STUDIES ON PHARMACOLOGICAL MEDIATION OF HELMINTH INFECTION IN SHEEP

A thesis submitted for the degree

of

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 previously submitted for the award of a degree to any university.

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ABBREVIATIONS

ml	-	millilitre
mg	-	milligramme
ug	-	microgramme
pg	-	picogramme
g	-	gramme
M	-	Molar
ED_{50}	-	Dose required to produce a response in 50% of individuals under test
p.s.i.	-	pounds per square inch
xg	-	times gravity
SRS-A	-	slow reacting substance of anaphylaxis

SUMMARY

The first section of this thesis deals with general methods of experimentation and materials used in this period of study.

The second section describes experiments to study the effects of a non-steroidal anti-inflammatory drug on the responses of sheep of various degrees of immunity to a challenge of Ostertagia circumcincta. Treatment with meclofenamic acid of adult immune sheep tended to reduce the number of parasites at necropsy, but did not alter the plasma pepsinogen response after challenge when compared to untreated controls.

A similar result was obtained in the study with previously parasite-naive lambs. Meclofenamic acid treated animals contained fewer parasites at necropsy, but plasma pepsinogen activities were similar to untreated controls.

Finally, previously parasite-naive lambs were infected with adult O. circumcincta parasites. All animals showed parasite eggs in the faeces indicating establishment. Those animals which received parasites from untreated donors, and were themselves untreated with meclofenamic acid, showed a rise in plasma pepsinogen activities, which was not observed in the other groups. Meclofenamic acid was subsequently found to inhibit parasite larval motility in vitro which could explain the above results.

Section three is concerned with the effects of various drugs on the responses of adult immune sheep to Ostertagia circumcincta challenge.

Treatment with a corticosteroid allowed larger numbers of parasites to establish and produced higher plasma pepsinogen activities. Leukotriene C_4 concentration in gastrointestinal mucus was lower than that measured in the other groups, producing evidence for a possible protective role of this substance against parasites.

Sheep treated with a mucolytic, bromhexine hydrochloride had fewer parasites at necropsy and lower plasma pepsinogen activities when compared to the control group.

Omeprazole, a drug that raises intra-abomasal pH produced unexpected results. Plasma pepsinogen activities rose towards the end of the experiment. However parasite numbers were very small at necropsy. Omeprazole treatment may have altered plasma pepsinogen activity, either directly by its action on abomasal pH or via stimulation of gastrointestinal hormones e.g. gastrin. Alternatively, its effect may have been to allow the larval challenge to establish, but when the adult parasites emerged, the omeprazole treatment having finished, the host was able to remove them.

The responses of isolated gastrointestinal smooth muscle were studied in section four.

Contractions of the tissue in response to acetylcholine was the consistent feature of both bovine and ovine abomasal and duodenal smooth muscle.

Histamine and prostaglandins E_1 and E_2 generally caused contraction of bovine duodenal preparations. In contrast, few ovine duodenal preparations contracted to prostaglandins E_1 and

E_2 and histamine. 5-hydroxytryptamine and leukotrienes B_4, C_4 and D_4 failed to produce a response in the smooth muscle of abomasal and duodenal preparations tested. Administration of O. circumcincta homogenates to ovine abomasal and duodenal tissues from immune animals failed to produce a Schultz-Dale reaction.

In vitro studies using parasitic larvae were presented in section five. Faecal and mucus homogenates from immune animals did suppress larval motility in the test system used. Prostaglandins E_1 and E_2 and leukotriene C_4 also appeared to have an adverse effect on parasite motility. The usefulness of these studies to investigate the effectiveness of possible anthelmintic drugs was also discussed.

The final section deals with studies carried out on benzimidazole-resistant strains of O. circumcincta.

One strain (HFRO resistant) was passaged through lambs and the effect of treatment with fenbendazole monitored using the egg hatch assay as a measure of the level of resistance of the strain.

Daily variations in the level of resistance were detected. Despite passaging of this strain through five lambs and treatment with fenbendazole on four occasions, the level of resistance did not alter. Another resistant strain (Moredun) was investigated and found to have a lower level of resistance than the HFRO strain.

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GENERAL MATERIALS AND METHODS

Faecal egg counting technique

Faecal samples were collected per rectum and examined for parasite eggs by a modified McMaster technique (Gordon and Whitlock, 1939).

3 g of faeces were weighed and added to 42 ml of water. This was homogenised and the resulting suspension was passed through a 250 um sieve (Endecotts Ltd.). The filtrate was collected, mixed and a 15 ml sample removed and centrifuged in a glass, flat bottomed tube for three minutes at 1500 x g. The supernatant was discarded and the remaining pellet agitated using a Vortex mixer.

Saturated salt solution was added to make a total volume of 15 ml and the faecal suspension was mixed. Only sufficient of this suspension was removed to fill both sides of a McMaster worm egg counting slide (Gelman Hawksley Ltd.). Eggs within both grids (0.15 ml) were counted using a light microscope and the result multiplied by 50 to obtain the number of eggs per gram of faeces.

Baermanisation technique to detect third stage larvae in faeces

The improved Baermann technique described by Henriksen (1965) was used.

10 grams of freshly passed faeces were weighed and placed on a gauze strip which was made into a bag. The bag was suspended in a sedimenting flask containing warm water and left for 24 hours. Larvae which migrated from the faeces into the water were collected in the bottom of the flask. The supernatant was carefully removed to leave 10 ml of the residue. 1 ml aliquots

were taken of the residue and examined under a light microscope using a larval counting slide. All the residue was examined. The number of larvae per gram of faeces was then calculated.

Salt flotation technique to detect third stage larvae in faeces

A technique similar to that employed for detecting parasite eggs in faeces was used as an additional method of larval detection. Saturated salt solution was added to the faecal homogenate and this was used to fill a larvae counting slide. Using a light microscope, the number of larvae were counted.

Abomasal parasite identification and estimation of parasite numbers

Processing of abomasal contents and mucosa

The abomasum was acquired at necropsy and opened along its greater curvature. The contents were collected and the pH estimated. The mucosal surface of the abomasum was gently washed with water to remove all food particles and the washings added to the contents.

The volume of the abomasal contents was made up to two litres with water. This was well mixed and 200 ml aliquots taken into labelled containers to which 20 ml of 40% formalin was added.

The abomasal mucosa was removed using the edge of a glass microscope slide and incubated at 42^{o}C in a pepsin-hydrochloric acid mixture for eight hours. Approximately 1 : 3 (v/v) was added. It was periodically shaken during this time. After digestion, the volume was made up to two litres with water, aliquots were taken and formalised as above.

The pepsin-hydrochloric acid mixture was made up as follows. 80 g of pepsin A (British Drug Houses) were added to seven litres of cold water. 240 ml of hydrochloric acid were added and the total volume made up to eight litres with water.

Estimation of parasite numbers

The abomasal contents and mucosal digests were well mixed and 2 ml aliquots were transferred to petri dishes marked with a grid. Several drops of a 45% iodine solution were added and decolourised with a 2.6% sodium thiosulphate solution.

Parasites were counted and differentiated into adults, male and female, fifth stage larvae (young adults) and fourth stage larvae.

The fourth stage larvae are distinguished by their smaller size and lack of mature reproductive system.

Fifth stage larvae, or young adults, are larger. The females have vulval flaps but no eggs in the uteri. The males have spicules, gubernaculum and bursal rays but they have not attained mature adult dimensions.

Adults are mainly distinguished on a size basis. Often the female uteri are filled with eggs.

Estimation of plasma pepsinogen activity

The concentrations of pepsinogen in plasma samples were estimated using a method modified from that described by Edwards, Jepson and Wood (1960). The plasma was incubated at 37^{o}C with bovine serum albumin substrate (Fraction V, Sigma Chemicals Ltd.) at pH 2 for 24 hours. The phenolic amino acid (tyrosine) liberated was estimated using Folin-Ciocalteu's reagent (BDH Chemicals Ltd.). Corrections were made for normal non-incubated,

concentration of tyrosine-like substances and also the release of these substances due to incubation of bovine serum albumin alone.

Method:

1. The pH of a 2% solution of bovine serum albumin (BSA) was adjusted to pH 1.5 using 2N HCl on the day of use.
2. Each plasma sample was divided, 0.5 ml to be incubated and 0.5 ml to be the non-incubated control. 2.5 ml of the acidified BSA was added to both samples.
3. The test sample was incubated at 37^{o}C for 24 hours in a sealed container.
4. The unincubated controls were immediately precipated by the addition of 5 ml of 4% trichloroacetic acid (TCA).
5. To calculate the release of tyrosine-like substances from incubation of BSA alone, BSA blanks were made up. These consisted of four containers all containing 2.5 ml BSA with 0.5 ml of deionised water. Two were incubated with the samples, two were immediately precipitated with 5 ml of 4% TCA.
6. The precipitated suspensions were allowed to stand for 30 minutes and were then filtered through Whatman No. 44 filter paper. The filtrate was stored in a refrigerator until the incubates were ready.
7. The incubated samples were precipitated and filtered as described above.
8. 1 ml of each filtrate was transferred to suitably labelled vessels containing 10 ml of 0.25 N NaOH.

9. 1 ml of each of the tyrosine standards, respectively 0.2, 0.4 and 0.6 umoles of tyrosine/ml, were also added to 10 ml of 0.25 N NaOH.

10. A reagent blank was set up containing 1 ml of water with 10 ml of 0.25 N NaOH.

11. To all these, 1.5 ml of diluted Folin-Ciocalteu's reagent was added. This had been diluted (1 : 2 v/v) with deionised water immediately prior to use.

12. Thirty minutes later the blue colour was read using a spectrophotometer at 725 nm.

Results

The reading for the reagent blank was substracted from all readings.

The readings for each unincubated sample was subtracted from that for the incubated sample.

The calculation of tyrosine released from incubation of BSA alone was subtracted from each sample value. From the tyrosine standards a factor was calculated to convert all the readings to umoles of tyrosine. This factor was used to convert all readings to umoles of tyrosine released on incubation due to action of activated pepsinogen in 0.125 ml plasma in 24 hours. The result was converted to umoles of tyrosine released by 1,000 ml of plasma per minute - International Units (I.U.).

Abomasal cannulae

The abomasal cannulae used in these experiments were made of a lightweight Teflon material. The design and dimensions are shown in Figures 1 and 2. The essential part was the barrel

which fitted into the fistula between the abomasal lumen and the skin. The barrel was threaded and it had an internal diameter of 13 mm and length of 55 mm.

An internal flange was permanently attached to the barrel and lay within the lumen of the abomasum. This was concave towards the abomasal lumen. The peritoneal flange lay between the perioneal surfaces of the viscera and the abdominal wall. This had large holes which allowed fibrous tissue to grow through and hold the cannula in place.

The external flange fitted onto the barrel externally against the skin to stop the external end of the barrel from slipping into the abdominal cavity.

The barrel was closed with a cap.

Cannulation of the ovine abomasum

The animal was anaesthetised with sodium pentobarbitone administered intravenously (Sagatal, May and Baker Ltd.) at a dose of about 20 mg/kg bodyweight. Anaesthesia was maintained by incremental doses as required.

The cannula was inserted by a technique based on that described Hecker (1974).

The animal was placed on its left side and the operative area clipped and cleaned. A right, (paracostal) incision was made in the lower third of the abdomen and the abomasum located. The fundus and pyloris of the abomasum were exteriorised as far as was possible. The area surrounding the abomasum was packed with sterile swabs.

A horizontal incision was made into the abomasal wall on the greater curvature at the fundic/pyloric junction. The incision

was just large enough to allow entry of the internal flange of the cannula. The barrel of the cannula was placed close to one end of the incision. Inverting sutures were placed to close the incision using sterile cat gut ending at the barrel of the cannula. The sutures were continued around the barrel, inverting the abomasal wall around it. The peritoneal flange was screwed onto the barrel to hold the abomasal wall between the peritoneal and internal flange of the cannula. The barrel of the cannula was stuffed with swabs to prevent escape of contents. The abomasum and cannula were returned to the abdomen and the abomasum allowed to lie in its normal position. The cannula was exteriorised through a separate stab incision just large enough to allow the barrel to pass through. This second incision was generally anteroventral to the first. The external flange was then added to lie against the body wall. The original incision was closed in several layers using standard surgical techniques. The swabs were removed from the cannula and the barrel was stoppered using a screw cap.

Preparation of larval inoculum

The animals that were to provide the parasitic Ostertagia circumcincta larvae (L_3) were examined from day 21 post-infection for the presence of parasite eggs in the faeces. When a positive faecal egg count was obtained all faeces from the animal were collected by means of a bag suspended over and below the anus by a harness. Male animals were used in this procedure to avoid urine contamination of the faeces. The faeces were collected from the bag twice daily and divided into aliquots of approximately 100 grams. Each aliquot was placed in a glass jar

for incubation. Where the faeces were very soft, a small amount of vermiculite was added to make a firmer mass.

The faeces were incubated for ten days at 22°C to allow development of the eggs through first and second larval stages to the infective, third-stage larvae (L_3) of O. circumcincta. At the end of the incubation, warm water was added to each of the jars to cover the faeces. This was left for three hours to allow the faeces to break up and the larvae to migrate into the water.

The faecal mixture was passed through a coarse sieve to remove the large faecal matter. The filtrates from the same animal were then pooled. The resultant liquid was further filtered using a Buchner apparatus and two 20 cm milk filter papers (Maxa filters, A. McCaskie) which allowed further removal of faecal matter.

Once all the fluid was removed, the filter paper was placed on top of a Baerman apparatus. This consisted of a glass funnel sealed at the narrow end with rubber tubing and a clip. The apparatus was filled with warm water. The filter paper was floated on the top of the water supported by a 150 um metal sieve. The larvae emerge into the warm water and by gravity are collected at the bottom of the funnel leaving the remainder of the faecal contamination on the filter paper. A minimum of six hours was allowed for this process. The larvae were then run off into a collecting vessel. The number of larvae present was estimated by counting 40 aliquots of 0.025 ml, the collecting vessel was vigorously shaken prior to withdrawal of each aliquot to prevent larval clumping. The larval concentration was

adjusted to less than 30 per 0.025 ml to allow more accurate counting and when the larval concentration had been estimated, the volume required to make the required inoculum was calculated. This volume was pipetted into universal bottles and the total volume made up to 20 ml with water, which was used to dose animals *per os*.

Statistical methods

Generally, results were analysed for statistically significant differences using the Student's t-test for unpaired data. The levels of significance are designated,

P (probability)	< 0.05	*
P	< 0.01	**
P	< 0.001	***

Data obtained from abomasal parasite counts were treated differently. Analysis of variance was carried out where there were more than two groups. With two groups, t-tests for populations with unequal variances were carried out after transformation of the data to logarithms.

The *in vitro* studies into benzimidazole resistant strains of *O. circumcincta* were analysed using probit transformation to estimate the ED_{50} (the dose of drug required to affect 50% of the individuals under test).

The proportion of individuals affected are converted into probit values, which are plotted against the log dose of the drug. The best straight line is calculated by using the method of maximum likelihood to weight the results. In this, the doses that give responses close to 50% are considered more important deterimants of the ED_{50} than those producing 0 or 100% response.

The method of least squares was then applied to the weighted values to produce a straight line. The dose of drug corresponding to a probit of 5 was then found, the ED_{50}.

FIGURE 1

The abomasal cannula used in these experiments

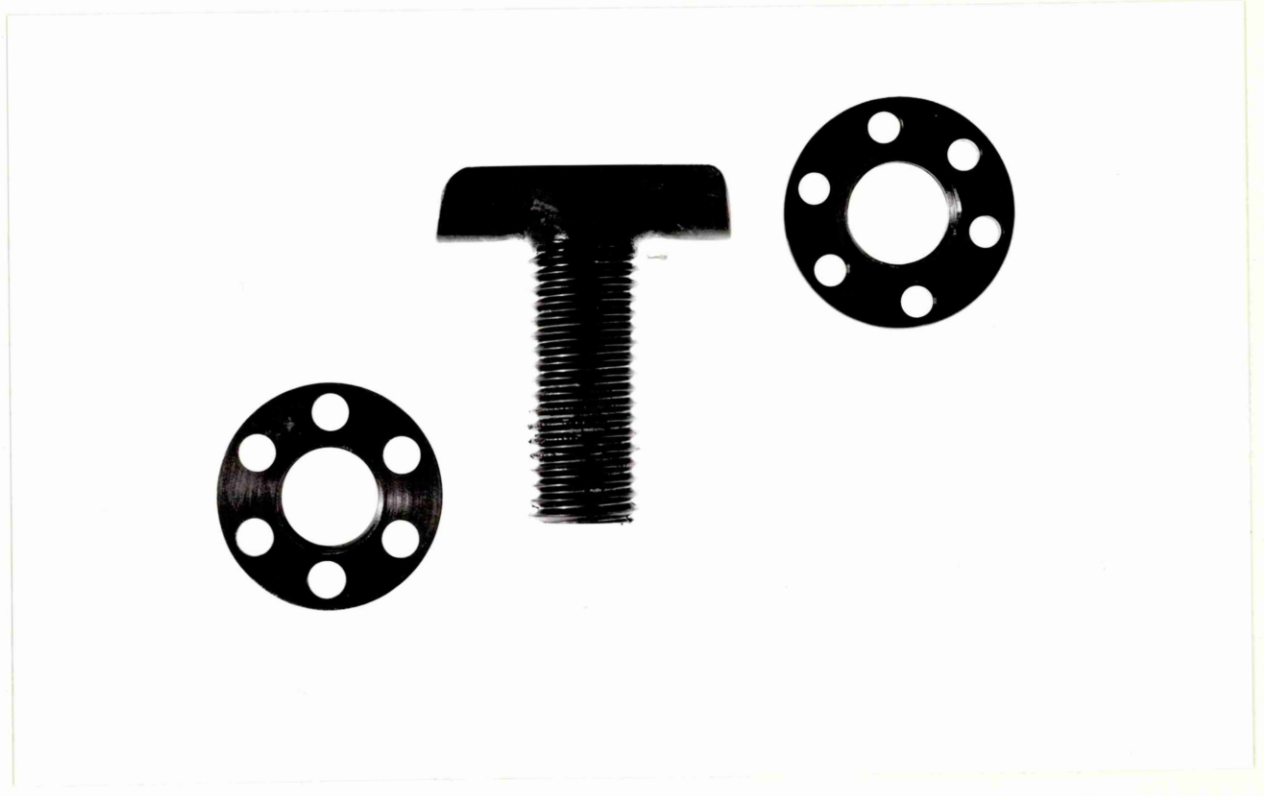
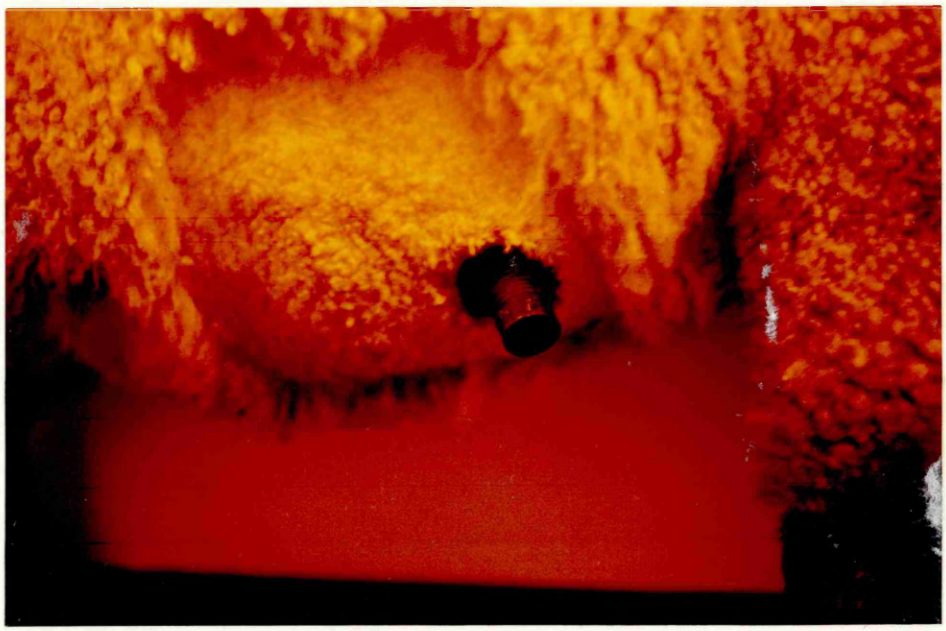


FIGURE 2

The abomasal cannula *in situ*



STUDIES INTO THE EFFECT OF A NON-STEROIDAL ANTI-INFLAMMATORY DRUG, MECLOFENAMIC ACID, ON THE RESPONSES OF SHEEP TO *Ostertagia circumcincta*

INTRODUCTION

Pepsinogen

Pepsinogen is produced by the zymogen cells within the gastric glands of the acid-producing stomach. These cells synthesise, store and secrete pepsinogen (Ito, 1981).

In histological sections, the basal cytoplasm stains strongly with basic dyes, indicating cytoplasm rich in rough endoplasmic reticulum (RER).

At the cells' luminal surface there are a few microvilli and the cell membrane has a thin glycoprotein coat. The lateral cell membranes are relatively smooth and are joined to adjacent cells at their apices by typical junctional processes. These consist of two areas, the tight junction (*zonula occludens*) in which the outer dense laminae of the adjacent cell membranes are fused and the intermediate junction (*zonula adherens*) where the membranes are separate. The intercellular space is filled with mucosubstance that acts as an adhesive. Adjacent cytoplasmic condensations contain numerous intermediate filaments which are anchored to the membrane by electron dense plaques (Banks, 1986a).

Desmosomes (*macula adherens*) are also present. These do not encircle the cell, but are scattered at discrete locations. These are similar to the intermediate junctions. The

intercellular space is filled with a sialic-acid rich material.

Gap junctions are also involved in joining the adjacent cells. These are like tight junctions but the small intercellular space present contains an orderly array of hexagonal subunits, which consist of proteins that extend through the cell membrane and are in contact with each other. These junctions act as diffusion barriers from the gland lumen to the blood vessels, facilitate diffusion between component epithelial cells and permit intercellular communication.

Langley in 1881 described the zymogen cell as the site of pepsinogen sythesis which is then stored in granules prior to release. This has remained as the basis of our understanding of the zymogen cell.

Many different types of pepsinogen have been isolated (Samloff, 1971). These, when released, are acted upon by acid and by pepsin itself to produce its active form, pepsin. This is the result of removal of a number of amino acid residues from the pepsinogen; some of the various peptides formed in this process can act as pepsin inhibitors.

Pepsin is formed within the gland lumen and acts within the stomach as a protease. The definition of pepsin is a protease that exists as an inactive stable zymogen and is formed in the presence of acid. It clots milk. It is active at acid pH and inactive at neutral or slightly alkaline pH.

The pepsinogen is released from the cell by exocytosis. A wide variety of agents have been shown to stimulate pepsinogen secretions (Hersey, Norris and Gilbert, 1984). Cholinergic

agents stimulate pepsinogen secretion, an action that is specifically inhibited by atropine indicating a muscarinic receptor mechanism.

