above trials this has not been shown.

Other factors may be involved in the development of resistance to anthelmintics including change in uptake of the drug, changes in drug metabolism (Sutherland *et al*, 1988), and physiological changes (Kelly *et al*, 1978). The use of the tubulin binding assay to detect resistance to benzimidazoles is a highly specific biochemical test directed at one area possibly involved in the mode of action of the benzimidazoles and therefore in changes associated with drug resistance.

The above studies involved experimental infections with a single species of nematode parasite. In the field situation infections are mixed and preparing a tubulin extract from a mixed population for the binding assays makes detection of resistance in a single species of nematode impossible. Other methods are therefore more appropriate where mixed infections predominate e.g. faecal egg count reduction test, egg hatch assay with culture of larvae to allow identification of species or inhibition of larval development.

DEVELOPMENT OF AN ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA) FOR THE DETECTION OF α AND β TUBULIN IN NEMATODES

Materials

Parasites - Infective larvae and first stage (L1) larvae of *O.circumcincta*, Bearsden and HFRO strains were prepared by the method given previously (see General Materials and Methods). Infective larvae were exsheathed (see General Materials and Methods). Adult *O.circumcincta* of the HFRO strain were obtained at necropsy of lambs in Experiment 2 of the Dose Titration Trial (see previously).

Antibodies - Monoclonal anti-α tubulin - mouse monoclonal antibody in ascites fluid, Monoclonal anti-β tubulin - mouse monoclonal antibody in ascites fluid and anti-mouse immunoglobulin horseradish peroxidase linked whole antibody (from sheep) affinity purified in buffered aqueous solution were obtained from Amersham International plc.

Phosphate buffered saline (PBS) - Prepared with 1.07g Na_2HPO_4 (anhydrous),0.39g $NaH_2PO_4.2H_2O$ and 8.5g NaCl in 1 litre of distilled water, pH7.2.

BSA-PBS - 3%(w/v) bovine serum albumin in PBS

Tween-PBS - 0.05%(v/v) Tween 20 in PBS.

BSA-Tween-PBS - 3%(w/v) bovine serum albumin in Tween-PBS.

Carbonate/bicarbonate buffer - Na_2CO_3 (anhydrous) 10.6g/l and $NaHCO_3$ 8.401g/l were mixed in proportions of 29.3ml Na_2CO_3 with 70.7ml $NaHCO_3$ to produce a solution with pH 9.6.

Sodium acetate/citric acid buffer - 0.1M sodium acetate (anhydrous) and 0.1M citric acid were mixed in proportions of approximately 99ml sodium acetate and 1ml citric acid to produce a solution with pH 6.0.

Enzyme substrate - 250ug 33'-diaminobenzidine-4HCl per ml, 0.5ul 30% H_2O_2 per ml in 0.05M trisHCl buffer, pH7.6.

TMB enzyme substrate - 100mg 3,3',5,5'-tetramethylbenzidine (TMB) dissolved in 10ml DMSO. 1ml of this was added to 100ml of 0.1M sodium acetate/ citric acid buffer, pH6 and H_2O_2 was added to a final concentration of 1.3mM/l.

MES buffer - 0.025M MES buffer containing 0.1mM EGTA and 0.5mM $MgSO_4$, pH 6.5.

MES +GTP buffer - MES buffer plus 0.1M GTP.

Nitrocellulose membrane.

96 well ELISA plates (Nunc)

Immunological Detection by Protein Blotting of Alpha Tubulin in Extracts from Infective Larvae of *O.circumcincta* Bearsden Benzimidazole Susceptible and HFRO Benzimidazole Resistant Strains. (Method modified from Ogata *et al*, 1983)

Method.

1. Samples containing 10,000 or 100,000 infective larvae of the Bearsden or HFRO strain of *O.circumcincta* were washed in MES buffer and centrifuged on 3 occasions.

2. The larvae were homogenised in MES +GTP buffer. With 10,000 larvae the total volume was 0.5ml and with 100,000 larvae the volume was 1ml.

3. The homogenates were placed in Eppendorf plastic centrifuge tubes and centrifuged at 12,500r.p.m. in an Eppendorf benchtop centrifuge for 15min.

4. The supernatant was removed as the tubulin extract and a Lowry protein Assay (see previously) was carried out on all the extracts.

5. Nitrocellulose membranes were soaked in MES buffer

6. Separate nitrocellulose membranes were loaded with tubulin extracts . The volume of

extract loaded onto the membrane was adjusted to add 4ug of protein for the extracts from 10,000 larvae and 19.8 ug of protein for the extracts from 100,000 larvae. A blank was prepared using MES +GTP spotted onto the membrane. The membranes were placed in an incubator at 37°C to dry the spots.

7. The nitrocellulose strips were soaked in BSA - PBS for 1h at 37°C.

8. The nitrocellulose membranes were incubated for 1h at 37°C surrounded by anti-tubulin antibody (antibody diluted 0.02ml in 100ml BSA - Tween-PBS).

9. The membranes were washed 4 times with Tween-PBS with agitation over a period of 30min.

10. The membranes were incubated for 1h at 37°C surrounded by anti-mouse antibody peroxidase linked (diluted 1:400 in BSA -Tween -PBS).

11. The membranes were washed 4 times with Tween -PBS with agitation over a period of 30min .

12. The membranes were soaked in enzyme substrate for 5min and the reaction was stopped by the addition of distilled water.

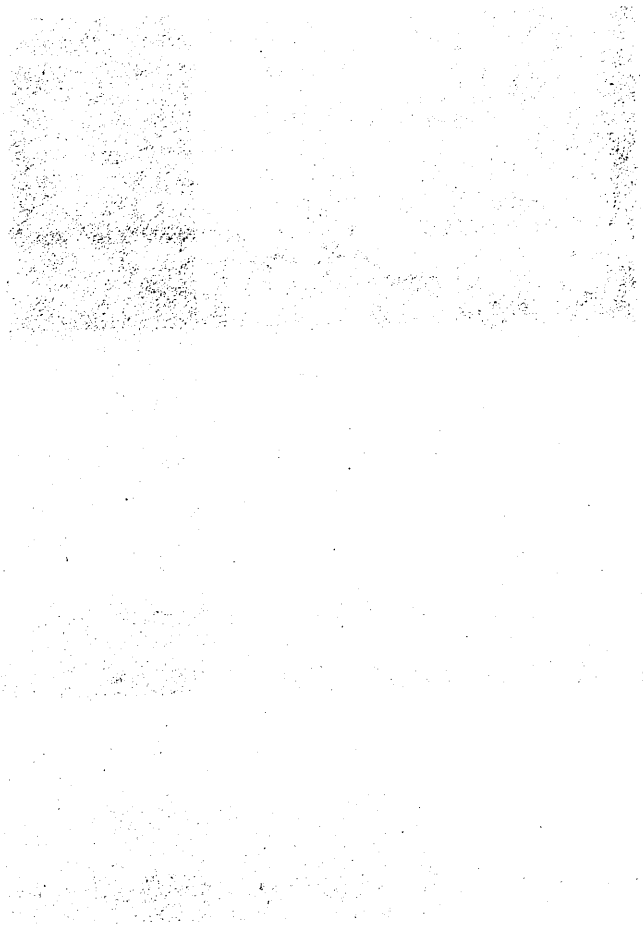
13. The membranes were dried in the incubator at 37°C and examined for development of a brown colour.

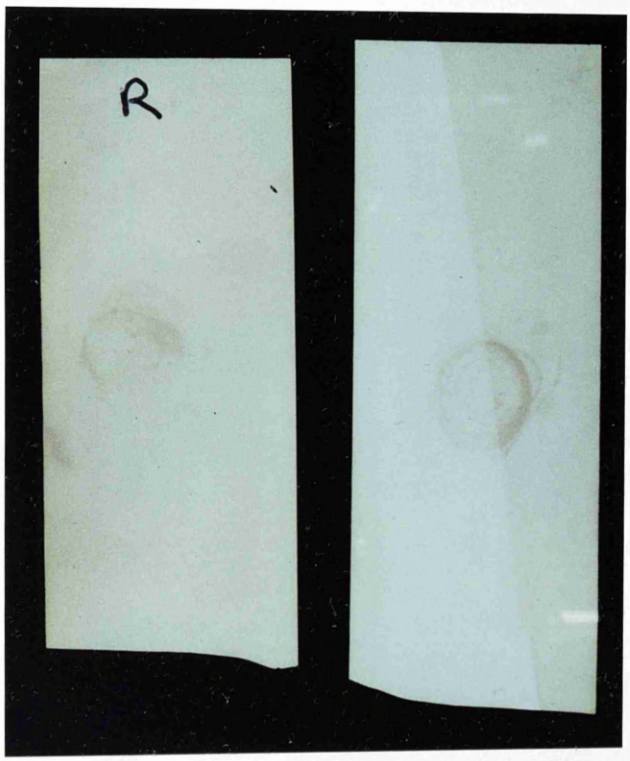
Results

The nitrocellulose strips were examined after the addition of substrate and a small brown circular spot was detected in the area where extracts from 100,000 infective larvae of both strains were loaded (see Fig.3.19). There was no colour change detected in the blank and on the nitrocellulose strips loaded with extracts from 10,000 infective larvae. The protein blots using extracts from 100,000 larvae contained 19.8ug protein and anti-α tubulin antibody bound to the protein blot. However, the blots from extracts from 10,000 larvae which contained 4ug protein were not detected with anti-α tubulin antibody.

Figure 3.19

Results of a protein blot onto nitrocellulose with tubulin extracts from infective larvae of the Bearsden and HFRO strains of *O.circumcincta* with detection of α tubulin using an immunological technique.




R

Discussion

The binding of anti-αtubulin antibody to extracts from 100,000 infective larvae which were blotted onto nitrocellulose strips was assumed to be due to binding to tubulin in the extracts. The nitrocellulose strips with buffer blotted onto the strip (blank) showed no change in colour after the addition of anti-α tubulin antibody , peroxidase linked second antibody and substrate which suggested that antibody was binding to a constituent of the extract from the 100,000 larvae and this was assumed to be tubulin. For the protein blot using extracts from 10,000 infective larvae the protein concentration added to the nitrocellulose strip was much lower and binding of anti-α tubulin antibody was not detected by examining the nitrocellulose strip with the naked eye. Using the protein blotting technique the full volume of extract loaded onto the nitrocellulose membrane may not have become bound to the membrane. The higher protein concentration in the extracts from 100,000 infective larvae may be necessary in order to ensure that sufficient tubulin bound to the strips.

In this experiment a constant protein concentration of the extracts from 100,000 infective larvae of each strain of *O.circumcincta* was added to the nitrocellulose membrane. However, because of the the diffuse nature of the colour spot on the nitrocellulose membrane it was impossible to measure the colour change quantitatively. Differences in the binding of anti-α tubulin antibody to the extracts from benzimidazole resistant and benzimidazole susceptible parasites could not be measured accurately using this method. To make the assay quantitative the peroxidase induced colour reaction would need to be eluted from the nitrocellulose strip and colour changes measured in a spectrophotometer. Alternatively, a similar reaction using ELISA plates would allow colorimetric analysis of the end products of the reaction.

These initial studies using nitrocellulose strips were useful to establish that tubulin extracts bound to the surface of the nitrocellulose membrane and binding of anti-αtubulin

antibody could be detected. This method was modified and used in an ELISA with anti-α tubulin antibody and anti-β tubulin antibody using 96 well plastic plates.

Development of an ELISA to Detectαtubulin and β tubulin in Extracts from *O.circumcincta* Bearsden and HFRO Strains

Method.

1. Tubulin extracts were made from infective larvae of the Bearsden and HFRO strains of *O.circumcincta* by the method described previously (see steps 1-4 in above method)

2. Using adult parasites, exsheathed infective larvae or first stage larvae the parasites were counted and a known number were sonicated in MES +GTP buffer. The parasites were exposed to several cycles of sonic waves(6 u) for 30 seconds with a 30 second rest period and the tubes were maintained in ice. The parasites were examined under a dissecting microscope to ensure they were fully destroyed.

3. The samples were placed in individual wells on the ELISA plate and were bound to the plate by incubating in the presence of carbonate/bicarbonate buffer for 1-2h at 37°C. For blanks MES + GTP buffer was added instead of the sample

4. The plate was washed with BSA-Tween-PBS and incubated for 1h at 37°C with BSA-Tween -PBS.

5. The BSA-Tween-PBS was removed from the wells and anti-αtubulin or anti-β tubulin antibody solution was added to each well (0.02ml antibody in ascites solution diluted with 100ml BSA-Tween-PBS). The plates were incubated for 1h at 37°C.

6. Plates were washed with Tween-PBS 4 times with agitation over a period of 30min.

7. To each well was added anti-mouse peroxidase linked antibody (diluted 1:400 in BSA-Tween-PBS). The plate was incubated for 1h at 37°C.

8. Plates were washed 4 times with Tween-PBS followed by 4 washes in distilled water.

9. TMB enzyme substrate (150ul) was added to each well and plates were incubated for

30min at 37°C. The reaction of horseradish peroxidase with TMB produced an intense blue colour and the absorbance was measured at 690nm. The reaction was stopped by adding 2M sulphuric acid (20ul) to each well and a further reading for absorbance at 450nm was taken. A more consistent reading for absorbance was obtained by using two filters of 690nm and 450nm in the plate reader.