Histamine stimulates secretion, although in some species it has only a weak effect (Hirschowitz and Sachs, 1969). B-adrenergic agonists, cholecystokinin-octapeptide (CCK-OP), gastrin, insulin and secretin can all stimulate pepsinogen release (Koelz et al, 1982; Hersey, May and Schyberg, 1983; Kasbekar, Jensen and Gardner, 1983; Raufman et al, 1983). Gastrin stimulates pepsin output in a number of species. It is thought that these all act via cyclic adenosine monophosphate (cAMP) or calcium ions (Ca^{2+}), these being secondary mediators (Raufman et al, 1983; Hersey, Norris and Gilbert, 1984). Therefore, the zymogen cell appears to possess a multitude of receptors, adrenergic, cholinergic and two types of peptide receptors (secretin and CCK-like). Prostaglandins can also inhibit pepsinogen output in response to histamine.

The association between plasma pepsinogen activity and parasitism

Pepsinogen has, for a long time, been recognised as present in the blood, the source being the gastric zymogen cells (Samloff, 1971). It is uncertain whether, in normal circumstances, it enters through endocrine release from the cells directly into the blood stream, or from degenerating cells.

In ostertagiasis in cattle and sheep a rise in plasma pepsinogen activity has been associated with the emergence of the young adults from the gastric glands (Anderson et al, 1964; Armour, Jarrett and Jennings, 1966; Jennings et al, 1967). This

was thought to be associated with structural damage and loss of the junctional processes joining adjacent cells, leading to an increased permeability of the gastric mucosa (Anderson et al, 1965). This would result in pepsinogen, present in the gastric lumen in greater concentration than normal due to the elevated abomasal pH, passing into the blood and plasma proteins from the blood, into the gastrointestinal tract. Mulligan, Dalton and Anderson (1963) showed that associated with the hypoalbuminaemia, there was a shortened half life of plasma albumin during ostertagiasis and, using radioactive labelling that this was associated with increased loss into the gut.

Holmes and MacLean (1971) also showed evidence of a plasma leak and increased albumin catabolism, but these were in decline 14 - 22 days post infection of sheep with O. circumcincta while the plasma pepsinogen remained elevated. They thought this could be due to a combination of a long biological half life of ovine pepsinogen, in addition to continuing rises in pepsinogen caused by developing inhibited larvae.

Murray (1969) and Murray, Jennings and Armour (1970) showed by electron microscopy, that the intercellular spaces in animals parasitised with Ostertagia spp were dilated and filled with electron-opaque material, probably protein.

In Nippostrongylus brasiliensis infestation of rats, there is also an increase in permeability of the intestinal wall which has been visualised by electron microscopy. This increase coincides with increased mast cell numbers and it is postulated that mediators released by these cells contribute to the

permeability (Murray, Jarrett and Jennings, 1971).

However, while plasma pepsinogen activity in young animals is found to correlate well with the number of parasites present, in older animals it is more variable (Mylrea and Hotson, 1969). In one study it was found that plasma pepsinogen tended to increase with age.

Anderson (1972; 1973) found that in older animals, the plasma pepsinogen activities rose associated with increased numbers of parasite larvae ingested but few established to cause damage. It was suggested that this could be due to a hypersensitivity reaction causing an increase in mucosal permeability. This was also discussed by Armour et al (1979) and Yakoob, Holmes and Armour (1983).

Stringfellow and Madden (1979) failed to demonstrate an increase in mucosal permeability in ostertagiasis using the marker horseradish peroxidase. They found that zymogen cells were denuded of pepsinogen granules and they concluded that their results indicated the high pepsinogen in plasma was due to it being retained in the circulation. Also that the zymogen cells released pepsinogen directly into the circulation rather than it passing from the gastric contents through the damaged mucosa. McKellar et al (1986; 1987) have also shown rises in plasma pepsinogen activities in calves transplanted with adult O. ostertagi. A transfer of a mainly adult population of Ostertagia spp into sheep also produced a pepsinogen rise (Anderson, Hansky and Titchen, 1985). Therefore, it would seem that the increased permeability of the gastric mucosa caused by

larval damage is not the only mechanism for causing the rise in plasma pepsinogen activities during ostertagiasis. It is possible that adult parasites themselves cause damage by moving in and out of the mucosa. It has been shown that the females of a related parasite O. leptospicularis lay eggs in gastric glands and therefore proving their capacity for mucosal damage (Al Saqur et al, 1980). However, there has not been any evidence for this occurring in O. circumcincta or O. ostertagi infections.

Alternatively, the adult parasites could affect the macromolecular leak, possibly mediated by their execretory/secretory products. It is possible that abomasal pH is similarly directly affected by the adult parasites (Eiler et al, 1981).

It has also been suggested that the zymogen cells could be stimulated by the adults to secrete pepsinogen directly into the circulation (McKellar et al, 1986; 1987).

Prostaglandins or other mediators could also be involved in these effects.

Eicosanoids

Eicosanoids are a group of substances that are formed from their precursor, arachidonic acid. They include prostaglandins, prostacyclin, leukotrienes and thromboxanes (Moncada, Flower and Vane, 1985). Of these compounds, prostaglandins have been the subject of many studies. Leukotrienes and thromboxanes were discovered later and much work on their actions has yet to be done.

Prostaglandins are among the most prevalent of autacoids and have been detected in almost every tissue and body fluid, except erythrocytes. They are produced as a result of a wide variety of stimuli, and cause, in minute amounts, a wide variety of effects.

Arachidonic acid is found as a component of the phospholipids of cell membranes, or in ester linkage in other complex lipids. The concentration of free arachidonic acid is low and the biosynthesis of eicosanoids depends on its release by phospholipases. Prostaglandins are not stored in cells (Piper and Vane, 1971) and therefore stimuli must influence their biosynthesis probably via the phospholipases. It would seem that an increase in intracellular calcium concentration (Ca^{2+}) and its action in combination with calmodulin acts as the secondary messenger within the cell and stimulates arachidonic acid metabolism.

Prostaglandins can be divided into several main classes, designated by letters. The main classes are then subdivided in accordance with the number of double bonds on their side chains. They are all 20-carbon unsaturated carboxylic acids with a cyclopentane ring.

Synthesis begins by the action of cyclo-oxygenase enzyme to form prostaglandin G_2 (PGG_2) and then prostaglandin H_2 (PGH_2). Both of these compounds are chemically unstable (see Figure 3). These are then isomerised enzymatically or non-enzymatically into different products PGE_2, PGF_2 or PGD_2 (Flower, 1978; Moncada, Flower and Vane, 1985). Prostaglandins A, B and C, which arise from the corresponding PGE are formed chemically during

extraction, probably none occurs biologically.

PGH_2 can also be metabolised into thromboxane A_2 (TxA_2) with the action of the enzyme thromboxane synthetase. This compound is unstable and breaks down non-enzymatically to the stable thromboxane TxB_2 (Hamberg, Svensson and Samuelsson, 1975). PGH_2 can also be used to produce the unstable compound prostacyclin (PGI_2) by action of the enzyme prostacyclin synthetase. PGI_2 has a double ring structure and is hydrolysed non-enzymatically to the stable compound 6-keto prostaglandin F1α.

The first step in the formation of the leukotrienes is the formation of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) by the action of the enzyme 5-lipoxygenase. 5-HPETE can be converted to 5-HETE (5-hydroxyeicosatetraenoic acid) or to leukotriene A_4 (LTA_4). Leukotriene A_4 can be converted to LTB_4 or LTC_4. LTD_4, LTE_4 and LTF_4 can all be formed stepwise from LTC_4.

A mixture of leukotrienes, containing LTC_4 and LTD_4 and LTE_4 make up the substance known as 'slow reacting substance of anaphylaxis' (Morris et al, 1980; Lewis and Austen, 1981).

The wide variety of effects caused by prostaglandins seem to be mediated by causing a rise of cyclic adenosine monophosphate (cAMP) within the affected cells (Bourne, 1974; Moncada, Flower and Vane, 1985). There appears to be strong evidence for the existence of specific membrane-bound receptors for the eicosanoids in many tissues.

Efficient mechanisms exist for the inactivation of most prostaglandins. This is important, as such potent, vasoactive compounds as these could cause widespread effects. The lungs

play an important role in the inactivation (Flower, 1978).

As previously stated, prostaglandins have a wide variety of effects, different prostaglandins often having opposite effects to others. Many articles have reviewed their actions (Higgs et al, 1981; Higgins, 1985) and those known for leukotrienes (Piper, 1983). Their most important actions are summarised below. Their effects on smooth muscle are discussed elsewhere.

Cardiovascular system - most prostaglandins cause vasodilation, but some, at selected sites cause vasoconstriction. Thromboxane A_2 (TxA_2) appears to be a powerful vasoconstrictor (Hamberg, Svensson and Samuelsson, 1975). Leukotrienes seem to act on microvasulature, to cause plasma exudation (Dahlén et al, 1981). They are also vasoconstricting (Piper, 1983).

Blood - Prostaglandins and prostacyclin exert powerful actions on platelets. Generally they inhibit aggregation, but prostaglandin PGE_2 exerts variable effects. TxA_2 induces platelet aggregation (Hamberg, Svensson and Samuelsson, 1975). Leukotriene B_4 (LTB_4) is a very potent chemotactic for polymorphonuclear leukocytes. 5-HETE and prostaglandin E_1 (PGE_1) are also (Higgs, McCall and Youlten, 1975; Goetzl and Sun, 1979; Palmer et al, 1980; Piper, 1983).

Gastrointestinal tract - Prostaglandins generally inhibit gastric acid secretion and stimulate bicarbonate secretion from the gastric mucosa. They also have effects on mucus secretion and are involved in maintaining blood flow to the stomach. It is thought leukotrienes can also affect mucus production. This is discussed in more detail elsewhere.

Prostaglandins also have effects on the kidney, afferent nerves and pain, the endocrine system, possibly the central nervous system and have metabolic effects.

Role of the eicosanoids in inflammatory and immune responses

Prostaglandins contribute importantly to the genesis and symptoms of inflammation (Moncada, Ferreira and Vane, 1978; Larsen and Henson, 1983).

They are released when tissues are mechanically or chemically stimulated. Initially the source of the prostaglandins is the injured tissue, then it could be augmented by polymorphonuclear leukocytes which produce prostaglandins during phagocytosis. Macrophages also could add to the prostaglandin concentration (Higgs, McCall and Youlten, 1975; Higgs et al, 1981). Prostaglandins, particularly PGE_2 have been detected in inflammatory lesions in sheep (Greenwood and Kerry, 1979).

Although prostaglandins do not appear to have direct effects on vascular permeability, both PGE_2 and PGI_2 markedly enhance oedema formation and leukocyte infiltration by promoting blood flow into the inflamed area (Williams and Morley, 1973; Williams and Peck, 1977).

5-HPETE and 5-HETE may be required for histamine release from basophils and may aid secretion of histamine and other mediators from mast cells.

But, PGEs suppress the secretion of mediators of inflammation by mast cells in anaphylactic reactions and inhibit participation of lymphocytes in delayed hypersensitivity

reactions. They also inhibit the release of hydrolases and lysosomal enzymes from human neutrophils and mouse peritoneal macrophages. Prostacyclin appears to inhibit leukocyte adherence to blood vessel walls.

Prostaglandins are also involved in producing the pain and hyperalgesia of inflammation. They may also have a role in the production of fever (Moncada, Ferreira and Vane, 1978; Feldberg and Milton, 1978).

Leukotrienes B_4, C_4 and D_4 all increase vascular permeability. LTB_4 can increase the adherence and margination of polymorphs (Dahlén et al, 1981). It also elicts release of B-glucuronidase and lysozyme from neutrophils.

Prostaglandins are also thought to play a role in the control of the immune system (Bourne, 1974; Pelus and Strausser, 1977). In summary, prostaglandin E_1, is thought to influence the functions of B lymphocytes. Humoral antibody response is decreased by PGE_1. Prostaglandins also affect T lymphocytes, particularly T killer cells, in inhibiting their action. PGE is also active in inhibiting production and release of lymphokines by sensitised T cells.

Inhibitors of eicosanoid production

There are a number of enzymes essential to eicosanoid production that drugs can act upon.

Phospholipase inhibitors

It is thought that glucocorticoid preparations act here and prevent the liberation of arachidonic acid. This is discussed in a later section.

Cyclo-oxygenase inhibitors

The non-steroidal anti-inflammatory drugs act here and prevent the formation of prostaglandins (Ferreira, Moncada and Vane, 1971; Smith and Willis, 1971; Vane, 1971; Higgs et al, 1981). The formation of leukotrienes is unaffected. It is thought that some pro-inflammatory effects caused by these drugs, for example, increased leukocyte migration, may be due to increased leukotriene formation, by increasing the amount of arachidonic acid available for lipoxygenases (Adams et al, 1977; Higgs et al, 1980; Morris et al, 1980; Myers and Siegel, 1983; Piper, 1983). This effect is abolished at higher doses of the anti-inflammatory drug.

Examples of cyclo-oxygenase inhibitors are aspirin, phenylbutazone, indomethacin and meclofenamic acid.

All these show similar toxicity signs associated with gastrointestinal damage and ulceration. There is evidence that some cyclo-oxygenase inhibitors are selective in inhibiting cyclo-oxygenase from different tissues. For example, sodium salicylate and the dual inhibitor BW755C have been shown to inhibit prostaglandin production generated by inflammation, but not to affect mucosal cyclo-oxygenase (Whittle et al, 1980).

Dual cyclo-oxygenase and lipoxygenase inhibitors

A number of pyrazoline analogues have been reported to inhibit both enzymes and therefore block prostaglandin and leukotriene production.

All of these substances are still at the experimental stage. BW755C and BW540 are examples. BW755C does not enhance leukocyte

migration at low doses like some selective cyclo-oxygenase inhibitors. It acts to decrease oedema, prostaglandin synthesis and leukocyte accumulation. Therefore the actions are similar to those of glucocorticoids, but do not share the same systemic side effects (Higgs, Flower and Vane, 1979). In most experimental models, cyclo-oxygenase inhibitors enhance antigen induced responses. Corticosteroids and these dual inhibitors do not. Therefore this is evidence that lipoxygenase activity is involved both in the generation of anaphylactic mediators and the sensitisatation of smooth muscle to their contractile actions.

Thromboxane synthetase inhibitors

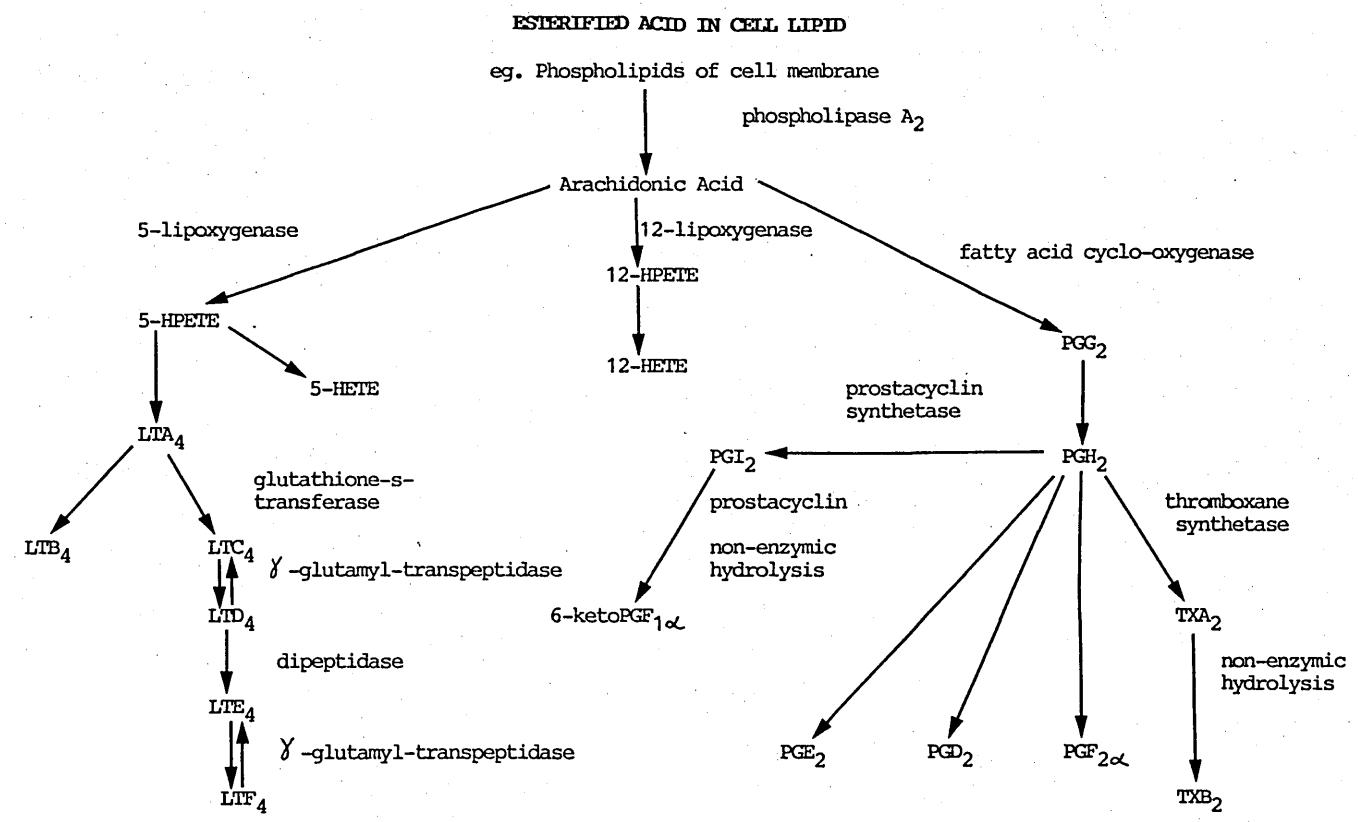
There is currently interest in analogues of imidazole which appear to inhibit thromboxane synthetase preferentially. One example of these is dazoxiben (Patrignani et al, 1984).

Lipoxygenase inhibitors

Work is still in the early stages with these inhibitors. FPL 55712 has been shown to be a selective lipoxygenase inhibitor (Augstein et al, 1973; Morganroth et al, 1984). Other products, piriprost, benoxaprofen and nordihydroguaiaretic acid (NDGA) have been investigated and found to have similar actions (Morris et al, 1980; Burka, 1985).

FIGURE 3

Biosynthetic pathway of the eicosanoids



List of abbreviations

HPETE	12-L-hydroperoxy-5,8,10, 14-eicosatetraenoic acid	LT	leukotriene	TX	thromboxane
		PG	prostaglandin		
HETE	12-L-hydroxy-5,8,10,14-eicosatetraenoic acid				

Meclofenamic Acid (N-(2,6,dichloro-m-tolyl) anthranilic acid)

Meclofenamic acid is a non-steroidal anti-inflammatory drug and a derivative of N-phenylanthranilic acid. Other members of this group, the fenamates, are mefenamic, flufenamic, tolfenamic and etofenamic acids (Flower, Moncada and Vane, 1985). They possess analgesic, antipyretic and anti-inflammatory properties. The structure of meclofenamic acid is shown in Figure 4.

It is marketed in Britain for the oral treatment of acute or chronic inflammatory conditions involving the musculo-skeletal system of the horse (Arquel granules, Parke Davis). It's pharmacokinetics have been studied in this species (Snow, Baxter and Whiting, 1981). It is not widely used in human medicine because of its frequent side effects, particularly diarrhoea, which can be severe (Flower, Moncada and Vane, 1985).

Activity

Like other non-steroidal, anti-inflammatory drugs, meclofenamic acid acts on the enzyme cyclo-oxygenase, which is necessary for the synthesis of prostaglandins from their precursor, arachidonic acid (Vane, 1971; Ferreira and Vane, 1979; Higgs et al, 1981). It is thought that it inhibits this enzyme by binding to the active site as it's aromatic moieties are similar to the polyene system in arachidonic acid (Shen, 1979). Meclofenamic acid is thought to bind irreversibly and therefore new enzymes have to be generated before further prostaglandins can be formed (Lees and Higgins, 1985).

Cyclo-oxygenase products synergise with other inflammatory mediators, therefore their removal reduces the effectiveness of

substances such as histamine. Prostaglandin production is not normally elevated unless there is tissue damage and this explains why cyclo-oxygenase inhibitors are not analgesic or antipyretic in the absence of inflammation (Higgs et al, 1981).

In addition, the fenamates have been found to be prostaglandin antagonists, as they interact with prostaglandin receptors (Collier and Sweatman, 1968).

Like other non-steroidal anti-inflammatory drugs, the fenamates are acidic and this is thought to aid their accumulation in inflamed tissue (Snow, 1983). Meclofenamic acid is also highly protein bound in plasma (Snow, Baxter and Whiting, 1981). It is probable, because of its high protein binding characteristics, that it remains in tissues after plasma concentrations are undetectable (Snow, 1983).

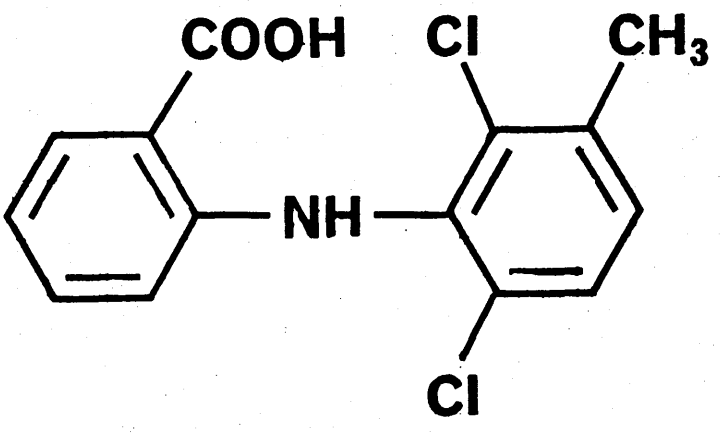
Snow, Baxter and Whiting (1981) have shown that unlike another non-steroidal drug, phenylbutazone, similar absorption in fasted and non-fasted ponies was obtained after oral administration of meclofenamic acid.

Toxicity

High doses have been shown to cause ulceration of the gastrointestinal tract and renal toxicity (Kaump, 1966). Ponies on low doses were also shown to produce a protein-losing gastroenteropathy but this was less marked than that seen with phenylbutazone. There has been some doubt expressed as to whether the plasma protein drop is attributable to the lesions in the gastrointestinal tract (Snow et al, 1983). Administration at the recommended dose rate for ten days was shown to cause a drop

FIGURE 4

Structure of meclofenamic acid


COOH
Cl
CH3
NH
Cl

in the total protein concentration of plasma, both albumin and globulin being reduced (P. Lees and W.E. Allen, cited Lees and Higgins, 1985).

Prostacyclin is produced in the gastric and intestinal mucosa where it has a vasodilator role which maintains local blood flow and prevents the development of ischaemic areas. The inhibition of cyclo-oxygenase activity in the gastric mucosa is closely related to ulcerogenicity (Whittle et al, 1980). A direct topical irritant action could also be involved after oral administration of the drug.