Results

ELISA 1

Tubulin was extracted from 100,000 infective larvae of the Bearsden and HFRO Strains of *O.circumcincta* A Lowry protein assay was carried out (for method see previously) and aliquots of each extract containing 50ug of protein were added to wells on the ELISA plate. One thousand larvae of each strain were sonicated and aliquots of equal volume of the sonicated material from each strain were added to wells on the ELISA plate. Results of the ELISA using anti-α tubulin and anti-β tubulin antibodies followed by peroxidase linked anti-mouse antibody and substrate are shown in Table 3.25. The peroxidase activity, which corresponds to the binding of antibody to the larval extracts, was measured spectrophotometrically at 450nm after the addition of substrates then sulphuric acid. The ratio of binding of anti-α tubulin to binding of anti-β tubulin for both sonicated larval material and tubulin extracts from larvae is shown in Table 3.25.

ELISA 2

Using infective larvae of the Bearsden and HFRO strains of *O.circumcincta* 2,000 larvae were sonicated in equal volumes of buffer and aliquots of the sonicated material were added to wells on an ELISA plate. Increasing concentrations of anti-tubulin antibody were used in the ELISA. The working concentration of anti-tubulin antibody recommended by Amershams was 1-2ug/ml. For this assay the normal concentration (1-2ug/ml) and 2, 5, 10, and 20 times this concentration were used. Results of the ELISA are shown in Table 3.26 and graphs of the peroxidase activity (absorbance units at 450nm) against the

concentration of anti-tubulin antibody used in the ELISAs with larvae of the Bearsden HFRO strains of *O.circumicincta* are shown in Figs. 3.20 and 3.21 respectively.

ELISA 3

The assay was carried out to compare the binding of anti-tubulin antibody to sonicated larval extracts of the Bearsden and HFRO strains of *O.circumcincta*. The larvae were untreated or incubated with ABZ for 24h at a concentration of 1ug/ml or 5ug/ml prior to sonication. Results of the ELISA expressed as peroxidase activity bound to the larval extracts are shown in Table 3.27. Using the Student's t-test a significantly higher level of anti-α tubulin and anti-β tubulin were bound to the material from larvae of the susceptible strain of *O.circumcincta* compared with material from larvae of the HFRO strain. There were some differences in binding of anti-tubulin antibodies between the sonicated material from larvae which were treated with ABZ or untreated but these differences were not consistent and not significant.

ELISA 4

Adult *O.circumcincta* were collected at necropsy from lambs in the dose titration trial Experiment 2 (see previously). The 5 lambs infected with the HFRO benzimidazole resistant strain of *O.circumcincta* had been treated orally with FBZ at dose rates of 5, 10, 20 and 40 mg/kg and one animal remained untreated as a control. From each lamb 10 female and 10 male adult parasites were obtained and sonicated. Binding of anti-αtubulin and anti-β tubulin to the samples measured as peroxidase activity is shown in Table 3.28.

ELISA 5

This assay was carried out using first stage larvae of *O.circumcincta*. The larvae were cultured from faeces using the method described previously (see General Materials and Methods). The faeces for culture of first stage larvae were obtained from Lamb DB 33 (see Fig.2.1), a lamb infected with the Bearsden strain of *O.circumcincta* and a lamb from

Table 3.25

Results of ELISA 1 comparing the binding of anti-tubulin antibodies to tubulin extracts from 100,000 infective larvae of the Bearsden and HFRO strains of *O.circumcincta* and extracts from 1,000 sonicated infective larvae of the same strains

Extract	Absorbance Units ± SEM at 450nm		
	α tubulin	β tubulin	α/β
Bearsden - tubulin extract	0.081±0.019	0.043±0.002	1.88
HFRO - tubulin extract	0.054±0.007	0.048±0.003	1.13
Bearsden - sonicated larvae	0.155±0.006	0.098±0.002	1.58
HFRO - sonicated larvae	0.079±0.003	0.049±0.002	1.61

Table 3.26

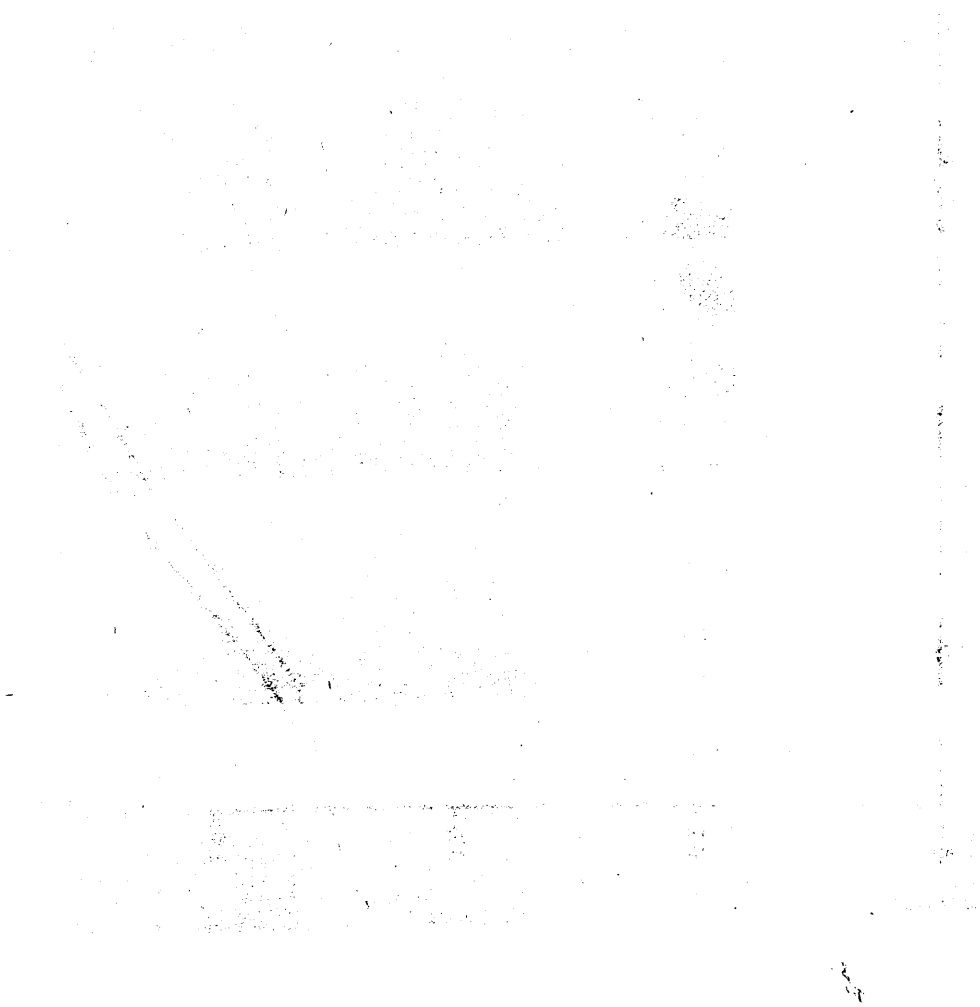
Results of ELISA 2 using varying concentrations of anti-tubulin antibody and extracts obtained after sonication of 2,000 infective larvae of the Bearsden benzimidazole susceptible and HFRO benzimidazole resistant strains of *O.circumcincta*

Antibody conc.*	Absorbance units ± SEM at 450nm			
	α tubulin		β tubulin	
	Susceptible	Resistant	Susceptible	Resistant
20 X	> 2	> 2	> 2	> 2
10 X	> 2	1.856±0.035	> 2	1.751±0.033
5 X	1.703±0.018	1.192±0.016	1.615±0.031	1.094±0.028
2 X	1.385±0.006	0.670±0.007	1.323±0.030	0.534±0.018
1 X	1.298±0.032	0.520±0.027	1.297±0.030	0 446±0.014

*Normal concentration of antibody 1-2ug/ml

Figure 3.20

Graph of peroxidase activity measured at 450nm against concentration of anti-tubulin antibody in ELISA 2 using sonicated infective larvae of the Bearsden strain of *O.circumcincta*



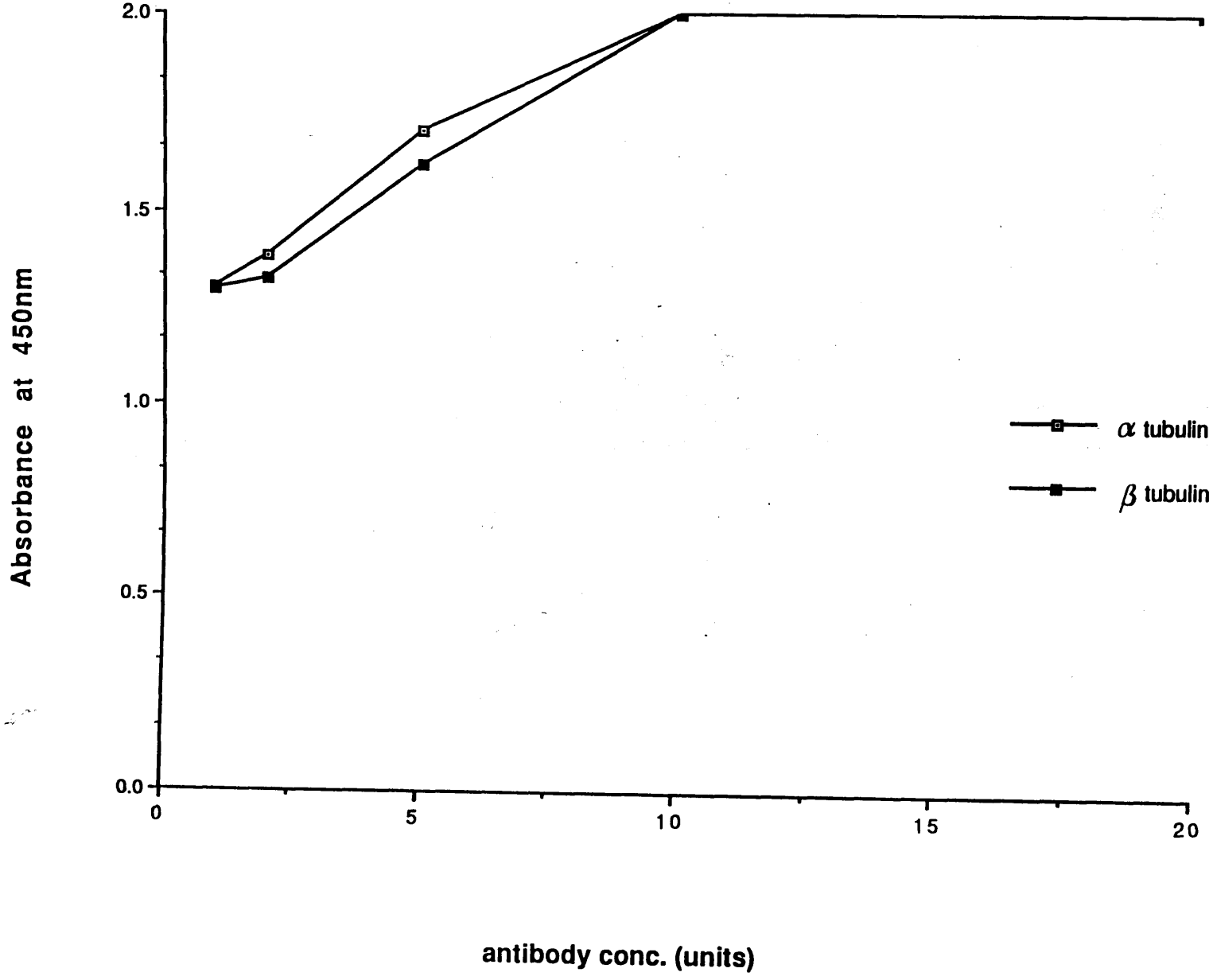
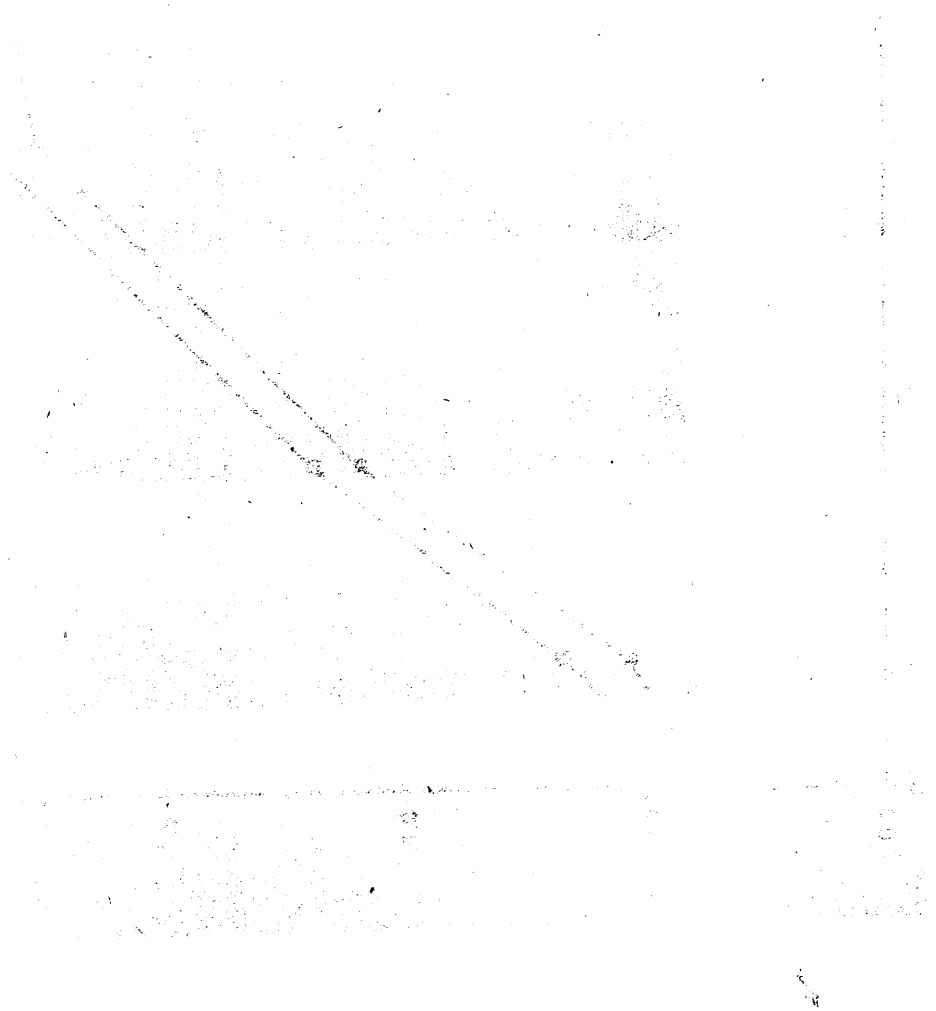