The effects caused by adult stages of Ostertagia spp on their host

Transplantation of adult parasites directly into the abomasum has been used by a small number of workers to study the effects caused directly by adult Ostertagia without the complication of larval development within the mucosa.

Anderson, Hansky and Titchen (1985) transferred a mainly adult population of Ostertagia spp into previously parasite-naive sheep. Large numbers of parasites were transferred. Rises in abomasal pH, plasma pepsinogen and plasma gastrin concentrations were observed. The inhibited fourth stage larvae generally remained inhibited after transfer.

Obviously the fact that larval stages were transferred could have damaged the mucosa in the same way as a larval infection to produce the changes observed.

However, studies by McKellar and colleagues (1986; 1987) in which adult populations of Ostertagia ostertagia were transferred into calves proved that a rise in plasma pepsinogen was a feature

of the adult parasite infection. Calves that had previously been infected with larval parasites also showed a similar rise. A rise in plasma gastrin concentrations also occurred in animals receiving the largest number of parasites. There was no significant change in abomasal pH however. This difference from the work of Anderson, Hansky and Titchen (1985) could be due to the fact that fewer parasites were transferred and a threshold number of adults are necessary to cause the pH rise, or to the fact that no larval stages were present.

MATERIALS AND METHODS

Animals

The adult sheep used in these experiments were Scottish Blackface ewes aged greater than five years. Their weights ranged between 50 - 60 kg. All had been reared and maintained since birth on a hill farm known to be infected with Ostertagia circumcincta (Q. McKellar, personal communication). They were therefore considered to be immune to O. circumcincta.

In contrast, the lambs used in these investigations had been born and reared indoors in conditions to exclude exposure to O. circumcincta. Female and castrated male lambs, aged approximately five months, were used. Their weights ranged between 20 - 30 kg. at the start of the experiment.

The lambs in the final experiment of this section were surgically prepared with abomasal cannulae four weeks prior to the start of the experiment.

Administration of O. circumcincta larvae (L_3)

For a period of nine weeks prior to the start of the second experiment, using adult immune sheep, each animal was dosed with 2,000 O. circumcincta L_3 once weekly, while also grazing contaminated pasture. This was carried out in order to ensure immunity to O. circumcincta. The animals were treated with an anthelmintic to remove any remaining gastrointestinal parasites one week before the start of the experiment. The adult sheep used in the preliminary study did not undergo this regime of larval administration.

The lambs in these studies remained parasite naive until the start of the experiment.

On day 0 of each experiment, the adult sheep were challenged with a single dose of 200,000 O. circumcincta L_3, the lambs with a single dose of 75,000 O. circumcincta L_3.

Administration of meclofenamic acid

Meclofenamic acid (Arquel granules, Parke-Davis) was administered orally as a slurry made up in 20 ml of water. The adult sheep received 500 mg of meclofenamic acid daily divided into two doses given at 9 a.m. and 5 p.m.

The lambs received 250 mg of meclofenamic acid daily, divided into two doses administered as above.

The average dose of meclofenamic acid was therefore 10 mg/kg bodyweight per day.

The administration of meclofenamic acid began one day prior to challenge with the O. circumcincta L_3 and continued, twice daily, throughout the experiment.

Sampling techniques

Abomasal fluid

The cannulated lambs in the final experiment of this section had samples of abomasal fluid taken every second day throughout the experiment. The animal was restrained in a standing position and the sample collected into a plastic beaker by allowing the normal passage of ingesta to push the abomasal contents through the cannula. The first portion of the sample was discarded as not representative, it was assumed to have been present in the barrel of the cannula for a period of time.

The abomasal pH was measured immediately after collection using a upH sensor (Whatman Labsales Ltd.). The sample was then centrifuged at 1,000 x g for ten minutes. The supernatant fluid was collected and stored at -20^{o}C for estimation of meclofenamate concentration.

Blood samples

Blood samples were obtained from the jugular vein using a 20 gauge 1" needle into heparinised syringes (Monovettes, Sarstedt). The blood was centrifuged at 1,000 x g for ten minutes. The plasma supernatant was removed and stored at -20^{o}C until assayed for plasma pepsinogen activity and meclofenamate concentration.

Faecal samples

Faecal samples were obtained per rectum. They were examined for the presence of parasite eggs using a modified McMaster technique.

Necropsy

Procedure at necropsy

All the animals were killed by intravenous administration of sodium pentobarbitone (Pentobarbitone Forte, Veterinary Drug Co. PLC) as an overdose. The lambs that were acting as donors of adult O. circumcincta were starved for one day prior to death to reduce the volume of the abomasal contents.

Sampling techniques at necropsy

Abomasal contents

Abomasal contents were collected at necropsy. The pH of the contents was measured using a upH sensor. The contents were then processed for estimation of parasite numbers as described previously.

In the experiment where lambs acted as donors of adult O. circumcincta, the above procedure was modified because of the volume of abomasal contents required to be transferred into recipient lambs.

In this case, the abomasal contents were collected from each lamb and the pH measured. The volume of contents of each abomasum was made up to two litres with water. This was well mixed prior to taking a 100 ml sample for the estimation of parasite numbers. 10 ml of 40% formalin was added to the 100 ml sample to preserve it until this was carried out.

The abomasal contents from group 1 lambs were pooled together, mixed well and divided equally between four 1,000 ml glass measuring cylinders. The contents were then allowed to settle.

The same procedure was carried out with the abomasal contents from the lambs of group 2.

Abomasal mucosa

The abomasal mucosa was collected and processed for estimation of parasite numbers as described previously.

Transfer of abomasal contents containing adult O. circumcincta

The supernatant was carefully poured from each cylinder and the residue transferred into each recipient lamb via the abomasal cannula. The recipients were restrained in standing position and a plastic tube was passed through the cannula into the abomasum. A funnel was attached to the other end of the tube and the abomasal contents containing the adult O. circumcincta were transferred into the recipient abomasum. The cylinder, funnel and tube were washed with phosphate buffered saline (PBS) to maximise the numbers of parasites entering the abomasum.

Assay to determine meclofenamic acid concentration in plasma and abomasal fluid

The method of Marriner and Bogan (1979) was used to measure the concentration of free drug in plasma and abomasal fluid. To 1 ml of plasma or abomasal fluid supernatant in a 15 ml stoppered test tube, 6 ml 0.25 M sodium chloride, 0.3 ml hydrochloric acid (1 M) and 5 ml carbon tetrachloride were added.

This was shaken on a rotary mixer for ten minutes and centrifuged for ten minutes at 1500 x g.

3 ml of the lower, carbon tetrachloride layer was removed to a 10 ml stoppered test tube. Four drops of trichloroacetic acid solution (5% w/v in carbon tetrachloride) were added and mixed

rapidly. The fluorescence (excitation 360 nm/emission 425 nm) was measured exactly ten minutes after the addition of the trichloroacetic acid using a spectrophotometer (Perkin-Elmer 2000). Solutions of sodium meclofenamate were made up. Six standards and blank plasma or abomasal fluid were added to each assay. Unknown samples were read from the calibration curve obtained from these standards. The results were converted to concentration of free drug i.e. meclofenamate.

EXPERIMENT 1

Investigation into the effect of meclofenamic acid on the responses of adult immune sheep to a single dose challenge of *O. circumcincta* L_3 - A preliminary study

Experimental design

The six adult sheep used in this experiment were divided into three groups of three, two and one sheep. The single sheep acted as a control that was treated with meclofenamic acid but was not challenged with *O. circumcincta* (L_3).

Group	Sheep Number	Procedure
1	96	Meclofenamic acid treatment (500 mg/animal/day)
	97	Challenged with 200,000 *O.circumcincta* (L_3)
	98	
2	47	Challenged with 200,000
	48	*O.circumcincta* (L_3)
3	38	Meclofenamic acid treatment (500 mg/animal/day)

The animals were not slaughtered at the end of this experiment.

Samples collected

Blood samples were taken daily throughout the experiment.

Results

Plasma concentrations of meclofenamate

The concentrations of meclofenamate in the plasma of sheep of groups 1 and 3 sheep are shown in Table 1.

Figure 5 shows the mean concentrations in group 1 sheep and the plasma concentration in the one sheep in group 3. As can be seen in Figure 5, the concentration of meclofenamate was greater than 1 ug/ml one day after the start of administration in group 1 sheep. The concentration remained between 1.0 to 1.6 ug/ml until day 7 of the experiment when it dropped to below 0.8 ug/ml. A small rise in concentration to 1 ug/ml occurred on day 12, and again on day 19 to 0.8 ug/ml.

The sheep that was treated with meclofenamic acid only, reached higher concentrations initially, greater than 2.0 ug/ml where it remained until day 9 of the experiment. The concentration of meclofenamate dropped sharply to below 0.5 ug/ml for the remainder of the experiment.

Plasma pepsinogen activity

The plasma pepsinogen activities measured throughout this experiment are shown in Table 2 the mean of each group shown in Figure 6. From Figure 6, it can be seen that after the challenge with 200,000 O. circumcincta (L_3) on day 0, the mean plasma pepsinogen activities of both groups of sheep rose. The maximum

concentration was 1.543 I.U. for the group receiving meclofenamic acid (group 1) and 1.915 I.U. for the untreated control group (group 2). Plasma pepsinogen activities then declined in both groups until day 11 of the experiment. The plasma pepsinogen of the group 1 sheep remained below 1.7 I.U., that of group 2 rose to reach a maximum of 4.115 I.U. on day 19 of the experiment.

The plasma pepsinogen activity shown by the sheep receiving meclofenamic acid only, was 1.9 I.U. at the start of the experiment. The activity fell to 1 I.U. and then below.

Discussion

Therefore, a difference was seen in the plasma pepsinogen activities of the two groups of sheep from day 10 of the experiment. Those animals that did not receive meclofenamic acid showed a rise in plasma pepsinogen activity probably indicating damage to the abomasal mucosa by the parasites that had established and were emerging as young adults from the gastric glands. Therefore the immunity of these sheep was called into question but as these animals were not slaughtered, no further steps could be undertaken to prove the establishment of parasites.

The lower plasma pepsinogen activity in the group of animals receiving meclofenamic acid could be due to two reasons:-

1. These animals were able to expel their parasitic challenge before entry into the gastric glands and subsequent damage to the abomasal mucosa.

2. The animals receiving meclofenamic acid were similar to the control group in that Ostertagia larvae were able to become established but the rise in plasma pepsinogen activity was blocked in some way by the meclofenamic acid.

The low plasma pepsinogen activities in the sheep receiving meclofenamic acid only, (Group 3) proved that administration of meclofenamic acid in sheep does not cause a significant rise in plasma pepsinogen by damage to the gastrointestinal tract, or by inhibiting prostaglandin production. Prostaglandins can have an inhibitory effect on pepsinogen secretion. The plasma pepsinogen activity of this sheep declined during the experiment. It is possible that loss of parasites and lack of parasite challenge indoors caused this.

The difference in the plasma concentration of meclofenamate at the start of the experiment compared to later, which was shown by every animal, may be due to weight increases in the sheep while remaining on the same dose of 500 mg meclofenamic acid per day. The purpose was to provide an adequate concentration of meclofenamic acid to be active as an anti-inflammatory agent and not to study the pharmacokinetics of this drug. An increased rate of metabolism of the meclofenamic acid by the animals could also be a reason for this decline in plasma concentration after seven to nine days of administration. This possibility is discussed later.

The experiment was therefore repeated in a larger number of sheep, which had been dosed for nine weeks previously with O. circumcincta larvae (L_3) in order to ensure immunity to this

parasite. The animals were slaughtered at the end of the experiment and abomasal parasite numbers estimated.

Investigation into the effect of meclofenamic acid on the responses of adult immune sheep to a single dose challenge of *O. circumcincta* L_3 - Study two

Experimental Design

Twelve adult sheep were used in this study. They were divided into three groups of four sheep.

Group	Sheep Number	Procedure
1	41 46 102 120	Meclofenamic acid treatment (500 mg/animal/day) Challenged with 200,000 *O. circumcincta* (L_3)
2	47 113 124 7496	Challenged with 200,000 *O. circumcincta* (L_3)
3	48 96 98 103	Meclofenamic acid treatment (500 mg/animal/day)

Animals of groups 1 and 2 were killed at the end of the experiment to estimate abomasal parasite numbers.

Samples collected

Blood samples

Blood samples were collected every three days throughout the experiment.

Faecal samples

Faecal samples were taken prior to the start of the experiment, day 12 and day 14 of the experiment, then every three days.

Necropsy

The animals were killed and abomasal samples taken as described previously.

Results

Faecal egg counts

The results are shown in Table 3.

Very few eggs were detected in the faeces of any of the sheep and no difference was detected between the groups.

Plasma concentrations of meclofenamate

The concentrations of meclofenamate in the plasma of sheep in groups 1 and 3 are shown in Table 4 and the mean of each group in Figure 7.

The mean concentrations of each group remained around 0.4 ug/ml of plasma during the experiment, the group that received meclofenamic acid only (group 3) having a slightly higher mean concentration. The high initial concentrations seen in the first study were not seen here. It was unlikely that the longer intervals between sampling allowed the high concentrations of meclofenamate to be missed, as on day 2 of the previous study, concentrations of meclofenamate were above 1 ug/ml of plasma.

Plasma pepsinogen activity

The results are shown in Table 5, and the mean plasma pepsinogen activities of each group in Figure 8.

There was a rise in mean plasma pepsinogen activity in both groups 1 and 2 after challenge. This reached a maximum on day 5 of the experiment (2.402 I.U. in group 1, 2.128 I.U. in group 2). Thereafter, plasma pepsinogen activity declined below 1.5 I.U. by day 11 in both groups. This was similar to that found in the previous study, but pepsinogen activities reached were generally higher than here.

However, by day 23 of the experiment, the mean pepsinogen activity of group 2 sheep had declined to 1.224 I.U., in marked contrast to the previous study.

The mean pepsinogen activity of group 1 sheep increased slightly from day 14 to reach 1.604 I.U. by day 23. Group 3 sheep, who only received meclofenamic acid, had mean pepsinogen activities below 0.5 I.U. throughout the experiment as seen previously. Therefore meclofenamic acid did not contribute any rise in plasma pepsinogen activity when administered alone.

Abomasal pH at necropsy

The results are shown in Table 6.

All animals had an abomasal pH within the normal range except for two sheep. The high abomasal pH in sheep number 124 in group 2 may be explained by the blood contamination of the abomasal contents. Sheep number 120 of group 1 had an abomasal pH of 6.6 which was also above the normal range. This animal had the highest numbers of parasites at necropsy (15,800) which would have increased the abomasal pH.

Numbers of abomasal parasites

These results are shown in Table 7 and the percentage establishment of the larval challenge in Table 8.

There was a large within-group variation in those animals receiving meclofenamic acid and the untreated controls.

In group 1, sheep number 120 had 15,800 parasites, many more than the rest of the animals in that group.

The reason for the increased establishment of the larval challenge in this sheep is unknown.

In group 2, two sheep, numbers 47 and 7496 had higher numbers of parasites than the other two animals. Both these animals showed evidence of inhibited development of Ostertagia, particularly number 7496. Again, the reason for this is not know.

When considering the mean of each group, however, there would appear to be a large difference between groups. A mean of 4,350 parasites in the sheep receiving meclofenamic acid, 13,588 in the control sheep, but this difference is not statistically significant.

Discussion

The results obtained for plasma pepsinogen activity and meclofenamate concentration in plasma in the preliminary study were not reproduced in the second experiment with adult sheep.

The plasma pepsinogen response to the parasite challenge was very similar between treated and untreated groups in the second study. The peak in plasma pepsinogen activity early in the experiment probably represents a hypersensitive response to the

incoming larvae as discussed earlier. It would seem unlikely from this experiment that prostaglandins are involved in this response. The difference in plasma pepsinogen activities in the first study was probably due to differences in the host's ability to expel the challenge of parasites.

The high meclofenamate concentration seen at the beginning of the preliminary study was also not repeated. This was probably due to the fact that the sheep in the second study were in reasonable body condition and their weights were maintained throughout the experiment. Weight increases in the animals of the preliminary study probably contributed to the different pattern of plasma meclofenamate concentrations.

There did appear to be a reduction in the number of parasites established in the animals treated with meclofenamic acid, although there was a great deal of individual variation. The differences between groups were not statistically significant however. The different numbers of parasites established also did not affect mean plasma pepsinogen activities. However, those animals with the highest number of parasites at necropsy did have high plasma pepsinogen activities towards the end of the experiment. There did not seem to be a relationship between parasite establishment and the possible 'hypersensitive' rise in plasma pepsinogen activity early in the experiment.

Most of the animals in this experiment allowed less than 3% of the parasites to establish. This is considered normal for animals immune to O. circumcincta (Yakoob, Holmes and Armour, 1983). Very few parasite eggs were detected, even in those

animals in which larger numbers of parasites established.

The reduction in parasite numbers in meclofenamic acid treated animals would seem to indicate that the host environment was made less suitable for parasite establishment by its action on prostaglandin synthesis. This would be contrary to the finding by Kelly and Dineen (1976) that prostaglandins can damage worms *in vitro* and that they are involved in protection against *N. brasiliensis* in the rat. Here suppression of prostaglandins led to a reduced establishment.

However, it is also possible that meclofenamic acid exerted a direct effect on the parasites. An experiment subsequent to these studies investigated the effect *in vitro* of meclofenamic acid on *O. circumcincta* L_3 motility (Table 24). It was found that motility was inhibited by the presence of the drug. In contrast, another non-steroidal anti-inflammatory drug, phenylbutazone, did not affect larval motility *in vitro* (Table 25). The animal in the group receiving meclofenamic acid that harboured the largest number of parasites (number 120) generally showed the lowest plasma concentrations of the drug. Therefore it is probable that meclofenamic acid exerted a direct effect on the parasites, and caused the reduction in establishment.

EXPERIMENT 2

Investigation into the effect of meclofenamic acid on the responses of parasite-naive lambs to a single dose challenge of *Ostertagia circumcincta*

Experimental design

Eight parasite-naive lambs were used in this experiment and were divided into two groups.

Group	Animal number	Procedure
1	49 57 104 133	Meclofenamic acid treatment (250 mg/animal/day) Challenged with 75,000 *O.circumcincta* (L_3)
2	52 63 64 85	Challenged with 75,000 *O.circumcincta* (L_3)

The animals were killed at the end of the experiment and acted as donors of adult *O. circumcincta* into two subsequent groups of parasite-naive lambs.

Samples collected

Blood samples

Blood samples were collected prior to the start of the experiment and every three days until death on day 24 of the experiment.

Faecal samples

Faecal samples were taken prior to the start of the experiment, day 12 and day 14 of the experiment, then every three days.

Necropsy

The animals were killed on day 24 of the experiment and samples were taken as described previously, prior to the transfer of abomasal contents containing adult parasites into other lambs.

Results

Faecal egg counts

The results are shown in Table 9 and Figures 9 and 10. Only one animal in group 1 had parasite eggs in its faeces. In contrast, all of the animals in group 2 passed parasite eggs.

Therefore, on the days sampled, the animals receiving meclofenamic acid produced fewer parasite eggs than the control group of lambs.

Plasma concentrations of meclofenamate

These results are shown in Table 10 and the mean in Figure 11. There was a large variation in plasma meclofenamate concentrations within the group 1 lambs.

Animal number 49 had concentrations of meclofenamate below the level of detection (0.1 ug/ml) from day 5 to day 17 of the experiment. This animal had diarrhoea on day 5 of the experiment, but it improved without treatment within two days. Animal number 104 also produced diarrhoea on the fifth day of the experiment which improved without treatment. Subcutaneous oedema between the rami of the mandible (bottle-jaw) and diarrhoea appeared in this animal on day 19 of the experiment (day 20 of meclofenamic acid administration). Biochemical analysis of plasma samples showed reduced total protein, albumin and globulin concentrations. A protein-losing enteropathy was diagnosed,

probably due to meclofenamic acid toxicity. The daily dose of meclofenamic acid was reduced to half and this was administered once daily from day 19 of the experiment. The diarrhoea quickly disappeared by day 21 but the subcutaneous oedema improved more slowly, a little remaining at necropsy.

Animal number 57 showed signs of bottle jaw on day 20 but no diarrhoea developed. Meclofenamic acid was administered as usual.

Plasma pepsinogen activity

The results are shown in Table 11, and the mean of each group in Figure 12. The mean plasma pepsinogen activities of both groups rose rapidly from five days post challenge, both reaching maximum concentrations at day 14 post challenge.

From day 8, the mean plasma pepsinogen activity of the animals receiving meclofenamic acid rose more sharply than the control group, reaching a maximum of 4.722 I.U. as compared to 3.422 I.U. of the control group. The mean values then fell quickly to below 2 I.U. by day 20 of the experiment.

Abomasal pH at necropsy

The results are shown in Table 12. All of the lambs had an abomasal pH above the normal range; although animal number 49 of group 1 had a lower abomasal pH than the others.

Numbers of abomasal parasites

These results are shown in Table 13, and the percentage establishment of the larval challenge in Table 14.

There appeared to be larger numbers of parasites established in those lambs not receiving meclofenamic acid. A mean value of

15,313 parasites in group 2 lambs compared to 3,763 in group 1 lambs. But this was not statistically significant.

In the lambs receiving meclofenamic acid, the lamb that showed severe signs of meclofenamate toxicity, number 104, only had 100 parasites compared to counts of greater than 3,500 in the other animals.

In the group 2 lambs there was a wider range in parasite numbers, two animals, numbers 64 and 85 having counts greater than 25,000; in contrast, lambs numbers 52 and 63 had less than 5,000 O. circumcincta.

Therefore meclofenamic acid appeared to have an effect on the numbers of parasites established. Mean values of 5.02% establishment in those animals receiving meclofenamic acid compared to 20.42% in the control lambs.

Discussion

There appeared to be little difference in the plasma pepsinogen activities between treated and untreated lambs. The plasma pepsinogen response was different from that seen with adult animals under challenge. The rise in plasma pepsinogen occurred later and reached high activities than seen previously. This is typical of non-immune sheep and is associated with emergence of the parasites from the gastric glands. It is interesting to note that in young, non-immune animals it has been reported that plasma pepsinogen activities correlate well with numbers of parasites established (Mylrea and Hotson, 1969). This did not seem to occur in this experiment. The highest pepsinogen activities were seen in the group 1 treated lambs although they

harboured fewer parasites at necropsy. At the end of the experiment, the situation had reversed, so that the group with the larger number of parasites (group 2) had the higher pepsinogen activity. It would have been interesting to monitor these animals for longer during this infection, but it was important to obtain as many adult parasites for transfer as possible. The number of parasites would have gradually fallen after day 24 of the experiment.

The reduction in parasite numbers in treated sheep was again seen, probably due to the direct effect of meclofenamic acid on the parasites.

The high abomasal pH seen in the majority of animals would be caused by the parasite infection with loss of acid-producing cells and possible additional inhibition of acid secretion. The fact that animals in both groups showed this indicates that the meclofenamic acid was not the cause of the pH rise. Prostaglandins can have an inhibitory action on acid secretion, therefore inhibition of prostaglandin production by the meclofenamic acid could cause a rise in acid production. This did not occur here.