Absorbance at 450nm
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
α tubulin
β tubulin
antibody conc. (units)

Figure 3.21

Graph of peroxidase activity measured at 450nm against concentration of anti-tubulin antibody in ELISA 2 using sonicated infective larvae of the HFRO strain of *O.circumcincta*



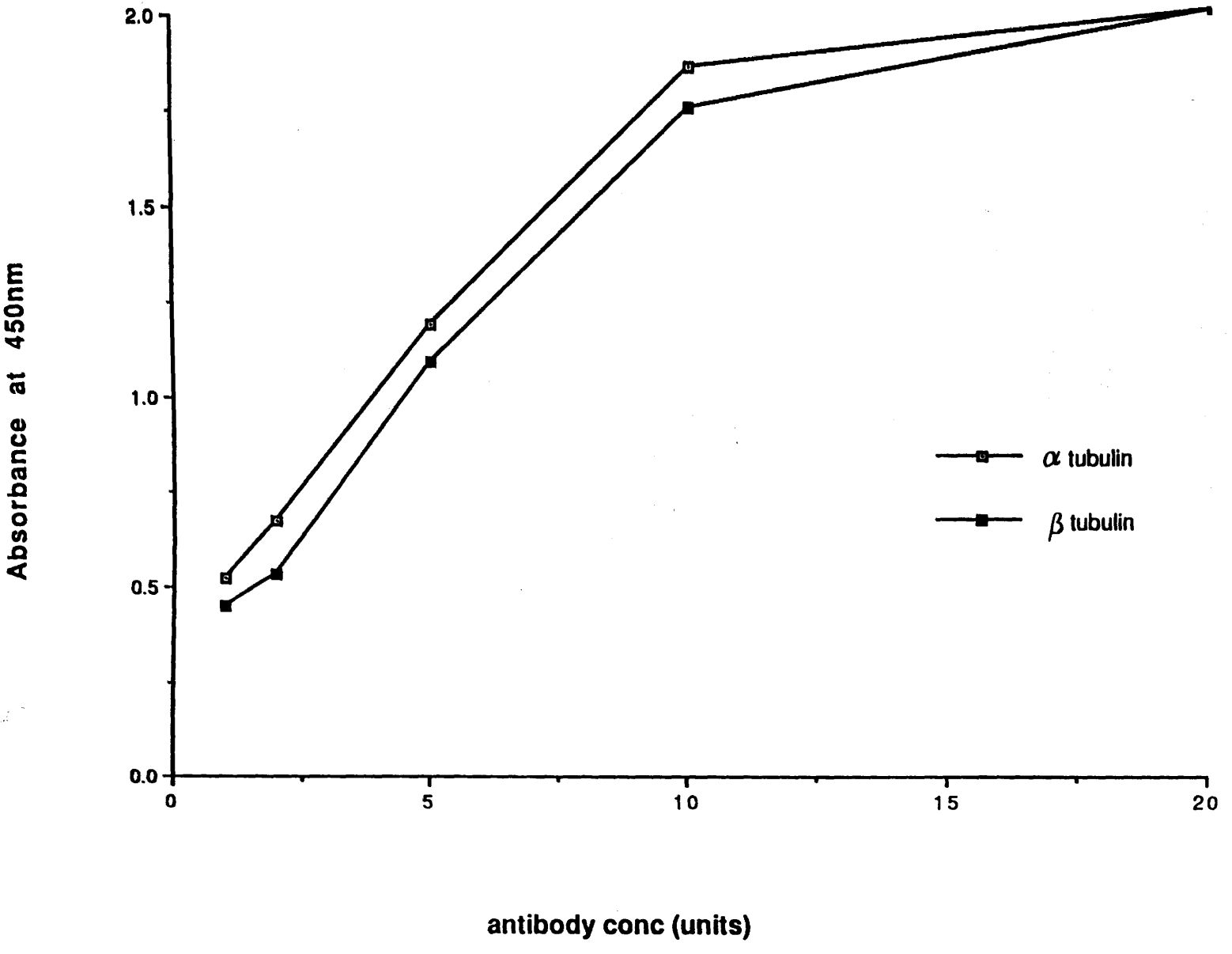

Absorbance at 450nm
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
α tubulin
β tubulin
antibody conc (units)

Table 3.27

Results of ELISA 3 using extracts from sonicated infective larvae of the Bearsden benzimidazole susceptible and HFRO benzimidazole resistant strains of *O.circumcincta* which were untreated or treated with ABZ for 24h at a concentration of 1ug/ml or 5ug/ml.