The toxicity seen in some of the animals was typical of that described by others (Snow et al, 1983; Lees and Higgins, 1985).

Interestingly, only one animal in the treated group passed parasite eggs in the faeces compared to all the animals of the untreated group, and this animal did not harbour the largest number of parasites within the group. It is possible that meclofenamic acid has an effect on the egg laying capacity of the

female parasites as it did not cause a preferential reduction in the number of female Ostertagia (Table 15). Too little information is available in this study to draw any conclusions on this subject.

Therefore it would seem unlikely, from this experiment, that prostaglandins play a role in the plasma pepsinogen rise in non-immune sheep challenged with O. circumcincta. Further evidence for a direct effect of meclofenamic acid on the parasites is also given.

EXPERIMENT 3

Investigation into the effect of meclofenamic acid on the responses of parasite-naive lambs, transplanted with adult Ostertagia circumcincta

Experimental design

Eight parasite-naive lambs were used in this experiment. All of the animals had abomasal cannulae fitted four weeks prior to the start of the experiment.

Group		Animal number	Procedure
3	A	1	
		51	Meclofenamic acid treatment (250 mg/animal/day)
	B	56	Transplanted with adult O.circumcincta
		93	
4	A	75	
		76	Transplanted with adult O.circumcincta
	B	79	
		86	

Groups 3B and 4B received adult O. circumcincta from donors treated with meclofenamic acid. All the animals were killed at

the end of the experiment.

Samples collected

Abomasal fluid samples

These were taken prior to, then every two days during the experiment.

Blood samples

Blood samples were taken prior to the start of the experiment and then every two days until slaughter.

Faecal samples

These were taken prior to the start, and every two days during the experiment.

Necropsy

The animals were killed 15 days after the transfer of the adult O. circumcincta.

Results

Numbers of adult O. circumcincta transferred

Table 16 shows the estimation of the number of adult parasites transferred to each of the lambs in groups 3 and 4.

Obviously, those animals receiving adult parasites from donors treated with meclofenamic acid received substantially fewer parasites than those from untreated donors, 3,450 parasites compared to 15,025.

Faecal egg counts

The results are shown in Table 17 and in Figures 13 and 14. All of the animals passed parasite eggs during the experiment. Parasite eggs were detected in the faeces of the majority of animals one day post transfer and continued until day 11 of the

experiment, after which the numbers of eggs declined.

In the group 3 lambs, very high numbers of eggs were detected, regardless of the origin and therefore the number of parasites transferred.

In the animals not receiving meclofenamic acid (group 4) egg counts were generally lower. The animals receiving adults from meclofenamic acid treated animals generally had the lowest number of parasite eggs.

Plasma concentration of meclofenamate

The results are shown in Table 18 and the mean in Figure 15. Meclofenamate concentrations were generally slightly higher than those in the previous experiment. There were no signs of meclofenamate toxicity.

Abomasal fluid concentration of meclofenamate

The results are shown in Table 19 and the mean concentration in Figure 15. Lower concentrations of meclofenamate were detected in abomasal fluid than in the plasma of these animals. Often the meclofenamate concentration was below the limit of detection. Meclofenamate was not detected in the abomasal fluid of animal number 56 throughout the experiment.

Abomasal pH

The abomasal pH measurements during the experiment are shown in Table 20 and the mean pH in Figure 16. The abomasal pH of the animals in both groups were very similar and remained around pH 3 throughout the experiment. There was a slight rise in abomasal pH after the transfer of adult parasites but this fell gradually throughout the experiment.

Plasma pepsinogen activity

The results are shown in Table 21 and the mean activity of the groups in Figure 17. Figure 18 shows the mean activity dividing the groups according to the origin of the adult parasites.

The mean plasma pepsinogen activity of the untreated animals (group 4) was higher than those receiving meclofenamic acid throughout the experiment. The animals that received untreated parasites and were themselves untreated (Group 4A), showed the highest plasma pepsinogen activities. The parasites that received meclofenamic acid either as larval stages or when adults produced lower plasma pepsinogen activities in their recipient sheep.

Numbers of abomasal parasites

The results are shown in Table 22, and the percentage establishment of the adults in Table 23. The mean number of parasites in the two groups were very similar (3,250 in group 3, 3,625 in group 4). When the origin of the parasites were taken into account and the different numbers transferred, it is obvious that those receiving adult parasites from meclofenamic acid treated donors had fewer parasites at necropsy. When the percentage establishment of the transferred adults is calculated however, there is a difference between those parasites from treated donors and transplanted into untreated sheep (Group 4B) compared to the rest. The establishment of these parasites was 24.64% compared to 42.60% in untreated recipients receiving

untreated parasites but due to the small number of animals this was not statistically significant.

There was little difference between the establishment of parasites in the other six sheep.

Discussion

The plasma pepsinogen rise in the lambs of Group 4A in response to the transplanted adult parasites is in agreement with responses found by Anderson, Hansky and Titchen (1985) and McKellar et al (1986; 1987).

The fact that the other lambs did not show a rise in plasma pepsinogen activity may be due to the smaller numbers of parasites transferred to lambs of groups 3B and 4B and the adverse effect of meclofenamic acid on parasite survival, as could affect parasites in group 3A lambs. The highest number of parasites was associated with the pepsinogen rise. Alternatively, meclofenamic acid treatment could be suppressing the pepsinogen rise. This is, however, impossible to determine given the direct toxic effect of meclofenamic acid on the parasites.

It is interesting that parasite eggs were detected in most animals one day after transfer indicating establishment of the parasites. There did not appear to be a suppressive effect on the egg counts in the group 3 lambs that were treated with meclofenamic acid, contrary to previous results. High numbers of parasite eggs were detected in the faeces of both groups of lambs. This might be due to the parasites being present in the lambs for a short time, no larval development having taken place,

and less opportunity for host immunity to act and cause a reduction in parasite egg output. The abomasal pH did rise slightly after the transfer. It is possible that the abomasal contents of the donor sheep directly caused the rise as the pH was higher than normal. However, quite small volumes were transferred. The meclofenamate concentrations were slightly higher in the plasma than in the previous study. The abomasal concentrations were low, but detectable and therefore able to affect the parasites.

Therefore it is possible that prostaglandins are involved in the pepsinogen response to adult parasites, but the fact that meclofenamic acid has a direct effect on Ostertagia larvae in vitro makes it impossible for conclusions to be drawn. This direct effect had not been anticipated at the start of these experiments.

TABLE 1

Concentrations (ug/ml) of meclofenamate in the plasma of two groups of adult sheep. Groups 1 and 3, each animal received 500 mg meclofenamic acid daily. Group 1, each animal received 200,000 *O. circumcincta* L_3 on day 0 as a single dose

Time (days)	Group 1 Animal number 96	97	98	Mean ± SEM	Group 3 Animal number 38
-1	0	0	0	0	0
0	1.65	1.08	1.00	1.24 ± 0.205	1.45
1	1.67	1.27	1.19	1.38 ± 0.149	2.02
2	1.88	1.50	0.82	1.40 ± 0.310	2.52
3	1.15	1.59	1.03	1.26 ± 0.170	2.21
4	1.39	1.83	1.59	1.60 ± 0.127	2.27
5	0.83	1.59	1.09	1.17 ± 0.223	2.38
6	1.15	1.66	1.19	1.33 ± 0.164	2.67
7	0.99	0.93	0.43	0.78 ± 0.178	2.05
8	0.89	0.63	0.37	0.63 ± 0.150	2.05
9	0.84	0.75	0.27	0.62 ± 0.177	1.30
10	0.73	0.70	0.37	0.60 ± 0.115	0.79
11	0.81	0.45	0.50	0.59 ± 0.113	0.51
12	0.99	1.06	1.00	1.02 ± 0.022	0.37
13	0.84	1.06	0.53	0.81 ± 0.154	0.25
14	0.75	0.82	0.71	0.76 ± 0.032	0.20
15	0.63	0.48	0.53	0.55 ± 0.044	0.20
16	0.67	0.52	0.50	0.56 ± 0.054	0.20

TABLE 1 (Cont'd)

Time (days)	Group 1 Animal number 96	97	98	Mean ± SEM	Group 3 Animal number 38
17	0.84	0.42	0.50	0.59 ± 0.129	0.22
18	0.58	0.48	0.70	0.59 ± 0.064	0.25
19	0.89	0.72	0.79	0.80 ± 0.049	0.34
20	0.75	0.69	0.47	0.64 ± 0.085	0.40
21	0.78	0.55	0.47	0.60 ± 0.093	0.34
22	0.70	0.42	0.53	0.55 ± 0.082	0.37
23	0.93	0.72	0.41	0.69 ± 0.151	NS
24	0.81	0.35	0.64	0.60 ± 0.134	NS

NS Not sampled

FIGURE 5

Mean concentrations (ug/ml) of meclofenamate in the plasma of two groups of adult sheep. Groups 1 and 3, each animal received 500 mg meclofenamic acid daily. Group 1, each animal received 200,000 O.circumcincta L_3 on day 0 as a single dose

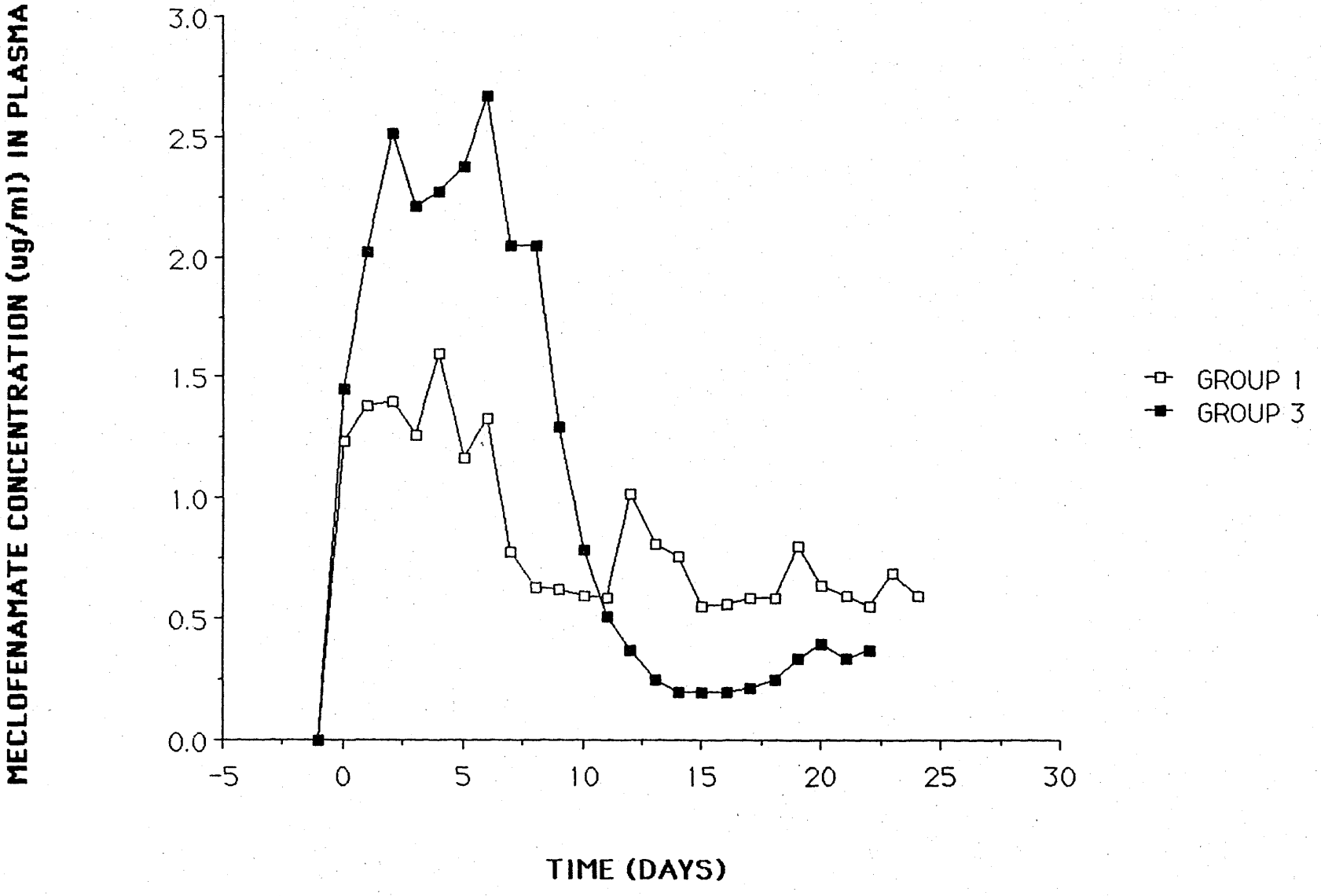

MECLOFENAMATE CONCENTRATION (ug/ml) IN PLASMA
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-5
0
5
10
15
20
25
30
TIME (DAYS)
GROUP 1
GROUP 3

TABLE 2

Plasma pepsinogen activities (I.U.) of three groups of adult sheep. Groups 1 and 2, each received 200,000 *O.circumcincta* L_3 on day 0 as a single dose. Groups 1 and 3, each animal received 500 mg meclofenamic acid daily

Time (days)	Group 1 Animal number 96	97	98	Mean ± SEM	Group 2 Animal number 47	48	Mean ± SEM	Group 3 Animal number 38
0	0.294	0.400	0.428	0.374 ± 0.041	0.697	0.381	0.539 ± 0.158	1.931
1	0.319	0.646	0.610	0.525 ± 0.103	0.782	0.504	0.643 ± 0.139	1.903
2	0.397	1.067	1.023	0.829 ± 0.217	1.102	0.826	0.964 ± 0.138	1.928
3	0.514	1.418	1.384	1.105 ± 0.296	1.374	1.185	1.280 ± 0.095	1.621
4	0.602	1.648	1.448	1.233 ± 0.320	1.599	1.420	1.510 ± 0.090	1.477
5	0.639	1.905	1.570	1.371 ± 0.379	1.588	1.274	1.431 ± 0.157	1.396
6	0.735	1.714	1.570	1.340 ± 0.305	2.100	1.035	1.568 ± 0.533	1.192
7	0.783	2.144	1.487	1.471 ± 0.393	2.577	0.989	1.783 ± 0.794	1.022
8	0.938	2.216	1.475	1.543 ± 0.371	2.841	0.988	1.915 ± 0.927	1.079
9	1.105	1.762	1.499	1.455 ± 0.191	2.422	1.028	1.725 ± 0.697	1.156
10	1.117	1.427	1.499	1.348 ± 0.117	1.912	1.120	1.516 ± 0.396	1.046
11	1.046	1.259	1.532	1.279 ± 0.141	2.069	1.359	1.714 ± 0.355	1.083
12	1.319	1.150	1.459	1.309 ± 0.090	2.261	2.081	2.171 ± 0.090	1.046
13	1.284	1.080	1.483	1.282 ± 0.117	2.225	2.332	2.279 ± 0.054	1.059
14	1.176	1.044	1.589	1.270 ± 0.164	2.429	3.365	2.897 ± 0.468	0.964
15	1.093	1.092	1.851	1.345 ± 0.253	2.645	3.918	3.282 ± 0.637	0.938
16	0.998	1.056	1.767	1.274 ± 0.247	2.562	4.097	3.330 ± 0.768	0.902

TABLE 2 (Cont'd)

Time (days)	Group 1 Animal number 96	97	98	Mean ± SEM	Group 2 Animal number 47	48	Mean ± SEM	Group 3 Animal number 38
17	0.945	1.175	1.877	1.332 ± 0.281	2.844	4.471	3.658 ± 0.814	0.866
18	1.042	1.078	2.072	1.397 ± 0.338	2.699	4.915	3.807 ± 1.108	0.829
19	1.006	1.139	2.072	1.406 ± 0.336	3.108	5.121	4.115 ± 1.007	0.782
20	0.981	1.090	2.327	1.466 ± 0.432	3.037	4.711	3.874 ± 0.837	0.831
21	1.006	1.212	2.715	1.644 ± 0.539	3.748	4.180	3.964 ± 0.216	0.741
22	0.945	1.224	2.678	1.616 ± 0.538	3.808	3.167	3.488 ± 0.321	0.887
23	0.898	1.224	2.549	1.557 ± 0.505	3.824	2.370	3.097 ± 0.727	NS
24	0.874	1.187	2.431	1.497 ± 0.476	4.391	2.082	3.237 ± 1.155	NS

NS Not sampled

FIGURE 6

Mean plasma pepsinogen activities (I.U.) of three groups of adult sheep. Groups 1 and 2, each animal received 200,000 O.circumcincta L_3 on day 0 as a single dose. Groups 1 and 3, each animal received 500 mg meclofenamic acid daily

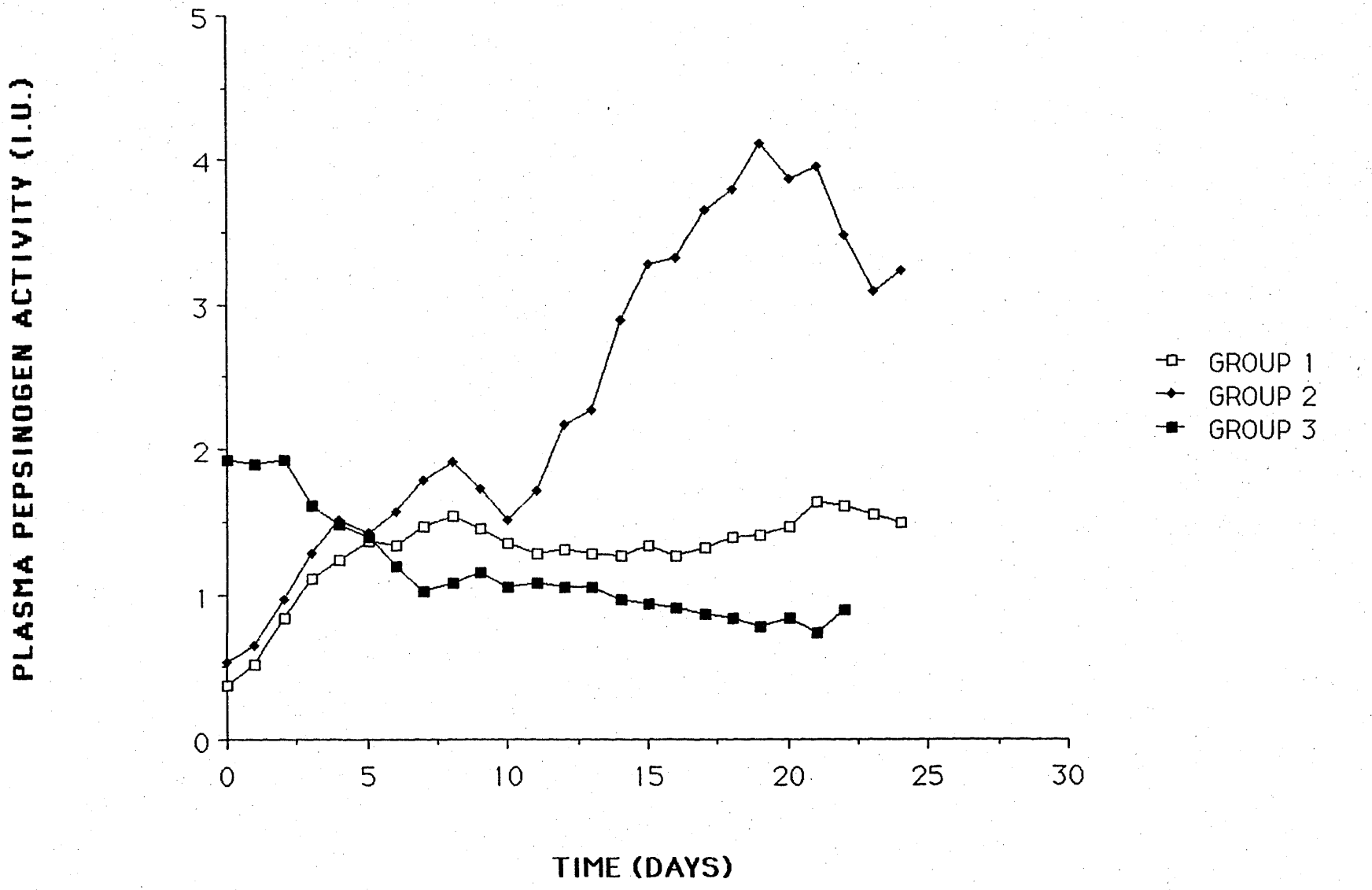

PLASMA PEPSINOGEN ACTIVITY (I.U.)
5
4
3
2
1
0
0
5
10
15
20
25
30
TIME (DAYS)
GROUP 1
GROUP 2
GROUP 3

TABLE 3

Numbers of parasite eggs per gram of faeces in three groups of adult sheep. Groups 1 and 2 sheep each received 200,000 O.circumcincta L_3 on day 0 as a single dose. Groups 1 and 3 sheep received 500 mg meclofenamic acid daily

Time	Group 1				Group 2				Group 3			
(days)	Animal number				Animal number				Animal number			
	41	46	102	120	47	113	124	7496	48	96	98	103
0	N	N	N	N	N	N	N	N	N	N	N	N
12	N	N	N	50	N	N	N	N	N	N	N	N
14	N	N	N	N	N	N	N	N	N	N	N	N
17	N	N	N	N	N	N	N	N	N	N	N	N
20	N	N	N	50	N	N	N	N	N	N	N	N
23	N	N	N	N	N	N	N	N	N	N	N	N

N = Negative

TABLE 4

Concentrations (ug/ml) of meclofenamate in the plasma of two groups of adult sheep. Groups 1 and 3, each animal received 500 mg meclofenamic acid daily. Group 1 animals each received 200,000 O.circumcincta L_3 on day 0 as a single dose

Time (days)	Group 1 Animal number 41	46	102	120	Mean ± SEM	Group 3 Animal number 48	96	98	103	Mean ± SEM
-1	0	0	0	0	0	0	0	0	0	0
2	0.47	0.34	0.67	0.21	0.42 ± 0.10	0.63	0.79	0.47	0.23	0.53 ± 0.12
5	0.30	0.53	0.60	0.21	0.41 ± 0.09	0.34	0.41	0.75	0.47	0.49 ± 0.09
8	0.25	0.29	0.39	0.14	0.27 ± 0.05	0.52	0.58	0.25	0.32	0.42 ± 0.08
11	0.32	0.33	0.64	0.17	0.37 ± 0.10	0.55	0.56	0.44	0.36	0.48 ± 0.05
14	0.29	0.30	0.55	0.23	0.34 ± 0.07	0.29	0.32	0.32	0.31	0.31 ± 0.01
17	0.27	0.49	0.55	0.14	0.36 ± 1.10	0.55	0.59	0.45	0.44	0.51 ± 0.04
20	0.29	0.48	0.7	0.17	0.43 ± 0.14	0.45	0.75	0.71	0.34	0.56 ± 0.10