Extract	Absorbance units ± SEM at 450nm			
	α tubulin		β tubulin	
	Susceptible	Resistant	Susceptible	Resistant
Untreated	1.427±0.016	0.918±0.066	1.474±0.007	0.626±0.010
Treated (1ug/ml)	1.435±0.035	1.086±0.037	1.340±0.024	1.047±0.014
Treated (5ug/ml)	1.690±0.143	0.887±0.053	1.779±0.012	0.794±0.008

Table 3.28

Results from ELISA 4 using sonicated adult *O.circumcincta* of the HFRO benzimidazole resistant strain. Parasites were obtained at necropsy of lambs used in dose titration trial Experiment 2 and had been treated orally with FBZ at dose rates of 0, 5, 10, 20 and 40mg/kg.

Lamb	Absorbance units ± SEM at 450nm	
Dose FBZ (mg/kg)	α tubulin	β tubulin
0	0.167±0.004	0.206±0.014
5	0.185±0.005	0.233±0.004
10	0.238±0.009	0.249±0.003
20	0.163±0.005	0.206±0.009
40	0.151±0.004	0.175±0.003

Table 3.29

Results of ELISA 5 using first stage larvae of *O.circumcincta* of the Bearsden and HFRO strains. Larvae were obtained from the faeces of a lamb infected with the Bearsden strain of *O.circumcincta*, Lamb DB33, which was infected with the HFRO strain and a lamb from dose titration trial Experiment 3 after treatment of the lamb with FBZ at a dose rate of 40mg/kg.

Source of Larvae	Absorbance units ± SEM at 450nm		
	α tubulin	β tubulin	α/β
Bearsden Strain	1.412±0.010	0.959±0.024	1.472
HFRO Strain (DB33)	1.092±0.032	0.670±0.017	1.630
Dose Titration Expt.3	1.237±0.087	0.830±0.035	1.490

dose titration trial Experiment 3 after treatment of the lamb with FBZ at a dose rate of 40mg/kg. Approximately 5,000 L1s of each strain were sonicated in an equal volume of buffer and aliquots were placed in wells in an ELISA plate. Results of the binding of anti-α tubulin and anti-β tubulin measured as peroxidase activity are shown in Table 3.29. In addition the ratio of binding of anti-α tubulin to binding of anti-β tubulin is shown in Table 3.29.

Discussion

Development of resistance by nematode parasites to the effects of benzimidazole anthelmintics may be due to an alteration in the binding of benzimidazoles to nematode tubulin (Lacey, 1985). This author used tubulin extracts from infective larvae of benzimidazole resistant and susceptible strains of *H.contortus*, *O.circumcincta* and *T.colubriformis* and found reduced binding of benzimidazoles to extracts from benzimidazole resistant strains compared with binding to benzimidazole susceptible strains. This tubulin binding assay can be used to detect benzimidazole resistant strains of nematodes (Lacey and Snowdon, 1988) but in experiments with this assay (see previously) tubulin extracts from parasites with a high protein concentration were required and results were inconsistent when used with the HFRO benzimidazole resistant and Bearsden benzimidazole susceptible strains of *O.circumcincta* Foster *et al.*, (1987) found that resistance to benzimidazoles exhibited by a strain of *Physarum polycephalum* was due to a mutant form of β tubulin.

An ELISA test for α and β tubulin was developed in order to compare the levels of these protein subunits in benzimidazole resistant and susceptible strains of *O.circumcincta* The assay was used to investigate if there were differences in the amount of tubulin in the two strains which may be implicated in the development of resistance to the effects of benzimidazoles. Differences which may be detected using anti-tubulin antibodies would include differences in the amount of tubulin in the parasites, differences in the ratio of α

and β tubulin or differences in the binding affinity of the antibodies to tubulin in the individual strains of *O.circumcincta*

In ELISA I the binding of anti-α tubulin and anti-β tuibulin was compared using tubulin extracts from infective larvae produced by homogenisation or by sonication of infective larvae(Table 3.25). For both the tubulin extract and the sonicated material the binding of anti-α tubulin was reduced for larvae of the benzimidazole resistant strain compared with binding to the susceptible strain. There was reduced binding of anti-β tubulin to sonicated material of the benzimidazole resistant strain compared with binding to sonicated material of the susceptible strain. However, there was no difference between binding of anti-β tubulin to the tubulin extracts from the two strains of *O.circumcincta*.

The resuts of the ELISA using sonicated larval material were similar to results obtained using tubulin extracts from the same strains of larvae (Table 3.25). For the tubulin extracts larger numbers of larvae were required. During extraction of tubulin by homogenisation there may be losses of tubulin in the pellet after centrifugation and losses due to binding of the material to the glassware. A small sample of the pellet discarded after centrifugation of the homogenised larvae during the production of the tubulin extract was used in the ELISA and a small amount of anti-tubulin antibody bound to this pellet material which suggested that there was some loss of tubulin during the extraction process. When material from sonicated larvae was used in the ELISA the peroxidase activity was higher than with tubulin extracts from a significantly greater number of larvae. There may be more non-specific binding of anti-tubulin antibody to the sonicated material but the differences in binding of anti-tubulin antibodies to resistant and susceptible strains of *O.circumcincta* appeared to be consistent.

The main advantage of using sonicated larvae as the test material in the ELISA was that similar numbers of larvae could be sonicated to give an accurate comparison between the binding of anti-tubulin antibodies to benzimidazole resistant and susceptible strains of

O.circumcincta When the tubulin extracts were used for the ELISA, although the same number of larvae were used initially, losses during homogenisation and centrifugation are not consistent between the preparations. Therefore the tubulin extracts had to be assessed for protein concentration in order to ensure that a similar concentration of extract was added to each ELISA well and that a true comparison of anti-tubulin antibody binding was being measured in the tubulin extracts. As mentioned previously the protein content of the tubulin extract gives an indication of the concentration of the tubulin extract but may not reflect the tubulin content in the extract because tubulin constitutes only 1-3% of the protein content. For a direct comparison of the tubulin content of parasites it was more convenient to use sonicated larvae.

The ELISA was also attempted using low numbers of infective larvae which were sonicated. Peroxidase activity was measured with sonicated material from 100 infective larvae but the difference in activity measured in the samples compared with the controls was small and therefore the test was more reliable with higher numbers of larvae (>1,000).

In the ELISA the levels of binding of anti-α and β tubulin to the larval extracts were compared. However the concentration of α tubulin and β tubulin in the larval extracts could not be calculated because standard concentrations of pure α tubulin and β tubulin were not used in the assay. The aim of the assay was to investigate possible differences in the tubulin components of benzimidazole resistant and susceptible parasites and therefore measurement of the exact amount of tubulin in the extracts was unnecessary. The ELISA could be used to measure the level of tubulin in the extracts from larvae and might be useful in combination with tubulin binding assays. In these binding assays the amount of benzimidazole bound to the extract from larvae was expressed as binding per mg of protein in the extract but the results may be more accurate if the binding per unit of tubulin in the extract was measured (see previous discussion). This use of the ELISA as a

quantitative assay for tubulin in extracts from larvae of *O.circumcincta* which were to be used in tubulin binding assays with tritiated benzimidazoles has not yet been investigated.

In ELISA 2 the effect of using increasing concentrations of anti-tubulin antibodies in the assays to try to improve the final peroxidase activity was investigated. Using sonicated infective larvae of the benzimidazole resistant and susceptible strains of *O.circumcincta* there was increasing peroxidase activity with incubation in the presence of increasing concentrations of anti-tubulin antibodies (Table 3.26). At 10 times and 20 times the normal concentration of antibody the peroxidase activity was too great, too high an intensity of colour, to allow measurement in the plate reader. In order to avoid this problem the plate should be read at 690nm prior to the addition of sulphuric acid. After the reaction of peroxidase with TMB substrate the addition of sulphuric acid changes the colour from blue to yellow and causes a two-fold increase in the colour intensity (Bos *et al.*, 1981). The intensity of the blue colour can be measured at 690nm prior to the addition of sulphuric acid.

In ELISA 2 similar results were obtained with anti-αtubulin and anti-β tubulin and the graphs (Figs. 3.20 and 3.21) of peroxidase activity at increasing concentrations of anti-tubulin had similar shapes for anti-α tubulin and anti-β tubulin. The antibody binding curves for ELISAs are usually sigmoidal in shape and ideally for the assay a concentration of antibody which corresponds to the steep portion of the curve should be used to maximise any differences in antibody binding. From Figs. 3.20 and 3.21 the best concentration of anti-tubulin antibodies for the ELISA would be 5 times the normal concentration (5-10ug/ml antibody).

In ELISA 2 the binding of anti-tubulin antibody to sonicated material from the HFRO strain of *O.circumcincta* was significantly lower than binding of anti-tubulin antibodies to sonicated larvae of the Bearsden strain at all antibody concentrations except 20 times normal where the absorbance was too high to give an accurate reading (Table 3.26).

The reduced binding of anti-tubulin antibody to material from the HFRO strain of *O.circumcincta* compared with binding to material from the Bearsden strain in ELISA 1 and 2 may be a result of changes in the affinity of the anti-tubulin antibody for the tubulin molecules or a reduced concentration of α and β tubulin in parasites of the HFRO strain. Lacey (1985) noted a reduction in the binding of tritiated benzimidazoles to crude tubulin extracts from resistant strains of nematodes compared with binding to benzimidazole susceptible strains. These changes in binding of benzimidazoles in the resistant strain of parasites may be due to changes in the tertiary structure of the tubulin molecules which cause an alteration in the benzimidazole binding site and therefore there is reduced binding of benzimidazoles to the resistant parasites when compared with binding to the susceptible parasites. A change in the tertiary structure of the individual α or β tubulin molecules or a change in the structure of the microtubules formed by these subunits may also cause a change in the antigenic determinant on the molecule. Any alteration in the antigenic determinant will cause a change in the binding of anti-tubulin antibody. Foster *et al.*, (1987) found changes in the β tubulin molecule in a benzimidazole resistant strain of *Physarum polycephalum*. No differences were detected by these authors in the α tubulin molecule between benzimidazole resistant and susceptible strains. However, in ELISAs 1 and 2 there were differences in the binding of both anti-α tubulin and anti-β tubulin. This may be more suggestive of an alteration in the overall tubulin concentration between larvae of the benzimidazole resistant and susceptible strains of *O.circumcincta*. The amount of tubulin in the larvae may be influenced by time of storage of the larvae. To try to eliminate differences in the time of development and storage which occur with infective larvae, first stage larvae were used in an ELISA (see later) There can be different sub-types of tubulin within a single species and differences in the binding of anti-tubulin antibodies may reflect differences in the tubulin sub-types found in various organelles within the parasite. These differences in

the binding of anti-tubulin antibodies between the Bearsden and HFRO strains of *O.circumcincta* may be due to strain differences in type of tubulin present in the parasites and may not have any relevance in dictating the level of resistance to the effects of benzimidazoles. Unfortunately other strains of parasites were not available to test if changes in the binding of anti-tubulin antibody were due to strain variation or linked with benzimidazole resistance.

In ELISA 3 there was no significant difference in the binding of anti-tubulin antibodies between larvae incubated in the presence of two concentrations of ABZ prior to sonication and untreated larvae (Table 3.27). Benzimidazoles are thought to act by binding to tubulin and preventing the formation of microtubules in nematodes (Borgers and De Nollin, 1975). Destruction of microtubules or disruption of the equilibrium between formation and breakdown of microtubules by benzimidazoles may not have any effect on the overall concentration of α and β tubulin within the parasite cells. The sonication of the larvae was carried out on ice and therefore most of the microtubules should have disassembled to tubulin subunits at low temperature. The ELISA measures the ability of anti-tubulin antibody to bind to tubulin rather than measuring the degree of polymerisation of the microtubules in the larvae. There were some differences between α tubulin and β tubulin levels between treated and untreated parasites of both the Bearsden and HFRO strains but these differences were not significant in most cases and were not consistent. As in other ELISAs there were differences between the binding of anti-tubulin antibodies to sonicated larval extracts from the Bearsden benzimidazole susceptible strain and the HFRO resistant strain. However, treatment of the larvae with ABZ did not have a significant or consistent effect on either strain. Using treatment of the larvae with benzimidazoles prior to an ELISA for tubulin concentration was therefore not a suitable test to detect resistance to benzimidazole in larvae of *O.circumcincta*

Adult *O.circumcincta* of the HFRO strain were obtained from lambs at necropsy in the

dose titration trial Experiment 2 after treatment of the lambs with FBZ administered orally at dose rates of 0, 5, 10, 20 and 40 mg/kg. Unfortunately adult *O.circumcincta* are not readily available because they are obtained at slaughter from the abomasum of the lambs. A lamb infected with the Bearsden susceptible strain of *O.circumcincta* was not available to allow a comparison of binding of anti-tubulin antibodies between the resistant and susceptible adult *O.circumcincta*. There were slight differences in the peroxidase activity measured in the ELISA for tubulin with the adult *O.circumcincta* which had been exposed to different concentrations of FBZ (Table 3.28). The adults exposed to 10mg/kg showed the highest binding of anti-α tubulin and anti-β tubulin but there were no significant differences in the binding of anti-tubulin antibodies among the selected adult parasites. Egg hatch assays carried out on the progeny of these differentially selected adult *O.circumcincta* showed similar ED50s for TBZ and ABZ (see previously). This ELISA gave an indication of the concentration of α and β tubulin in the adult parasites and the similarity in the levels of tubulin in the parasites suggested that there was a high level of homogeneity in the population. The ELISA was carried out using specific numbers of male and female worms to ensure that any differences detected in the binding of anti-tubulin antibodies would not be due to the possibly higher level of tubulin in female parasites which contain eggs.