FIGURE 7

Mean concentrations (ug/ml) of meclofenamate in the plasma of two groups of adult sheep. Groups 1 and 3, each animal received 500 mg meclofenamic acid daily. Group 1, each animal 200,000 *O. circumcincta* L_3 on day 0 as a single dose

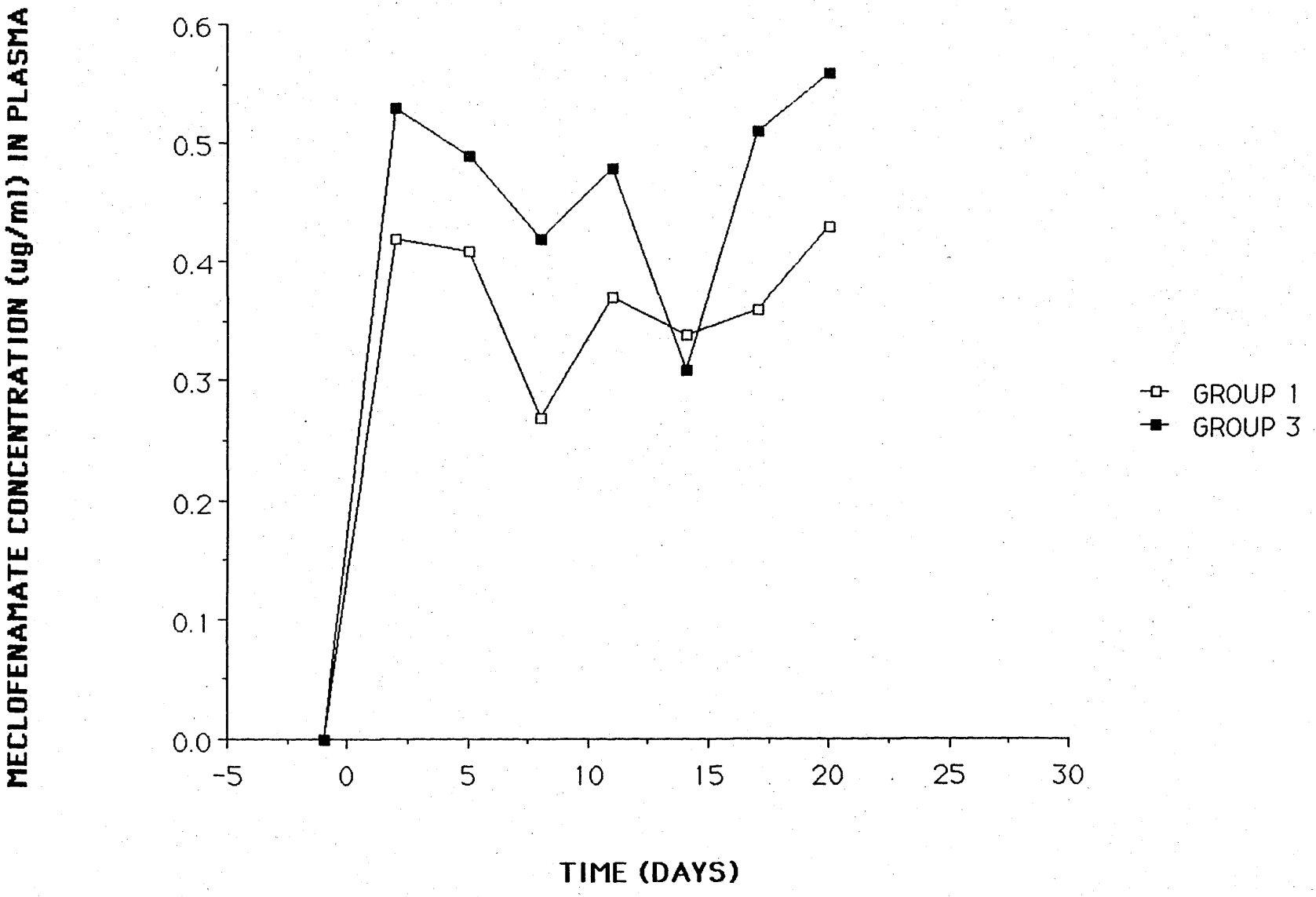

MECLOFENAMATE CONCENTRATION (ug/ml) IN PLASMA
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-5
0
5
10
15
20
25
30
TIME (DAYS)
GROUP 1
GROUP 3

TABLE 5

Plasma pepsinogen activities (I.U.) of three groups of adult sheep.

Groups 1 and 2, each animal received 200,000 O.circumcincta L_3 on day 0 as a single dose.

Groups 1 and 3, each animal received 500 mg meclofenamic acid daily

Time (days)	Group 1				Mean ± SEM	Group 2				Mean ± SEM	Group 3				Mean ± SEM
	Animal number					Animal number					Animal number				
	41	46	102	120		47	113	124	7496		48	96	98	103	
0	0.108	0.592	0.876	0.410	0.497 ± 0.161	0.980	0.317	0.395	0.339	0.508 ± 0.158	0.731	0.184	0.466	0.211	0.398 ± 0.128
2	0.184	2.339	3.040	0.859	1.606 ± 0.657	2.126	0.790	1.974	0.981	1.468 ± 0.340	0.576	0.379	0.434	0.255	0.411 ± 0.067
5	0.108	3.752	4.296	1.451	2.402 ± 0.982	3.657	1.279	2.437	1.139	2.128 ± 0.587	0.576	0.317	0.455	0.333	0.420 ± 0.060
8	0.227	2.463	2.597	0.869	1.539 ± 0.588	3.949	0.934	1.974	0.993	1.963 ± 0.704	0.609	0.367	0.413	0.432	0.455 ± 0.053
11	0.173	1.948	1.309	0.754	1.046 ± 0.380	2.110	1.056	0.925	1.151	1.311 ± 0.271	0.576	0.489	0.434	0.443	0.486 ± 0.033
14	0.216	1.871	1.233	0.897	1.054 ± 0.345	1.869	1.251	1.286	1.489	1.474 ± 0.142	0.520	0.350	0.391	0.310	0.393 ± 0.046
17	0.238	2.225	1.309	1.394	1.292 ± 0.407	1.794	1.045	1.309	1.264	1.353 ± 0.158	0.432	0.306	0.466	0.255	0.365 ± 0.050
20	0.108	1.795	1.309	2.578	1.448 ± 0.517	2.136	0.728	0.869	1.241	1.244 ± 0.317	0.532	0.267	0.370	0.133	0.326 ± 0.084
23	0.032	2.559	1.169	2.654	1.604 ± 0.624	2.040	0.634	0.947	1.275	1.224 ± 0.302	0.587	0.267	0.423	0.221	0.375 ± 0.083

FIGURE 8

Mean plasma pepsinogen activities (I.U.) of three groups of adult sheep. Groups 1 and 2, each animal received 200,000 *O. circumcincta* L_3 on day 0 as a single dose. Groups 1 and 3, each animal received 500 mg meclofenamic acid daily

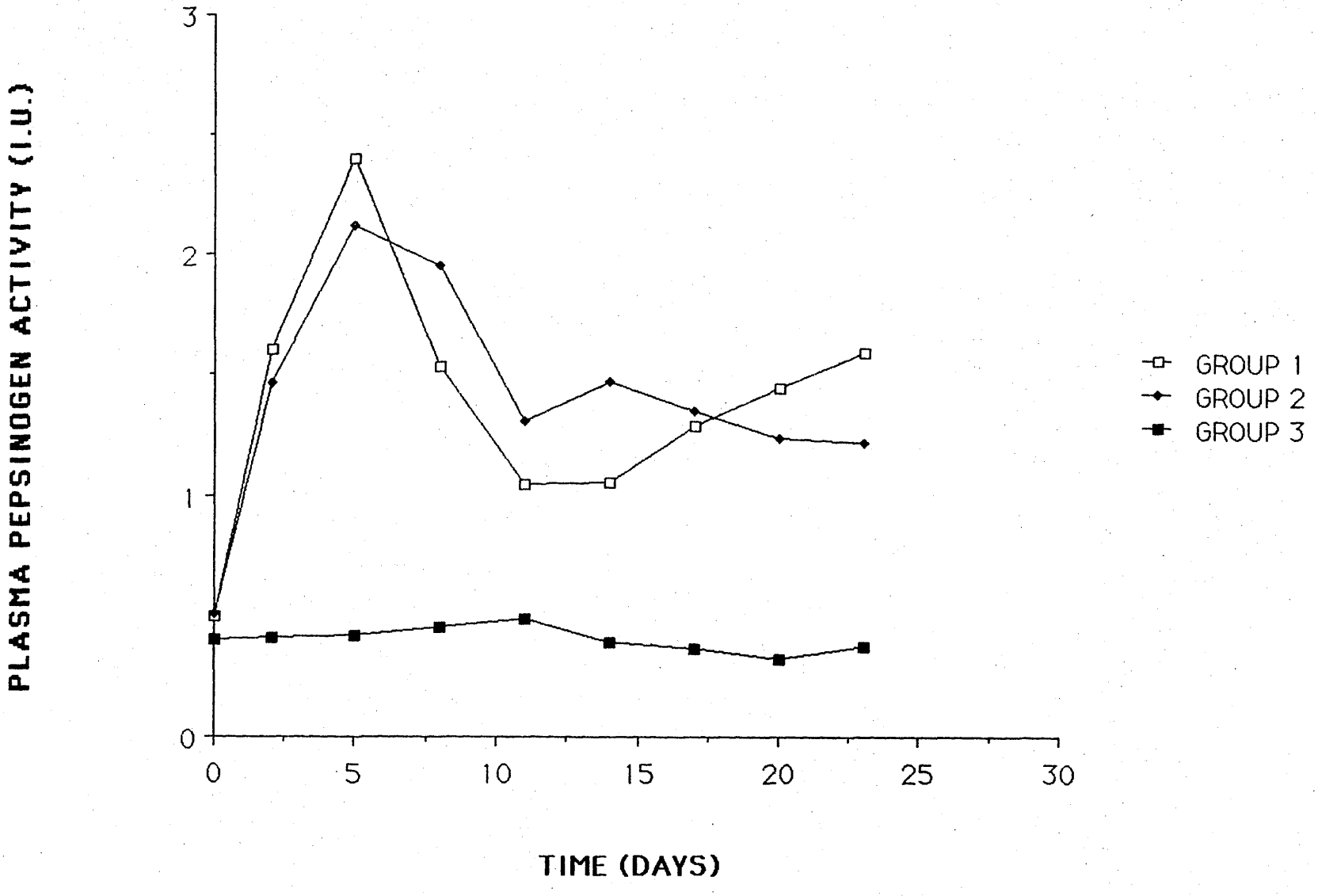

PLASMA PEPSINOGEN ACTIVITY (I.U.)
3
2
1
0
0
5
10
15
20
25
30
TIME (DAYS)
GROUP 1
GROUP 2
GROUP 3

TABLE 6

Abomasal pH at necropsy on day 23 of the experiment of two groups of adult sheep. Groups 1 and 2, each animal received 200,000 O.circumcincta L_3 on day 0 as a single dose. Group 1 animals each received 500 mg meclofenamic acid daily

Group	Sheep number	Abomasal pH
1	41	3.6
	46	3.0
	102	3.6
	120	6.6
2	47	3.0
	113	4.0
	124	4.7+
	7496	3.2

+ blood contamination

TABLE 7

The number of parasites remaining in the abomasa of two groups of adult sheep at necropsy on day 23 of the experiment. Groups 1 and 2, each animal received a challenge of 200,000 O.circumcincta L_3 on day 0 as a single dose. Group 1 sheep also received 500 mg of meclofenamic acid daily

Group number	Animal number	Abomasal contents					Abomasal digest					Total number in abomasum
		L_4	L_5	Adults ♂	Adults ♀	Total	L_4	L_5	Adults ♂	Adults ♀	Total	
1	41	0	0	100	0	100	0	0	0	0	0	100
	46	200	200	0	200	600	400	0	0	0	400	1,000
	102	100	100	100	100	400	100	0	0	0	100	500
	120	0	200	6,600	9,000	15,800	0	0	0	0	0	15,800
											Mean	4,350
											± SEM	± 3,821
2	47	0	2,700	2,900	4,700	10,300	0	300	100	350	750	11,050
	113	200	0	0	100	300	0	0	0	0	0	300
	124	200	0	0	0	200	0	0	0	0	0	200
	7496	18,000	20,000	1,600	1,200	40,800	1,400	600	0	0	2,000	42,800
											Mean	13,587.5
											± SEM	± 1,0064.75

TABLE 8

Percentage establishment of a single dose of 200,000 *O.circumcincta* L_3 at necropsy, 23 days post-challenge of two groups of adult sheep. Group 1 sheep also received 500 mg meclofenamic acid daily

Group number	Animal number	Total number of parasites	Percentage establishment
1	41	100	0.05%
	46	1,000	0.50%
	102	500	0.25%
	120	15,800	7.90%
		Mean	2.175%
		± SEM	± 1.91
2	47	11,050	5.50%
	113	300	0.15%
	124	200	0.10%
	7496	42,800	24.40%
		Mean	6.79%
		± SEM	± 5.03

TABLE 9

Numbers of parasite eggs per gram of faeces of two groups of previously parasite-naive lambs. Groups 1 and 2, each animal received a challenge of 75,000 O. circumcincta L_3 on day 0 as a single dose. Group 1 lambs also received 250 mg of meclofenamic acid daily

Time (days)	Group 1				Group 2			
	Animal number				Animal number			
	49	57	104	133	52	63	64	85
0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	100	0
17	0	0	0	0	0	0	500	50
20	0	400	0	0	0	0	750	300
23	0	150	0	0	50	0	650	450
24	0	100	0	0	300	50	350	1750

FIGURE 9

Numbers of parasite eggs per gram of faeces in one group of previously parasite-naive lambs. Group 1, each animal received 75,000 O.circumcincta L_3 on day 0 as a single dose and 250 mg meclofenamic acid daily. No parasite eggs were detected in the faeces of lambs 49, 104 and 133

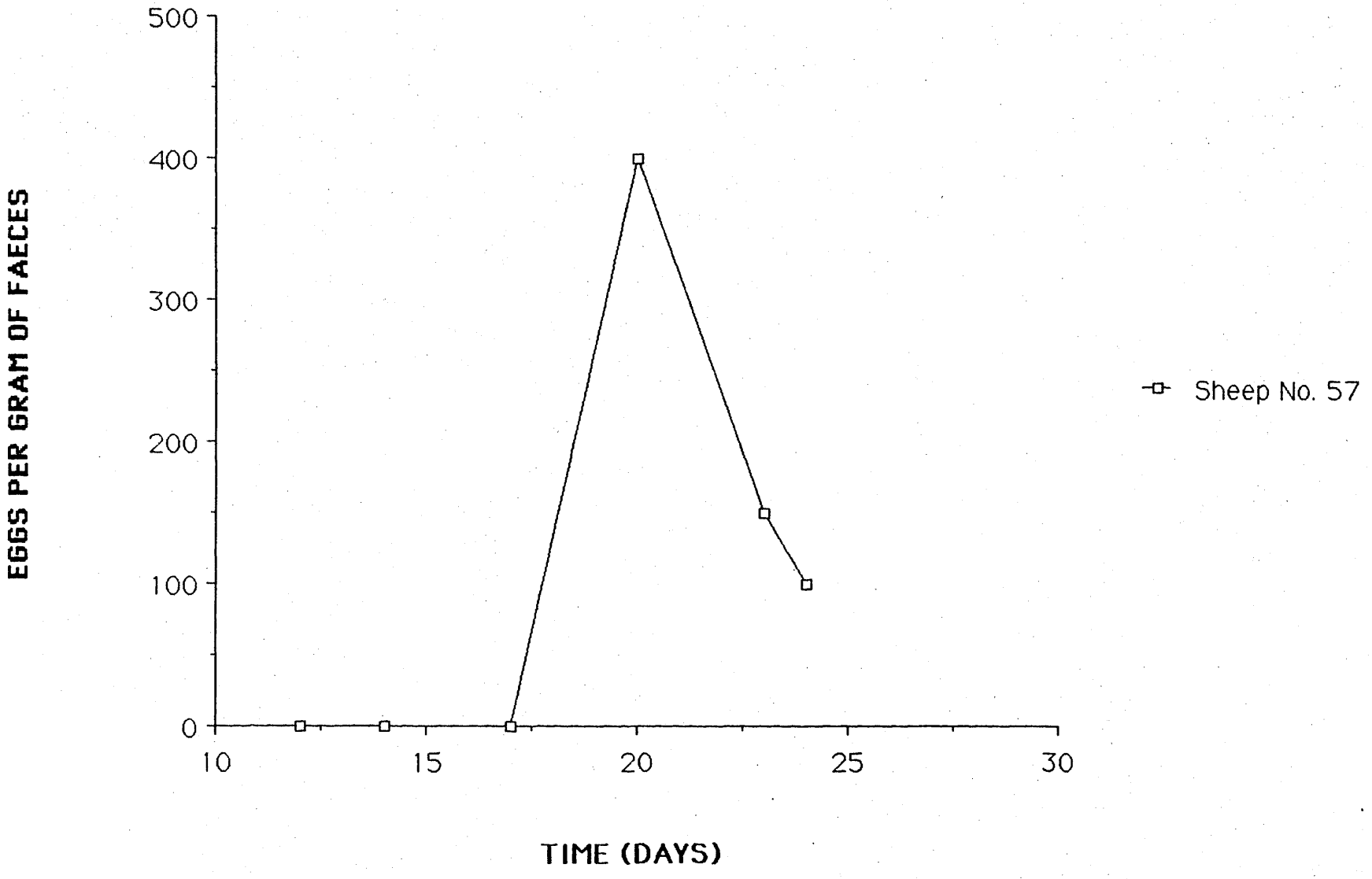

EGGS PER GRAM OF FAECES
500
400
300
200
100
0
10
15
20
25
30
TIME (DAYS)
Sheep No. 57

FIGURE 10

Numbers of parasite eggs per gram of faeces in one group of previously parasite-naive lambs. Group 2, each animal received 75,000 *O. circumcincta* L_3 on day 0 as a single dose

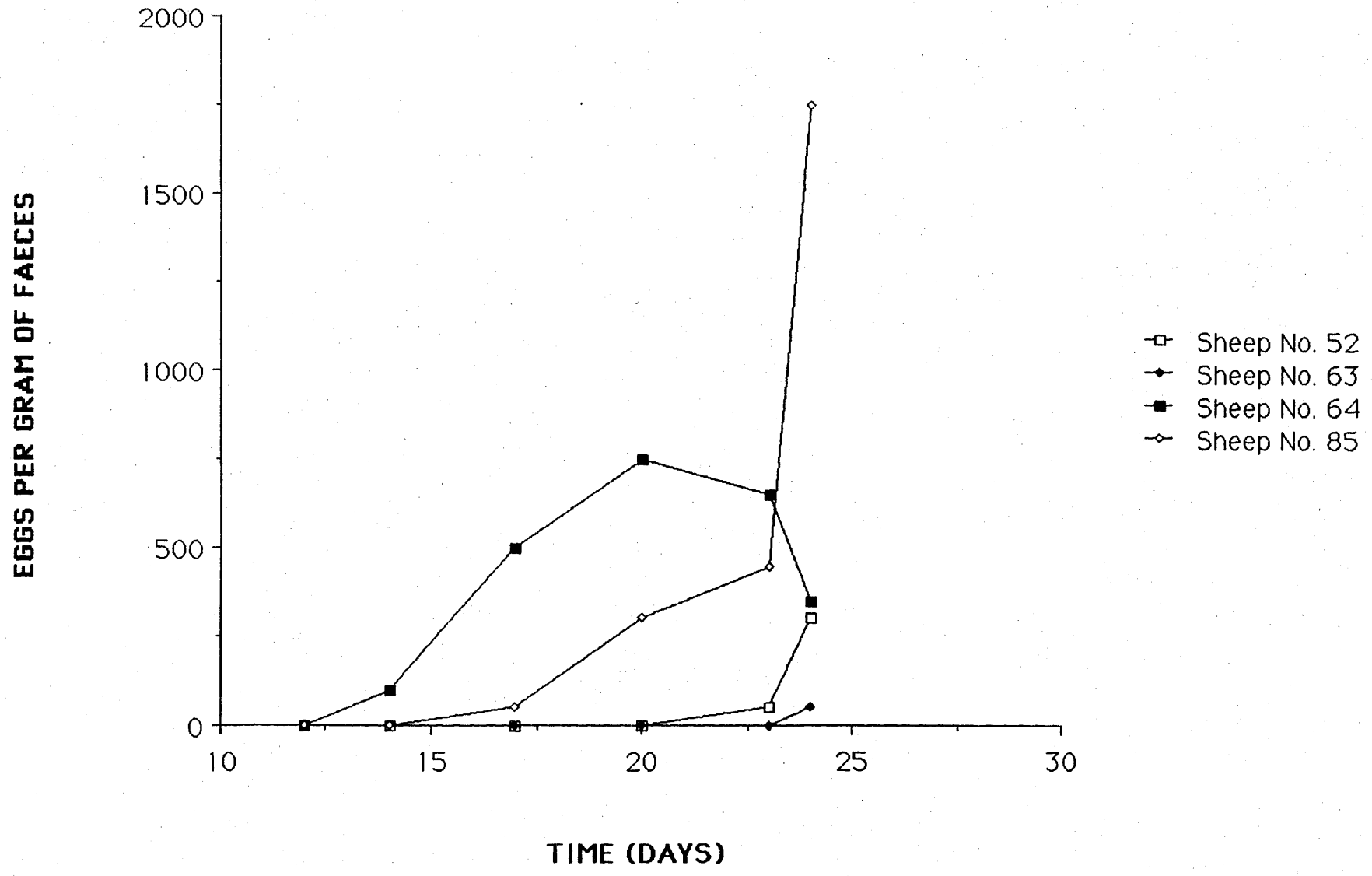

2000
1500
1000
500
0
10
15
20
25
30
EGGS PER GRAM OF FAECES
TIME (DAYS)
Sheep No. 52
Sheep No. 63
Sheep No. 64
Sheep No. 85

TABLE 10

Concentrations (ug/ml) of meclofenamate in the plasma of one group of previously parasite-naive lambs. Group 1, each animal received 250 mg meclofenamic acid daily and 75,000 *O. circumcincta* L_3 on day 0 as a single dose

Time (days)	Group 1 — Animal number 49	57	104	133	Mean ± SEM
-1	0	0	0	0	0
2	0.17	0.57	0.22	0.48	0.36 ± 0.10
5	+ 0	0.36	+ 0	0.50	0.22 ± 0.13
8	0	0.47	0.22	0.50	0.30 ± 0.12
11	0	0.34	1.01	0.51	0.47 ± 0.21
14	0	0.28	0	0.30	0.15 ± 0.08
17	0	0.61	0	0.29	0.23 ± 0.15
20	0.38	0.55	++ 0	0.40	0.33 ± 0.12
23	0.22	0.71	++ 0	0.72	0.41 ± 0.18
24	0.22	0.38	++ 0	0.43	0.26 ± 0.10

\+ diarrhoea, but improved without treatment.