First stage larvae were used in ELISA 5 to try to ensure that parasites of the Bearsden and HFRO strain were at exactly the same stage of development and had been produced under the same conditions to try to minimise possible environmental factors which could influence the level of tubulin in the parasites. With first stage larvae there was increased binding of anti-tubulin antibodies to sonicated larval extracts from the susceptible strain compared with sonicated extracts from the HFRO benzimidazole resistant strain of *O.circumcincta* (Table 3.29). The first stage larvae cultured from eggs passed by female *O.circumcincta* after treatment of the host with FBZ at 40mg/kg showed binding of

anti-tubulin antibodies higher than the first stage larvae cultured from eggs of untreated adult parasites but lower than binding of anti-tubulin antibodies to first stage larvae of the susceptible strain. All the larvae were produced under the same conditions therefore differences in the level of α and β tubulin should not be due to environmental influences. These differences in the binding of anti-tubulin antibodies may indicate small differences in the amount of tubulin in different strains or groups of parasites. The ratio of α tubulin to β tubulin in the first stage larvae was similar in all cases (Table 3.29) and resembled the ratio obtained in ELISA 1 using sonicated infective larvae (Table 3.25).

Overall in the ELISAs there was a lower level of binding of anti-tubulin antibodies to the HFRO resistant strain of *O.circumcincta* compared with binding to the Bearsden strain. These results suggested that the tubulin concentration in resistant parasites was not increased compared with benzimidazole susceptible parasites. Therefore an increase in the amount of available tubulin in resistant parasites was not the mechanism by which these strains of parasites could resist the effects of benzimidazoles. There may be changes in the tertiary structure of the microtubules or tubulin subunits which could result in decreased binding of anti-tubulin antibodies because of a change in the structure of the antigenic determinant. Alternatively, the microtubules which form in the resistant nematodes may be more stable than microtubules in susceptible parasites and are therefore less liable to disassemble at low temperatures, during sonication, during tubulin extraction and under the influence of benzimidazoles. This may explain the reduced concentrations of available α tubulin and β tubulin which were measured in resistant parasites during ELISAs.

There may be differences in the ability of sonicated material from the two strains of *O.circumcincta* to bind to the ELISA plate. Most protein molecules bind to plastic and it was assumed that the extracts from parasites would act in a similar way and bind to the plate in a consistent manner. A method to eliminate this possible problem would be to bind

anti-tubulin antibody onto the plate surface. The antigen, sonicated larvae or tubulin extract, could be added followed by anti-tubulin antibody, then peroxidase linked antibody. This method would add another layer to the sandwich ELISA. The main problem with this technique is that the anti-tubulin antibody added to the plate and the anti-tubulin antibody added after the antigen must be raised in different species. This is to prevent cross reaction on addition of the peroxidase labelled antibody.

In these ELISAs and the protein blotting experiment (see previously) monoclonal anti-tubulin antibodies produced against mammalian tubulin bound to nematode tubulin derived from all stages of the parasites. This underlines the amount of conservation of the tubulin molecule throughout the evolutionary process (Dales, 1972).

General Discussion and Conclusions

These ELISAs showed differences in the binding of anti-αtubulin and anti-β tubulin antibodies to parasites of different strains of *O.circumcincta*. However, there were no consistent differences in the anti-tubulin antibody binding between parasites treated with benzimidazoles or untreated. Similar results were obtained when first stage and infective larvae of *O.circumcincta* were used in the assays. The differences in binding of anti-tubulin antibodies may reflect differences in the affinity of the antibodies for tubulin or differences in the concentration of tubulin in the two strains of *O.circumcincta*. There were differences between the HFRO benzimidazole resistant strain of *O.circumcincta* and the Bearsden benzimidazole susceptible strain detected using the ELISA for tubulin. However, this test is not suitable for detecting resistance to benzimidazoles in nematode parasites because there is insufficient evidence on the exact biochemical mechanism of action of the benzimidazole anthelmintics including the role of tubulin and the possible changes in the tubulin molecule responsible for resistance to the effects of benzimidazoles.

SUMMARY

A possible mode of action of the benzimidazole group of anthelmintics is binding to nematode tubulin causing depletion of microtubular structures with disruption of cellular transport systems. Tubulin was extracted from the pig intestinal nematode *Ascaris suum* and the sheep nematodes *O. circumcincta* and *Haemonchus contortus*. The benzimidazoles albendazole and mebendazole were shown to inhibit the binding of colchicine to tubulin extracts from *A.suum*. Tritiated albendazole, fenbendazole and mebendazole were synthesised and were used to study the binding of these drugs to tubulin extracts from *A.suum*, *O.circumcincta* and *H.contortus*. Binding of these benzimidazoles was variable and was influenced by the protein content of the tubulin extract. High concentrations of tritiated benzimidazoles bound to extracts synthesised using low numbers (<100,000) of infective larvae of *O.circumcincta* and *H.contortus*.

The differential binding of tritiated benzimidazoles to tubulin extracts from nematodes as a test for resistance in these parasites to the effects of benzimidazole anthelmintics was investigated using tritiated fenbendazole, albendazole and mebendazole. Extracts from *H.contortus* larvae which were resistant to the benzimidazoles bound less mebendazole and albendazole than extracts from benzimidazole susceptible larvae. With tubulin extracts from different strains of *O.circumcincta* results were more variable and depended on the ligand used in the assays.

Displacement of albendazole binding to tubulin extracts from *H.contortus* by albendazole and its metabolites , albendazole sulphoxide and albendazole sulphone, was found with albendazole proving to be the most potent inhibitor. However, in assays using *O.circumcincta* tubulin extracts albendazole, albendazole sulphoxide, albendazole sulphone and colchicine failed to inhibit, consistently the binding of tritiated albendazole.

An ELISA to measure binding of anti-alpha and anti-beta tubulin antibodies to extracts from *O.circumcincta* larvae found reduced binding of both antibodies to extracts from

first stage and third stage larvae of the benzimidazole resistant strain compared with similar extracts from the benzimidazole susceptible strain. Treatment of the larvae with benzimidazoles prior to the ELISA for tubulin did not alter the antibody binding to extracts from both the resistant and susceptible strains of *O.circumcincta*

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