++ signs of possible meclofenamic acid toxicity from day 19. Dose reduced to half from day 19 until end of experiment.

FIGURE 11

Mean concentration (ug/ml) of meclofenamate in the plasma of one group of previously parasite-naive lambs. Group 1, each animal received 250 mg meclofenamic acid daily and 75,000 *O. circumcincta* L_3 on day 0 as a single dose

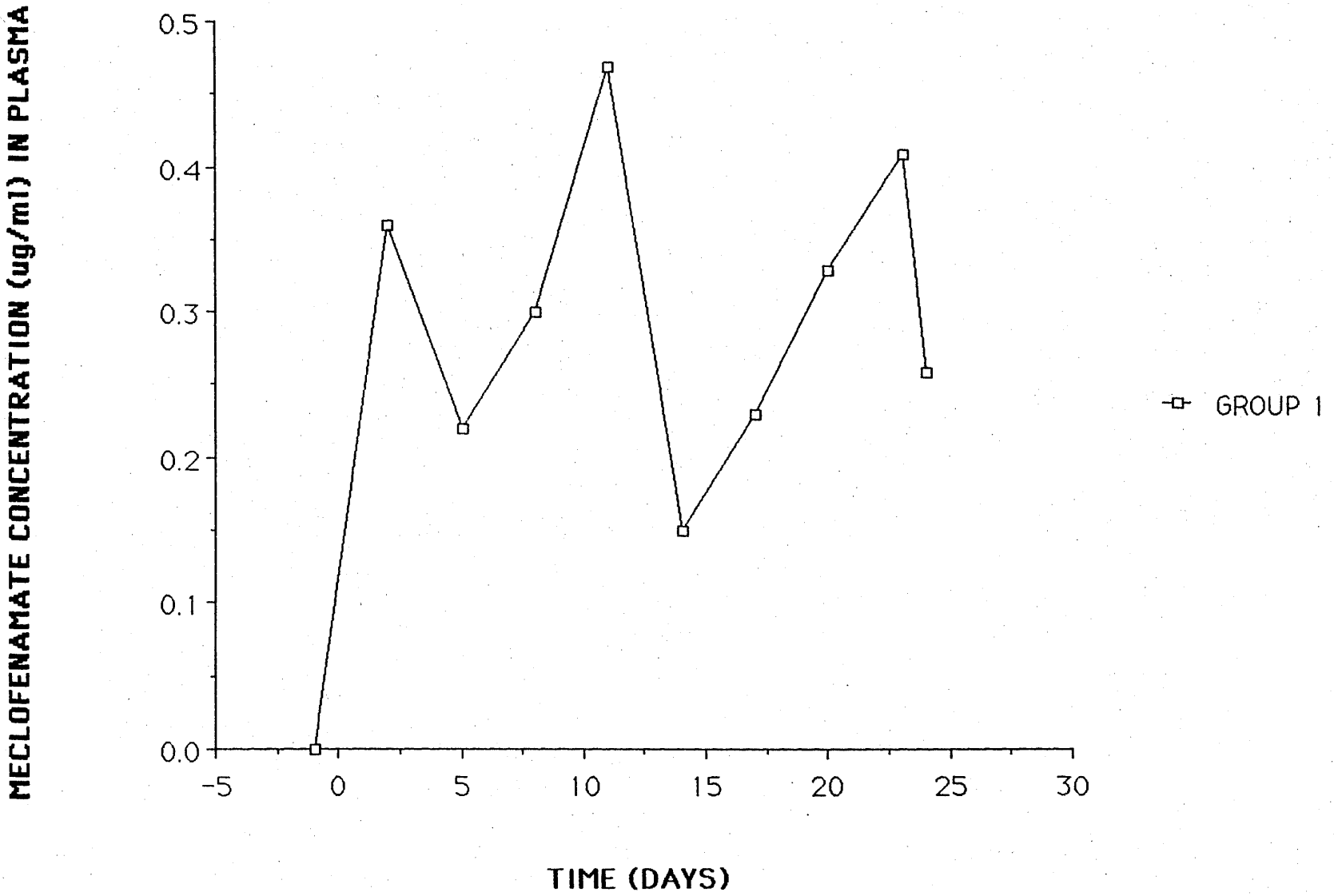

MECLOFENAMATE CONCENTRATION (ug/ml) IN PLASMA
0.5
0.4
0.3
0.2
0.1
0.0
-5
0
5
10
15
20
25
30
TIME (DAYS)
GROUP 1

TABLE 11

Plasma pepsinogen activities (I.U.) of two groups of previously parasite-naive lambs. Groups 1 and 2, each animal received a challenge of 75,000 *O. circumcincta* L_3 on day 0 as a single dose. Group 1 lambs also received 250 mg of meclofenamic acid daily

Time (days)	Group 1				Mean ± SEM	Group 2				Mean ± SEM
	Animal number					Animal number				
	49	57	104	133		52	63	64	85	
0	0.488	0.316	0.484	0.536	0.456 ± 0.048	0.191	0.446	0.376	0.218	0.308 ± 0.062
2	0.534	0.489	0.769	0.334	0.532 ± 0.090	0.226	0.519	0.527	0.353	0.406 ± 0.072
5	0.718	0.638	0.684	0.345	0.596 ± 0.085	0.330	0.591	0.619	0.321	0.465 ± 0.081
8	1.430	1.235	1.569	0.610	1.211 ± 0.212	0.701	2.116	1.106	1.079	1.251 ± 0.303
11	4.003	3.774	4.349	1.565	3.423 ± 0.630	2.450	3.443	2.172	1.617	2.421 ± 0.382
14	5.198	5.359	5.971	2.360	4.722 ± 0.805	2.160	3.650	5.275	2.603	3.422 ± 0.692
17	6.657	2.786	2.433	1.003	3.220 ± 1.209	1.233	2.924	5.009	2.551	2.929 ± 0.782
20	3.751	1.625	1.727	0.652	1.939 ± 0.651	0.666	1.877	2.994	2.292	1.957 ± 0.488
23	2.682	1.316	1.011	0.684	1.423 ± 0.439	0.689	1.680	2.241	1.960	1.643 ± 0.338
25	2.073	1.109	0.885	0.504	1.143 ± 0.334	0.689	1.711	2.033	2.084	1.629 ± 0.324

FIGURE 12

Mean plasma pepsinogen activities (I.U.) of two groups of previously parasite-naive lambs. Groups 1 and 2, each animal received 75,000 *O.circumcincta* L_3 on day 0 as a single dose. Group 1 lambs each received 250 mg meclofenamic acid daily

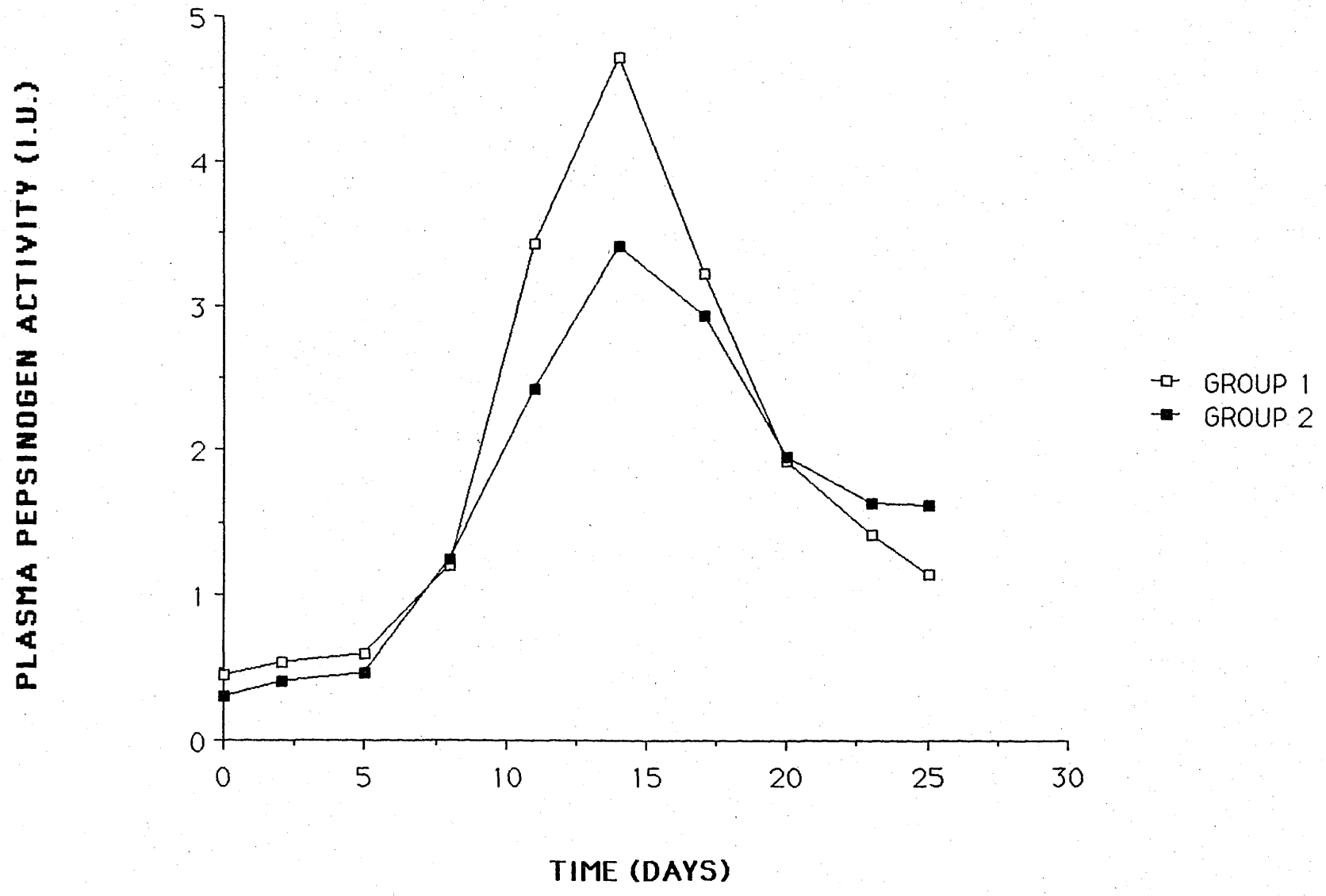

PLASMA PEPSINOGEN ACTIVITY (I.U.)
0
1
2
3
4
5
0
5
10
15
20
25
30
TIME (DAYS)
GROUP 1
GROUP 2

TABLE 12

Abomasal pH at necropsy on day 24 of the experiment of two groups of previously parasite-naive lambs

Groups 1 and 2, each animal received a challenge of 75,000 *O. circumcincta* L_3 on day 0 as a single dose.

Group 1 lambs each received 250 mg meclofenamic acid daily

Group number	Animal number	Abomasal pH
1	49	3.8
	57	6.6
	104	6.7
	133	6.1+
2	52	6.6
	63	5.6
	64	6.6
	85	6.3

+ blood contamination.

TABLE 13

The number of parasites remaining in the abomasi of two groups of previously parasite-naive lambs. Groups 1 and 2, each animal received a challenge of 75,000 O. circumcincta L_3 on day 0 as a single dose. Group 1 lambs each received 250 mg of meclofenamic acid daily

Group number	Animal number	Abomasal contents					Abomasal digest					Total number in abomasum
		L_4	L_5	Adults ♂	Adults ♀	Total	L_4	L_5	Adults ♂	Adults ♀	Total	
1	49	0	50	1,300	1,650	3,000	0	0	450	250	700	3,700
	57	0	0	2,450	1,400	3,850	0	0	50	0	50	3,900
	104	0	50	0	50	100	0	0	0	0	0	100
	133	0	100	2,550	4,200	6,850	0	100	100	300	500	7,350
											Mean	3,762.5
											± SEM	± 1,480.6
2	52	0	0	550	1,600	2,150	0	100	50	0	150	2,300
	63	0	100	1,600	2,550	4,250	0	0	0	50	50	4,300
	64	0	0	15,850	9,250	25,100	150	50	250	150	600	25,700
	+ 85	0	0	13,350	15,250	28,600	0	0	100	250	350	28,950
											Mean	15,312.5
											± SEM	± 6,979.0

+ large numbers of parasites in clumps in the aliquots counted.

TABLE 14

Percentage establishment of a single challenge of 75,000 *O. circumcincta* L_3 in two groups of previously parasite-naive lambs. Group 1 lambs received 250 mg meclofenamic acid daily

Group number	Animal number	Total number of parasites	Percentage establishment
1	49	3,700	4.933
	57	3,900	5.200
	104	100	0.133
	133	7,350	9.800
		Mean	5.017
		± SEM	± 1.974
2	52	2,300	3.067
	63	4,300	5.733
	64	25,700	34.267
	85	28,950	38.600
		Mean	20.417
		± SEM	± 9.305

TABLE 15

Numbers of male and female parasites in two groups of previously parasite-naive lambs. Groups 1 and 2, each animal was challenged with 75,000 *O. circumcincta* L_3. Group 1, each animal received 250 mg meclofenamic acid daily

Group number	Animal number	Total number of adults ♂	♀	Ratio Male : Female
1	49	1,750	1,900	0.92
	57	2,500	1,400	1.79
	104	0	50	-
	133	2,650	4,500	0.59
			Mean	1.1
			± SEM	± 0.64
2	52	600	1,600	0.38
	63	1,600	2,600	0.63
	64	16,100	9,400	1.71
	85	13,450	15,500	0.87
			Mean	0.90
			± SEM	± 0.29

TABLE 16

Estimation of numbers of adult *O. circumcincta* transferred into lambs of groups 3 and 4 from groups 1 and 2. Group 1 donor lambs received 250 mg meclofenamic acid daily throughout the infection

Group number	Animal number	Total number of parasites in abomasal contents	Mean ± SEM
1	49	3,000	3,450 ± 1,389
	57	3,850	
	104	100	
	133	6,850	
2	52	2,150	15,025 ± 6,878
	63	4,250	
	64	25,100	
	85	28,600	

TABLE 17

Numbers of parasite eggs per gram of faeces in two groups of previously parasite-naive lambs. Groups 3 and 4, each animal received adult O.circumcincta via abomasal cannulae on day 0. Animals in groups 3B and 4B received parasites from donors treated with meclofenamic acid. Animals in groups 3A and 4A received parasites from untreated donors. Group 3, each animal received 250 mg meclofenamic acid daily

Time (days)	Group 3A		3B		Group 4A		4B	
	Animal number 1	51	56	93	75	76	79	86
0	N	N	N	N	N	N	N	N
1	500	350	100	N	250	350	N	N
3	2,000	1,700	150	900	1,100	2,500	450	400
5	950	3,100	950	1,300	1,100	1,850	700	650
7	450	4,500	350	1,200	200	950	500	400
9	N	1,350	5,000	900	650	1,600	550	600
11	N	N	N	400	N	150	N	N
13	100	N	N	100	N	100	N	N
15	N	150	50	N	50	150	50	250

N - Negative

FIGURE 13

Numbers of parasite eggs per gram of faeces in one group of previously parasite-naive lambs. Group 3, each animal received adult O.circumcincta via abomasal cannulae on day 0, and 250 mg meclofenamic acid daily

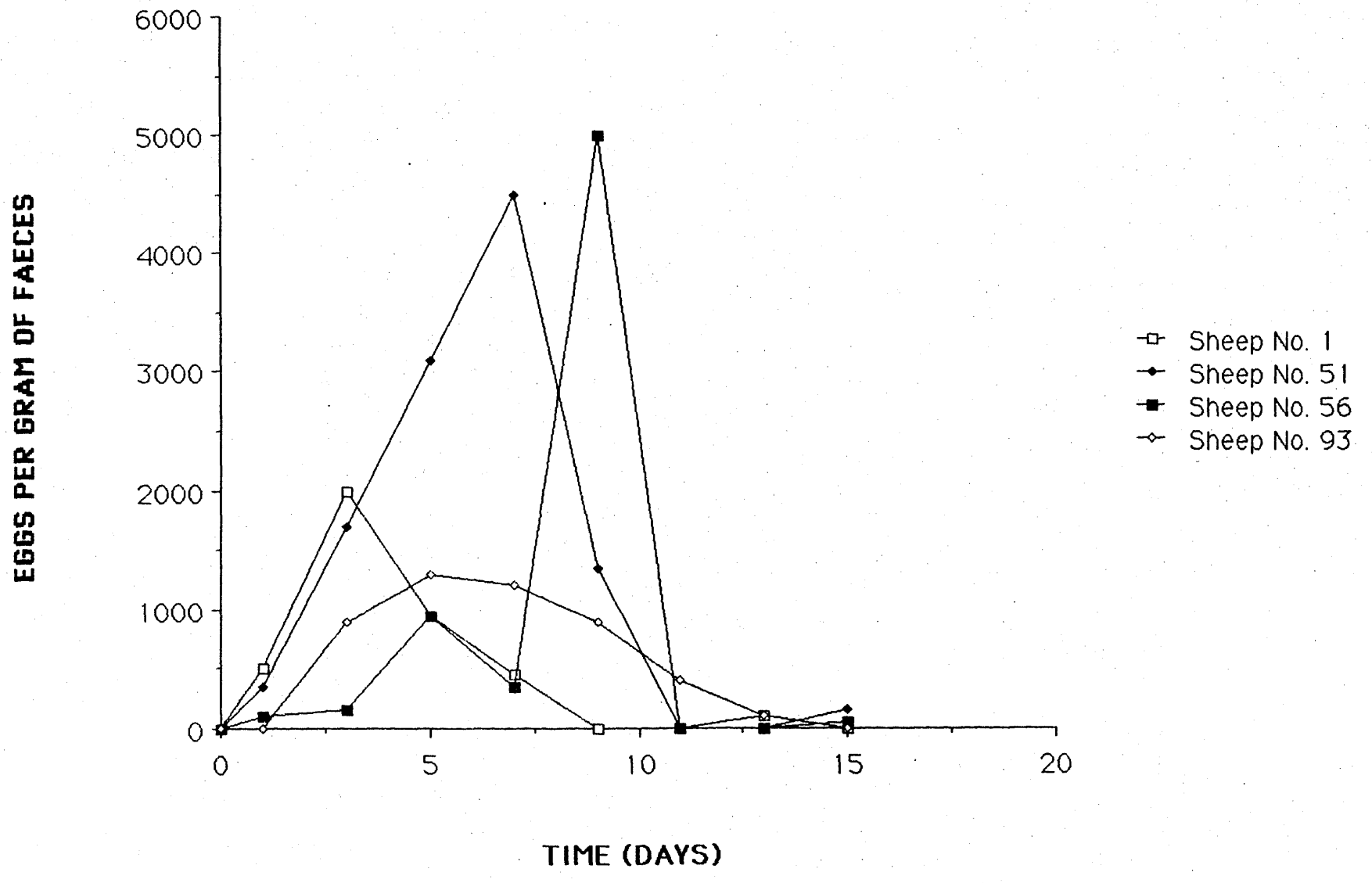

EGGS PER GRAM OF FAECES
6000
5000
4000
3000
2000
1000
0
0
5
10
15
20
TIME (DAYS)
Sheep No. 1
Sheep No. 51
Sheep No. 56
Sheep No. 93

FIGURE 14

Numbers of parasite eggs per gram of faeces in one group of previously parasite-naive lambs. Group 4, each animal received adult O.circumcincta via abomasal cannulae on day 0

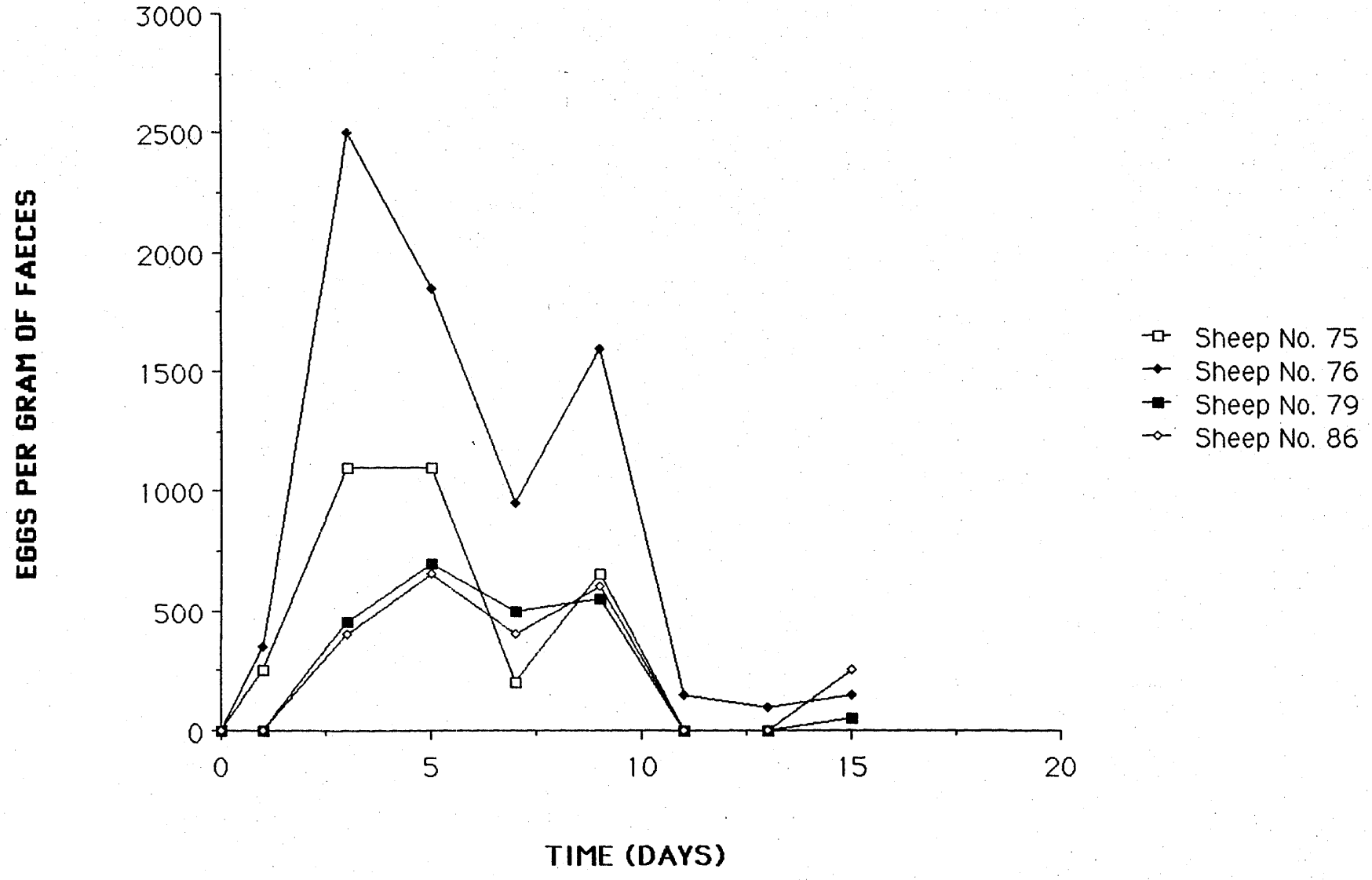

EGGS PER GRAM OF FAECES
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
TIME (DAYS)
Sheep No. 75
Sheep No. 76
Sheep No. 79
Sheep No. 86

TABLE 18

Concentrations (ug/ml) of meclofenamate in the plasma of one group of previously parasite-naive lambs. Group 3 lambs received 250 mg meclofenamic acid daily and adult O.circumcincta via abomasal cannulae on day 0

Time (days)	Group 3				Mean ± SEM
	Animal number				
	1	51	56	93	
-1	0	0	0	0	0
1	0.34	0.29	0.18	0.47	0.32 ± 0.06
3	0.52	0.57	0.26	0.68	0.51 ± 0.09
5	0.40	0.60	0.28	0.65	0.48 ± 0.09
7	0.34	0.40	0.24	0.91	0.47 ± 0.15
9	0.23	0.29	0.21	0.46	0.30 ± 0.06
11	0.23	0.21	0.18	0.61	0.31 ± 0.10
13	0.45	0.29	0.24	0.62	0.40 ± 0.09
15	0.30	0.49	0.34	0.31	0.36 ± 0.04

TABLE 19

Concentrations (ug/ml) of meclofenamate in the abomasal fluid of one group of previously parasite-naive lambs. Group 3 lambs received 250 mg meclofenamic acid daily and adult *O.circumcincta* via abomasal cannulae on day 0

Time (days)	Group 3 Animal number 1	51	56	93	Mean ± SEM
-1	0	0	0	0	0
1	0	0	0	0.12	0.030 ± 0.03
3	0.10	0	0	0.15	0.063 ± 0.04
5	0	0.14	0	0.38	0.130 ± 0.09
7	0	0	0	0	0 0
9	0	0	0	0	0 0
11	0	0.14	0	0	0.040 ± 0.04
13	0	0	0	0.10	0.030 ± 0.03
15	0	0	0	0	0 0

FIGURE 15

Mean concentrations (ug/ml) of meclofenamate in the plasma and abomasal fluid of one group of previously parasite-naive lambs. Group 3, each animal received 250 mg meclofenamic acid daily and adult O. circumcincta via abomasal cannulae on day 0

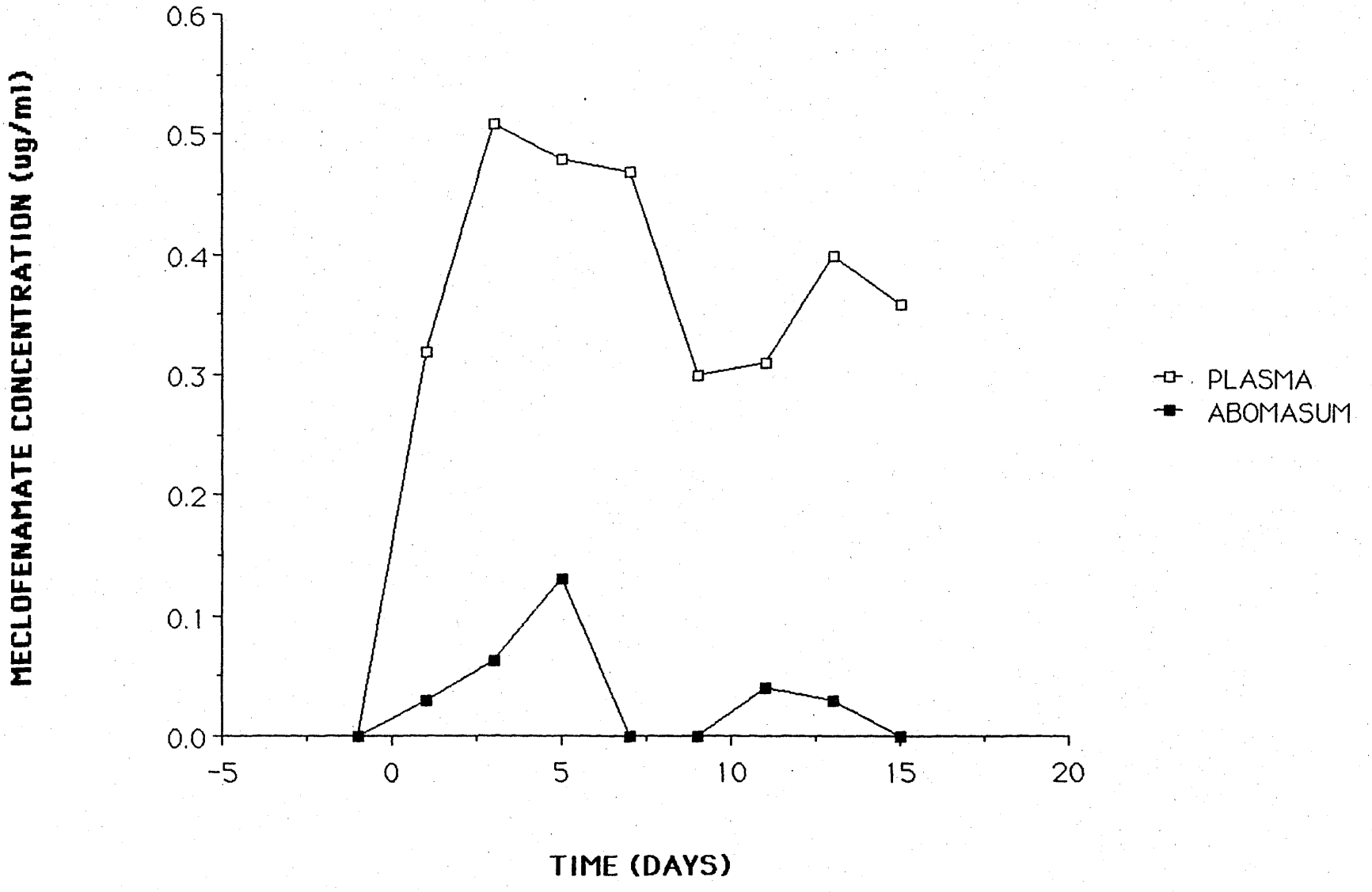

MECLOFENAMATE CONCENTRATION (ug/ml)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-5
0
5
10
15
20
TIME (DAYS)
PLASMA
ABOMASUM

TABLE 20

Abomasal pH in two groups of previously parasite-naive lambs. Groups 3 and 4, each animal received adult O. circumcincta via abomasal cannulae on day 0. Group 3, each animal received 250 mg meclofenamic acid daily

Time (days)	Group 3A Animal number 1	51	3B 56	93	Mean ± SEM	Group 4A Animal number 75	76	4B 79	86	Mean ± SEM
0	3.5	2.7	2.9	3.0	3.025 ± 0.170	3.3	3.4	3.2	2.6	3.125 ± 0.180
1	3.4	3.0	3.1	3.8	3.325 ± 0.180	3.4	3.6	2.9	2.9	3.200 ± 0.180
3	3.5	2.7	2.9	3.0	3.025 ± 0.170	3.3	3.4	3.2	2.6	3.125 ± 0.180
5	3.4	3.2	2.8	2.8	3.050 ± 0.150	3.25	3.2	3.1	2.9	3.110 ± 0.080
7	2.9	3.2	2.8	2.8	2.295 ± 0.095	2.85	3.0	2.8	2.7	2.840 ± 0.063
9	2.9	2.9	2.9	2.8	2.875 ± 0.025	2.7	3.1	2.5	2.8	2.780 ± 0.125
11	3.1	3.0	2.7	2.8	2.900 ± 0.090	2.8	3.4	2.9	3.1	3.050 ± 0.132
13	2.9	2.9	2.4	2.9	2.775 ± 0.125	2.8	3.2	2.5	3.0	2.880 ± 0.150
15	2.8	2.7	3.3	3.0	2.950 ± 0.132	3.3	2.4	2.3	2.8	2.700 ± 0.230

FIGURE 16

Mean abomasal pH of two groups of previously parasite-naive lambs. Groups 3 and 4, each animal received adult O.circumcincta via abomasal cannulae on day 0. Group 3, each animal received 250 mg meclofenamic acid daily

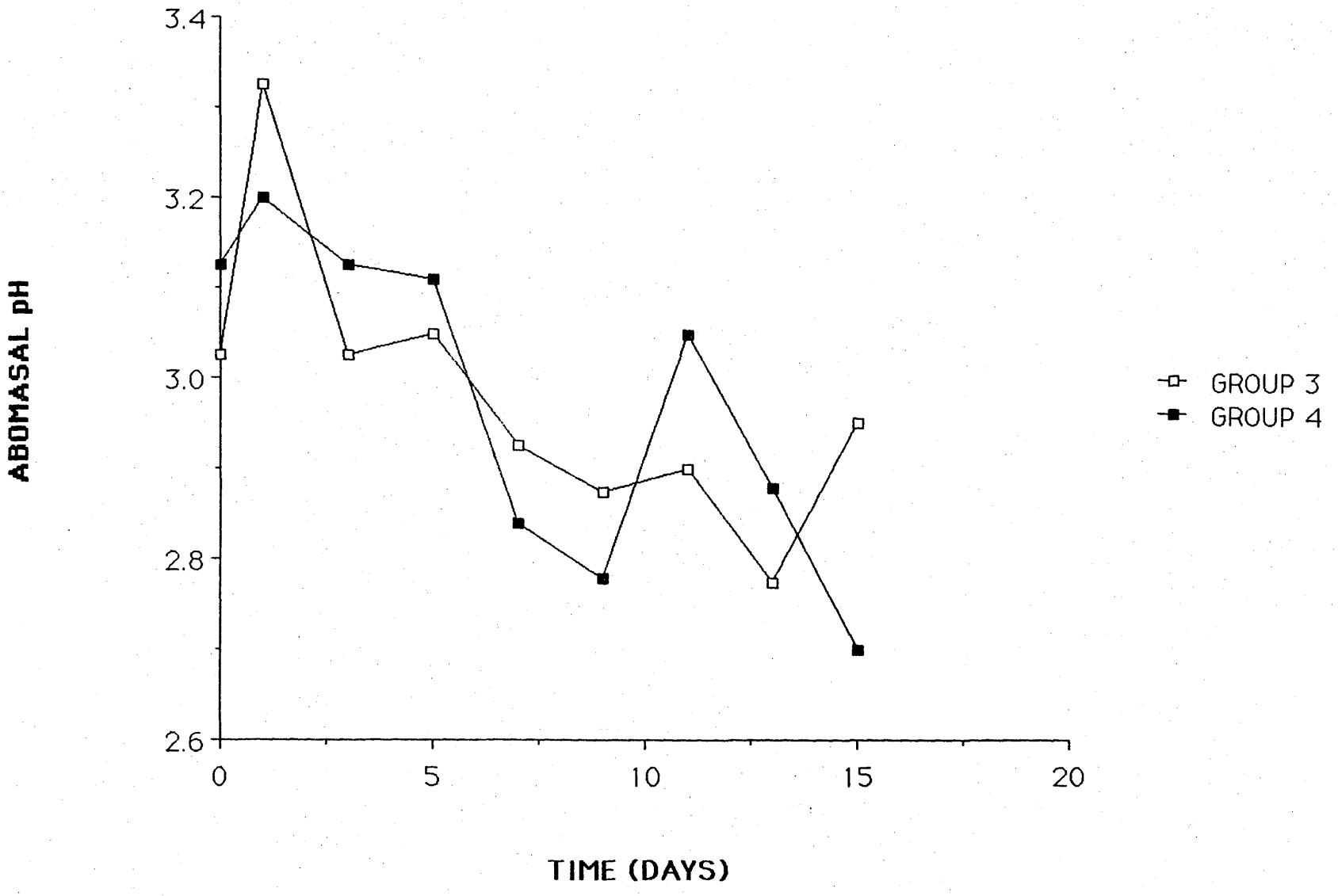

ABOMASAL pH
3.4
3.2
3.0
2.8
2.6
0
5
10
15
20
TIME (DAYS)
GROUP 3
GROUP 4

FIGURE 17

Mean plasma pepsinogen activities (I.U.) of two groups of previously parasite-naive lambs. Groups 3 and 4, each animal received adult O.circumcincta via abomasal cannulae on day 0. Group 3, each animal received 250 mg meclofenamic acid daily

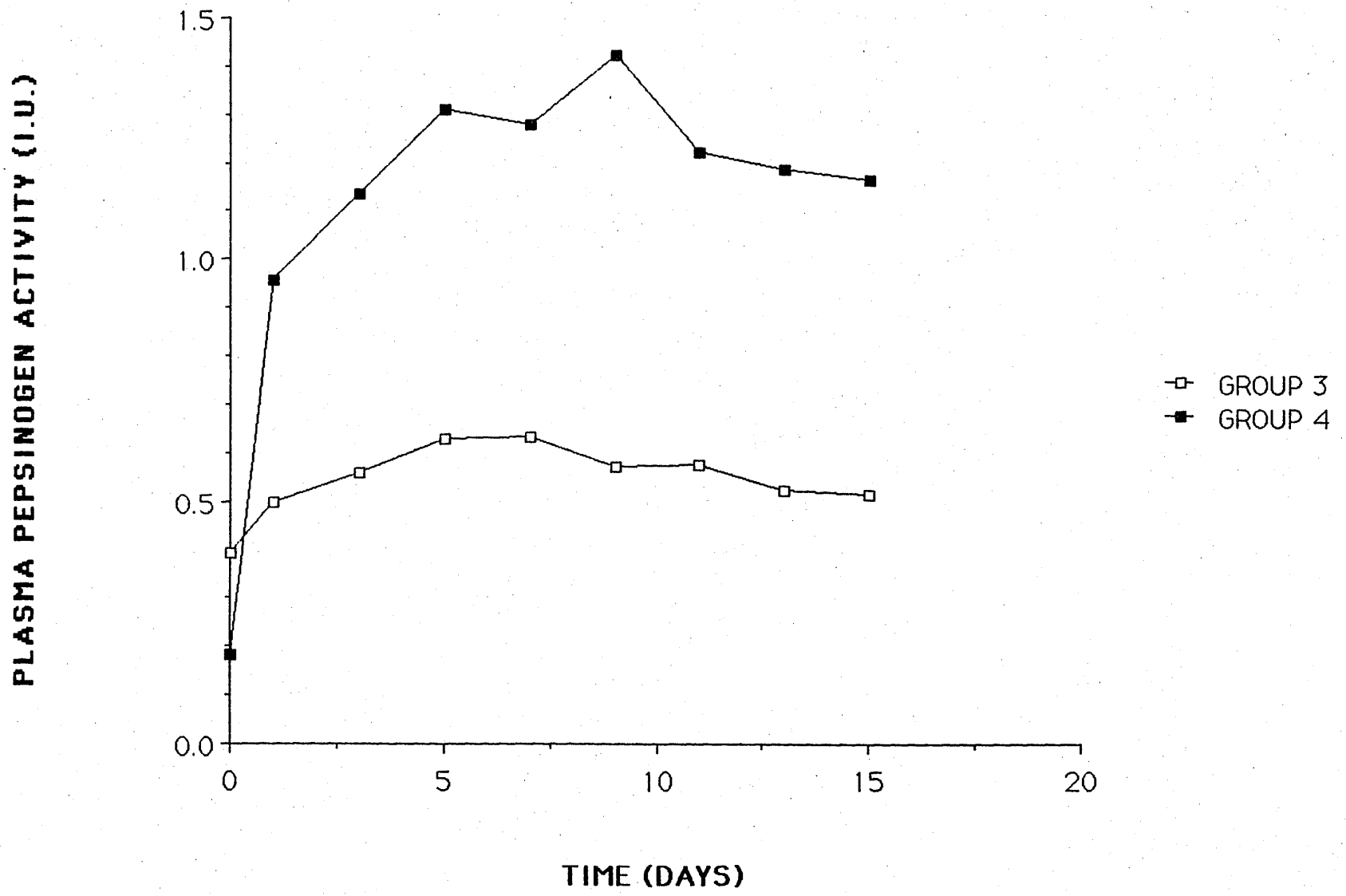

PLASMA PEPSINOGEN ACTIVITY (I.U.)
1.5
1.0
0.5
0.0
0
5
10
15
20
TIME (DAYS)
GROUP 3
GROUP 4

TABLE 21

Plasma pepsinogen activities (I.U.) of two groups of previously parasite-naive lambs. Both groups received adult O.circumcincta on day 0. Groups 3B and 4B received parasites from donors treated with meclofenamic acid. Group 3 also received 250 mg meclofenamic acid daily

Group	Animal number	Time (days post-challenge) 0	1	3	5	7	9	11	13	15
3A	1	0.505	0.346	0.346	0.408	0.383	0.274	0.274	0.286	0.310
	51	0.113	0.651	0.950	0.950	0.902	0.914	0.794	0.687	0.691
B	56	0.383	0.323	0.310	0.323	0.323	0.383	0.323	0.383	0.334
	93	0.579	0.675	0.627	0.842	0.926	0.723	0.914	0.735	0.735
	Mean (of Group 3)	0.395	0.499	0.558	0.631	0.634	0.574	0.576	0.523	0.518
	± SEM	0.102	0.095	0.149	0.156	0.163	0.148	0.163	0.111	0.113
	Mean (of A)	0.309	0.499	0.648	0.679	0.643	0.594	0.534	0.487	0.501
	± SEM	0.196	0.153	0.302	0.271	0.260	0.320	0.260	0.201	0.191
	Mean (of B)	0.481	0.499	0.469	0.583	0.625	0.553	0.619	0.559	0.535
	± SEM	0.098	0.176	0.159	0.260	0.302	0.170	0.296	0.176	0.201
4 A	75	0.080	1.109	1.726	1.473	1.552	1.204	0.998	0.966	0.808
	76	0.239	1.981	2.157	2.946	2.433	2.583	2.620	2.696	2.783
B	79	0.239	0.489	0.527	0.614	0.878	1.617	0.865	0.727	0.702
	86	0.174	0.254	0.142	0.222	0.269	0.301	0.412	0.364	0.380
	Mean (of Group 4)	0.183	0.958	1.138	1.314	1.283	1.426	1.224	1.188	1.168
	± SEM	0.038	0.386	0.479	0.604	0.464	0.474	0.482	0.518	0.546
	Mean (of A)	0.160	1.545	1.942	2.210	1.993	1.894	1.809	1.831	1.796
	± SEM	0.080	0.436	0.216	0.737	0.441	0.690	0.811	0.865	0.988
	Mean (of B)	0.207	0.372	0.335	0.418	0.574	0.959	0.639	0.546	0.541
	± SEM	0.033	0.118	0.193	0.196	0.305	0.658	0.227	0.182	0.161

FIGURE 18

Mean plasma pepsinogen activities (I.U.) of two groups of previously parasite-naive lambs. Groups 3 and 4, each animal received adult O. circumcincta via abomasal cannulae on day 0. Animals in groups 3B and 4B received parasites from donors treated with meclofenamic acid. Animals in groups 3A and 4A received parasites from untreated donors. Group 3, each animal received 250 mg meclofenamic acid daily

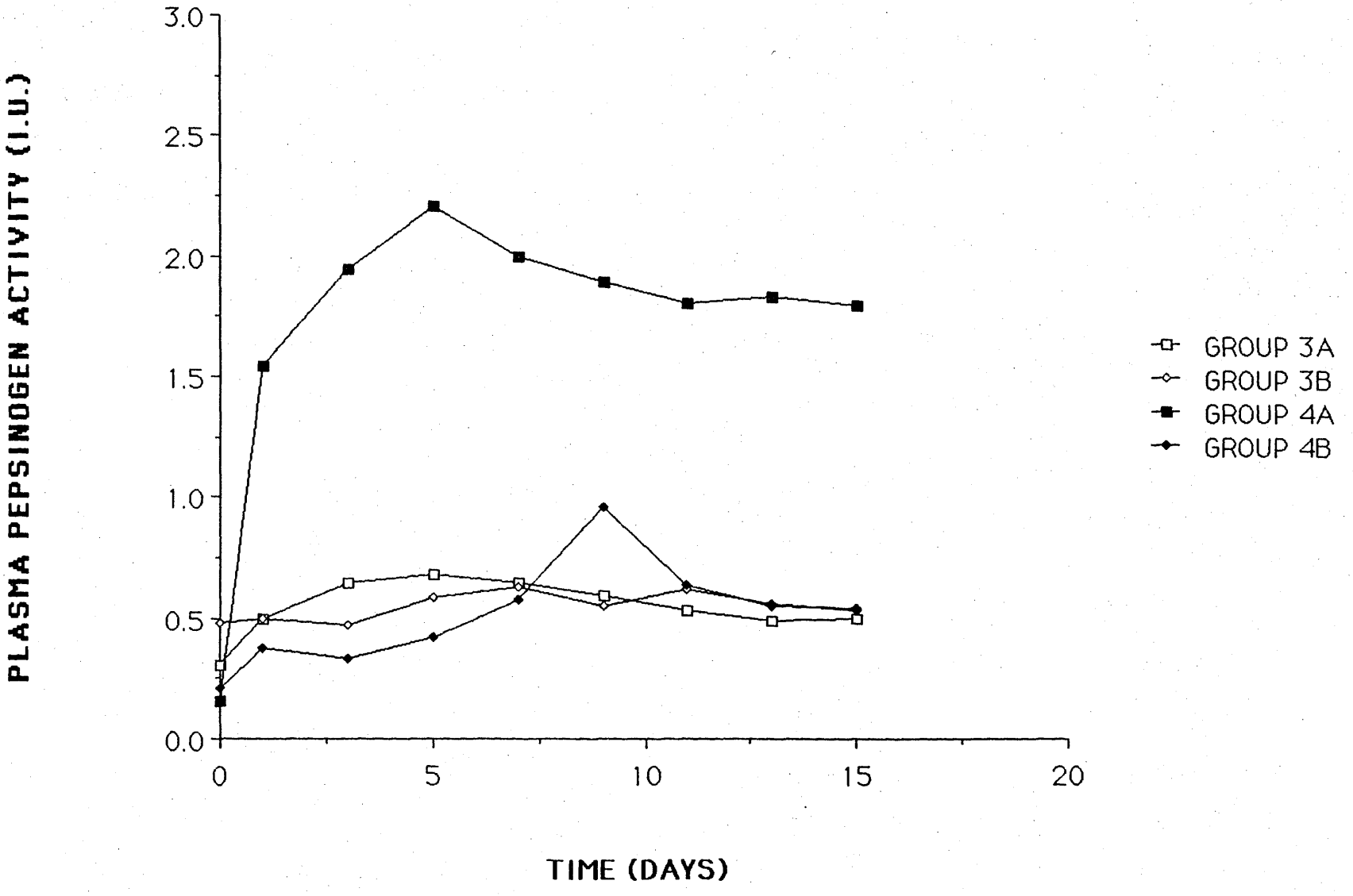

PLASMA PEPSINOGEN ACTIVITY (I.U.)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
TIME (DAYS)
GROUP 3A
GROUP 3B
GROUP 4A
GROUP 4B

TABLE 22

Numbers of parasites in the abomasi of group 3 and 4 lambs, at necropsy on day 15 of the experiment. Both groups of lambs received adult O.circumcincta on day 0. Groups 3B and 4B received parasites from donors treated with meclofenamic acid. Groups 3A and 4A received untreated parasites.

Group 3 lambs received 250 mg meclofenamic acid daily

Group number	Animal number	Abomasal contents					Abomasal digest					Total number in abomasum
		L_4	L_5	Adults ♂	Adults ♀	Total	L_4	L_5	Adults ♂	Adults ♀	Total	
3 A	1	0	0	2,000	1,400	3,400	0	0	100	100	200	3,600
	51	0	0	2,400	3,600	6,000	0	0	200	0	200	6,200
B	56	100	0	500	1,100	1,700	0	0	0	200	200	1,900
	93	0	0	500	800	1,300	0	0	0	0	0	1,300
											Mean	3,250
											± SEM	1,097.3
4 A	75	0	0	3,200	2,500	5,700	0	0	0	100	100	5,800
	76	0	0	3,000	3,800	6,800	0	0	200	0	200	7,000
B	79	0	0	500	400	900	0	0	0	0	0	900
	86	0	0	0	800	800	0	0	0	0	0	800
											Mean	3,625
											± SEM	1,620.9

Total number in abomasum -

Mean ± SEM	3A	4,900 ± 1,300.2
	3B	1,600 ± 300.0
Mean ± SEM	4A	6,400 ± 600.0
	4B	850 ± 50.0

TABLE 23

Percentage establishment of adult O. circumcincta in group 3 and 4 lambs. Group 3B and 4B received parasites from donors treated with meclofenamic acid. Group 3 lambs also received 250 mg meclofenamic acid daily

Group number	Animal number	Total number of parasites	Percentage establishment	Mean
3 A	1	3,600	23.96	32.62
	51	6,200	41.27	
B	56	1,900	55.07	46.38
	93	1,300	37.68	
4 A	75	5,800	38.60	42.60
	76	7,000	46.59	
B	79	900	26.09	24.64
	86	800	23.19	

TABLE 24

The motility of O. circumcincta L_3 in vitro as a percentage of control motility when incubated with various concentrations of meclofenamate

Concentration of meclofenamate (ug/ml)	Time (hours)				
	1	3	6	24	30
10	86.10	56.47	63.35	58.76	61.90
1	100	59.86	69.64	68.42	72.13
0.1	88.03	52.16	74.61	63.78	74.91
10	79.89	59.64	51.86	67.43	64.54
1	85.17	82.12	64.06	78.07	71.32
0.1	82.61	59.73	48.90	69.45	74.61
Mean ± SEM					
10	83.00 ± 3.11	58.06 ± 1.59***	57.61 ± 5.76***	63.10 ± 4.35***	63.22 ± 1.32**
1	92.59 ± 7.44	70.99 ± 11.16**	66.85 ± 2.80**	73.25 ± 4.84**	71.73 ± 0.41*
0.1	85.32 ± 2.72	55.95 ± 5.35***	61.76 ± 12.89**	66.62 ± 2.84**	74.76 ± 0.15*

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

indicating levels of significant difference from control values.

TABLE 25

The mean motility of *O. circumcincta* L_3 *in vitro* as a percentage of control motility during incubation with various concentrations of phenylbutazone

Time (hours)	Concentration phenylbutazone (ug/ml)		
	20	2	0.2
1	95.0	95.3	94.0
3	82.5	77.0	98.2
6	99.7	85.4	101.9
24	94.4	80.0	88.7
30	92.7	77.6	99.0

STUDIES INTO THE EFFECT OF A MUCOLYTIC, BROMHEXINE HYDROCHLORIDE, A CORTICOSTEROID, DEXAMETHASONE ACETATE; AND A SUBSTITUTED BENZIMIDAZOLE, OMEPRAZOLE, WHICH RAISES INTRAGASTRIC pH; ON THE RESPONSES OF IMMUNE SHEEP TO *Ostertagia circumcincta*

INTRODUCTION

The environment of the abomasal parasite in the sheep is made up of many components.

In order for the incoming *O. circumcincta* larvae (L_3) to mature in the abomasal glands, the mucus layer lining the gastrointestinal tract has to be penetrated.

When the parasite has reached the young adult stage, it emerges from the gland and maintains itself in close apposition to the mucosal surface and the mucus layer for the remainder of its life.

The abomasal mucus lining is therefore a very important component of the parasite's environment.

Gastrointestinal mucus

Structure

The mucus layer is a continuous lining to the gastrointestinal tract but there is a large variation in thickness along the tract as well as between species (Garner, Flemström and Allen, 1983).

Mucus contains large molecular weight glycoproteins which are responsible for its gel forming and viscous nature (Hollander, 1954; Allen, 1978).

Mucus contains 1% to 10% of its weight as the glycoproteins, 95% of its weight is water (Creeth, 1978). Dialyzable salts, 1%

by weight, give it an electrolyte composition close to plasma (Hollander, 1963). Immunoglobulins, plasma proteins, for example albumin and secretory IgA, some which is bound to the glycoproteins, DNA and enzymes can also be found (Forstner, Wesley and Forstner, 1982).

The mucus glycoproteins are very large molecules varying from 2×10^5 to 15×10^6 molecular weight and generally consist of a protein core surrounded by carbohydrate side chains (Allen, 1978; 1981).

In most cases, the glycoprotein polymers can be dissociated into subunits which do not possess the same viscous and gel forming properties (Allen, 1978). For example, native pig gastric mucus glycoprotein (molecular weight 2×10^6) can be dissociated into four subunits of equal weight (5×10^5) which are joined together by disulphide bridges. The sugar constituents of the side chains are:-

N-acetylglucosamine

N-acetylgalactosamine

galactose (a neutral hexose)

fucose (a deoxyhexose)

and various neuraminic (sialic) acids

The sugar constituents do not include uronic acid, a sugar characteristically found in the proteoglycans of connective tissue, or mannose which is found in serum and some membrane glycoproteins.

There is considerable variation in the carbohydrate side chains obtained from different regions of the gastrointestinal tract both in the same species and between different species

(Allen, 1978; 1981).

The glycoproteins possess a strong negative charge because of the presence of carbohydrate-bound ester sulphate and sialic acid residues.

The proteins contain large amounts of the amino acids, serine, threonine and proline when compared to typical globular proteins (Allen, 1981). Threonine and serine are involved in the α-O-glycosidic linkage to the carbohydrate side chains through N-acetylgalactosamine. This linkage is also found in other "non-mucus" glycoproteins including immunoglobulins (Carlson, 1977). Therefore the protein core is surrounded by closely packed carbohydrate chains which provide a protective sheath against proteolysis. However, not all of the protein core is covered and a protein similar in composition to a typical globular protein has been isolated from pig gastric mucus when subunits have been produced by breaking disulphide bonds. Human gastric mucus glycoprotein is thought to be similar (Allen, 1981).

Mucus is a viscoelastic gel, i.e. it has properties intermediate between a solid gel and a liquid (Litt, Wolf and Khan, 1977; Silberberg et al, 1977). Once fully hydrated it will remain in aqueous solution without swelling and when sectioned it will anneal.

All three properties can be explained at the molecular level and are due to the polymer-polymer interactions and polymer-solvent (water) interactions (Morris and Rees, 1978). The glycoproteins are "sticky" molecules with respect to themselves and others. They also bind to cell membranes (Allen and

Minnikin, 1975).

At dilute concentrations of glycoproteins, each molecule exists as an approximately spherical molecule. As the concentration is increased the glycoproteins begin to fill the whole volume (this filling occurs at a glycoprotein concentration of 20 mg/ml) and the viscosity starts to increase sharply (Allen, Pain and Robson, 1976; Allen, 1978). As the concentration increases still further, the molecules overlap more and more, the noncovalent interactions between the molecules increase as do the viscoelastic properties and a gel forms at a concentration of glycoprotein of approximately 50 mg/ml. If, at this concentration, the polymers are split into subunits by breaking the disulphide bonds, the viscoelastic properties are lost. This is found in many types of mucus. However, sheep and cattle submaxillary mucus and rat small intestinal mucus do not possess a structure dependent on disulphide bonds. Instead, the gel formation is due to the interlinking of the negatively-charged long-chain polymers (Holden et al, 1971, Forstner, Jabbal and Forstner, 1973; Hill, Reynolds and Hill, 1977).

Biosynthetic pathway of production

The gastrointestinal mucus layer is maintained by the secretions of the following cells:-

Oesophageal mucus glands

Cardiac, fundic and pyloric glands of the stomach/abomasum

Brünners glands of the duodenum

Goblet cells of the small and large intestine

Within these cells the following biosynthesis takes place.

The protein core of the glycoprotein is formed at membrane-bound ribosomes by the translation of messenger ribonucleic acid (mRNA). The carbohydrate side chains are added as the material passes through the rough endoplasmic reticulum (RER) and then the smooth endoplasmic reticulum (SER) to the Golgi apparatus. The carbohydrate side chains begin with the attachment of N-acetylgalactosamine to serine or threonine and sugars are added one by one. In the Golgi appartus the glycoprotein is packed into secretory vesicles and secreted into the lumen of the gastrointestinal tract by fusion of the vesicles with the plasma membrane of the cell (Carlson, 1977).

Control of secretion

The depth of the mucus layer is the result of the balance between mucus secretion and erosion.

The rate of mucus secretion can be controlled at two levels.

(i) the secretion of preformed mucus.

(ii) the biosynthesis of the mucus.

Studies in the dog gastric mucosa show three mechanisms by which mucus is released (Zalewsky and Moody, 1979).

(i) continuous exostosis of a small number of vesicles at one time.

(ii) explosive release of the apical portion of mucus-producing cells. This was followed by <u>in situ</u> degeneration of the cell. The explosive release was observed in the oldest cells forming crests in the interfoveolar area (i.e. the area between gastric pits).

(iii) cell exfoliation in which the entire cell was extruded into the gastric lumen. This is a relatively rare event, but probably occurs when irritants e.g. mustard oil are administered experimentally (Hollander, 1954).

Neural and hormonal influences can affect mucus output. Acetylcholine administered topically, or its release by stimulation of splanchnic or vagal nerves, stimulates gastric mucus production in the dog (Hollander, 1954; Florey, 1955). In the rabbit and human colon, acetylcholine increases the rate of mucus secretion but does not increase biosynthesis. Carbachol also increases mucus secretion (Black, Bradbury and Wyllie, 1979). Luminal pH may also play a part in mucus secretion. Acidification of the canine gastric antrum was found to be a most potent stimulation of mucus secretion (Menguy, 1969). The hormone secretin increases the amount of sugar molecules in the gastric mucus of man, cats and dogs (André, Lambert and Descos, 1972; Vagne, 1974; Kowalewski, Pachkowski and Kolodej, 1978) in addition to its effects on gastric secretions and motility.

Prostaglandins have been found to increase the production of a viscous mucus in man (Domschke et al, 1978). Bolton, Palmer and Cohen (1978) reported a similar effect in rats but only measured the amount of soluble glycoprotein in the luminal contents and not the adherent mucus gel. The effect of prostaglandins is possibly mediated by an increase in cyclic AMP (adenosine 3', 5' monophosphate) within the mucus-producing cells (Parke and Symons, 1977).

Prostaglandins E_1 and E_2 administered orally to rats produced an increased incorporation of N-acetyl [^{3}H] glycosamine

into gastric mucosal glycoprotein (Johston, Symons and Parke, 1975) indicating an increase in mucus biosynthesis as well as secretion. This is interesting in view of prostaglandins' possible cytoprotective effect in the gastric mucosa and their stimulatory effect on bicarbonate secretion by gastric and duodenal mucosal cells.

Leukotrienes have also been implicated in mucus secretion. Leukotrienes C_4 and D_4 increase mucus secretion in the canine trachea, leukotriene D_4 being the more potent (Johnson and McNee, 1983). Coles et al (1983) found a similar effect using human bronchial mucosa, the leukotrienes C_4 and D_4 being one hundred times more effective on a molar basis than cholinergic agonists. The effect on the mucus producing cells was not thought to be mediated through specific receptors as leukotriene analogues were also active.

The anaphylatoxin C3a is also a potent secretagogue for airway mucus (Marom et al, 1985). 5-hydroxytryptamine (5-HT) also increases mucus production in the rat colon (Menguy, 1969; Black, Bradbury and Wyllie, 1979).

In human bronchi, in vitro, histamine has been shown to cause an increase in mucus secretion, both by application of histamine and by mast cell degranulation. This effect could be blocked by cimetidine, a histamine H_2 receptor agonist (Shelhamer, Marom and Kaliner, 1980).

A number of anti-inflammatory agents have been studied in their relationship to mucus biosynthesis and release. Many anti-inflammatory drugs decrease mucus biosynthesis (Menguy, 1969;

Glass and Slomiany, 1977; Parke, 1978).

Salicylates decrease mucus production in the rat and dog stomachs and decrease glycoprotein biosynthesis in the sheep colon in vitro (Kent and Allen, 1968). Indomethacin and phenylbutazone also decrease the biosynthesis of mucus glycoprotein (Shillingford, 1975).

Corticosteroids and adrenocorticotropic hormone (ACTH) decrease the amount of stainable mucus in the mucosa and the amount of glycoprotein sugars in gastric mucus (Desbaillets and Menguy, 1967; Glass and Slomiany, 1977). Desbaillets and Menguy (1967) suggested that this reduction in carbohydrate may make the glycoproteins more subject to proteolytic enzymes and therefore less protective.

In the dog, corticosteroids initially produce hyperplasia and increase glycoprotein production which is then reversed (Glass and Slomiany, 1977).

Immunological reactions have also been shown to affect mucus secretion. Shelhamer, Marom and Kaliner (1980) showed that immunoglobulin IgE andantigen produced an increase in mucus production, probably mediated partly through histamine release i.e. anaphylaxis stimulates mucus release (Lake et al, 1980). Lake et al (1979) found that rats sensitised to bovine serum albumin produced a release of goblet cell mucus when bovine serum albumin was administered. Also, the addition of IgA to purified dog gastric glycoproteins caused a significant increase in viscosity (Murty et al, 1984).

Degradation of the mucus layer

There are two main forces which act on gastrointestinal

mucus.

Mechanical erosion takes place and reduces the depth of the mucus layer. Also the action of proteolytic enzymes (e.g. pepsin and trypsin) degrade the surface of the mucus (Hoskins, 1978). (The structure of mucus will not allow large molecular weight substances through, therefore pepsin {molecular weight 35,000} can only act on the gel surface). The results of the continuous erosion of the mucus are soluble glycoproteins which are further degraded by bacterial enzymes to low molecular weight sugars and amino acids which are utilised by enteric flora or the body itself.

The turnover of gastric mucus can be summarised as in Figure 19.

Histochemistry of mucus secretions

Mucus secretions can be divided on the basis of their histochemical staining.

(a) neutral - these stain only with the Periodic Acid Schiff (PAS) technique

(b) sialic acid containing] negatively charged, acid
(c) ester sulphate containing] secretion. These stain only with basophilic dyes e.g. Alcian blue. They can be distinguished by the use of staining solutions of different pH (Glass and Slomiany, 1977; Lev, 1977, Filipe, 1979).

Functions of gastrointestinal mucus

Mucus fulfils many functions.

(i) Protection against mechanical damage.

(ii) Water retention. It prevents dehydration of the mucosal surface.

(iii) It has a role in protection against autodigestion.

(iv) Possible role in protection against ulceration.

(v) It may help to protect the host from gastrointestinal parasites.

(vi) Mucus has antibacterial and antiviral functions.

(vii) Nutritional. Mucus helps to maintain gastrointestinal flora.

The first five functions will be discussed in more detail.

Protection against mechanical damage

The mucus gel layer is present as a continuous layer over the gastrointestinal mucosa. The nature of the mucus allows it to lubricate the passage of food material along the tract (Florey, 1955).

Water retention

The mucus layer is composed 95% by weight of water most of which is contained between the glycoprotein molecules. The carbohydrate chains of the glycoproteins ensure a strong attachment to the water molecules. This therefore provides a perpetual aqueous environment for the underlying mucosa (Silberberg et al, 1977).

Protection against autodigestion

As stated previously, the mucus structure does not allow large molecular weight substances e.g. proteolytic enzymes to penetrate and therefore protects the underlying mucosa from their effects.

Hydrogen ions (H^+) are able to diffuse across the mucus layer so it does not simply act as a barrier to the acid produced in the gastrointestinal tract (Williams and Turnberg, 1979) It does however, because of its dense molecular structure containing water, provide a barrier to gross mixing up to the mucosal surface (Heatley, 1959). In addition to this, the gastric and duodenal mucosa secretes bicarbonate ions which, because of the unstirred mucus layer, are restricted to the surface and incoming hydrogen ions are neutralised (Garner, Flemström and Allen, 1983). Support for this is given by the fact that there is a pH gradient across the mucus layer. In the rabbit, a pH of 2.36 was measured at the luminal side of the mucus layer while in the same animal a pH of 7.59 at the mucosal surface was recorded (Williams and Turnberg, 1979). A similar pH gradient has also been detected in the frog gastric mucus gel (Takeuchi et al, 1983).

The importance of this role is underlined by the fact that isolated gastric mucus-secreting cells cease to respire at pH 5 or less in vitro. In vivo, the mucus layer is the only structure separating these cells from a pH of 1 to 2 (Snary and Allen, 1972).

Protection against ulceration

This role is based mainly on circumstantial evidence. Gastric mucus production is inhibited by various ulcerogenic

agents e.g. salicylate (Kent and Allen, 1968) but anti-ulcer agents e.g. prostaglandins are reported to increase mucus production (Bolton, Palmer and Cohen, 1978). The greater the depth of the mucus gel, the greater its protective effect. Also alterations in the glycoprotein structure may alter its protective effect. In human gastric ulcer patients, there is a decrease in the amount of native polymeric glycoprotein in the gastric mucus and an increase in low molecular weight (degraded) glycoproteins. Patients with duodenal ulcers show similar, but not so marked, changes in the gastric mucus (Allen et al, 1981). Some workers have described a hydrophobic phospholipid layer below the mucus gel in the stomach that may also be involved in cytoprotection (Lichtenberger et al, 1983; Szelenyi and Engler, 1986). Prostaglandins may be involved in the maintenance of this layer.

Role of gastrointestinal mucus in protection against parasites

A number of studies have shown that mucus plays an important role in the immunity of animals to gastrointestinal parasites. It is also thought that the mucus could provide chemical or physical signals that direct the parasites to their environmental niche within the gastrointestinal tract (Miller, 1987).

In rats immune to the intestinal parasite, Nippostrongylus brasiliensis, the incoming larvae are excluded from the mucosal surface and further down the intestine become trapped within the mucus layer and expelled within two to three hours (termed 'rapid expulsion') (Miller, Huntley and Wallace, 1981). The mucus trapping was not necessarily the cause of

expulsion from the mucosa as the parasites only became enveloped in the mucus further down the gastrointestinal tract.

A similar phenomenon has been observed with Trichinella spiralis infection of rats where it was reported that the property of immune mucus could be transferred, to some extent, to parasite naive animals by intraperitoneal injection of immune serum at the same time as the larval challenge (Lee and Ogilvie, 1982).

Miller and Huntley (1982b) disrupted the mucus of immune rats by the use of a mucolytic and in doing so prevented the exclusion and expulsion of the Nippostrongylus larvae. Work by the same authors (Miller and Huntley, 1982a) also showed that treatment of immune rats with corticosteroids prevented the exclusion and expulsion of the parasites. A similar result was obtained with sheep immune to Haemonchus contortus.

The role of abomasal mucus in the immunity of sheep to H. contortus is not as clear as that in N. brasiliensis infection. Hyperimmune sheep challenged with H. contortus larvae allow them to penetrate the mucus layer to the mucosa, but they are prevented from entering the abomasal glands. Many larvae are enveloped in the superficial mucus (Miller et al, 1983).

In vitro work with mucus has underlined its possible role in immunity. Dobson (1966a, b; 1967) showed that mucus from sheep immune to the parasite Oesophagostomum columbianum inhibited the oxygen uptake of larvae in vitro. The mucus contained high titres of haemagglutinating antibody but did not precipitate parasite antigens. However, intestinal homogenates did contain precipitating antibodies. The effect on oxygen uptake was found

not to be specifically against one parasite, but to affect another, unrelated parasite, Nematospiroides dubius.

Other work concerning gastrointestinal mucus from sheep by Douch et al (1983) showed that mucus from immune sheep inhibited the migration of parasitic larvae. Again, this was not directed specifically against the parasite to which immunity was expressed. The substance within the mucus that appeared to produce this effect had the characteristics of slow reacting substance of anaphylaxis (SRS-A) known to consist of the leukotrienes C_4, D_4 and E_4. The authors suggest that the leukotrienes may produce paralysis of the larvae and prevent them establishing in the host tissues.

In contrast, Lee and Ogilvie (1982) implicated complement and non-IgA antibodies as being involved in the mucus-trapping of T. spiralis. Bell, Adams and Ogden (1984) suggest that IgG is involved, associated with systemic sensitisation that occurs during the tissue stages of the parasite.

Sheep, hyperimmune to H. contortus, have been found to contain significant concentrations of IgG in mucus, even after partial purification (Dawson and Miller, cited Miller, 1987).

Studies with N. brasiliensis have shown, by immunoperoxidase staining, that the parasite ingests luminal mucus during the infection, but this was reduced as the host's immunity developed (Miller, Huntley and Dawson, 1981). This is therefore another possible way in which substances in the gastrointestinal mucus could affect the parasite.

The number of mucus-producing cells and their association with the establishment of immunity in animals has also been investigated.

Ostertagia circumcincta infection in naive, non-immune sheep produces a hyperplasia of the mucus-producing cells early in the infection. This takes place in the abomasal glands that previously housed the developing larvae before the young adults emerged onto the mucosal surface (Armour, Jarrett and Jennings, 1966). This is also observed in calves infected with Ostertagia ostertagi (Ritchie et al, 1966).

N. brasiliensis infection in rats produces an increase in the number of goblet cells, which is associated with the expulsion of the parasite (Miller and Nawa, 1979a, b) and this response could also be produced in parasitised rats by the transfer of thoracic duct lymphocytes, particularly those lacking surface immunoglobulin, from immune rats (Miller, Nawa and Parish, 1979). This would suggest a T-cell dependent event. Therefore evidence is provided for the intestinal immune system having control over the intestinal mucosa.

This theory is also supported by Castro (1982) where it was found that in animals developing immunity to T. spiralis, there is an alteration in the mucosal brush-border enzymes, as measured by the reduced binding of wheat-germ agglutinin. The binding remained lowered for at least three months after the parasites were eliminated. This finding could also be an alternative mechanism for immune exclusion of parasites in contrast to a role for the mucus layer.

The phenomenon of exclusion of parasites from immune animals seems to depend on the size of the challenge dose. A lower percentage of parasites are excluded when challenged with a small number of parasites when compared to a higher number. This is the case for O. circumcincta (Smith et al, 1985) and H. contortus (Jackson, Newlands and Miller, cited Miller, 1987) but not for the rat challenged with N. brasiliensis (Miller, Huntley and Wallace, 1981). It would seem that in some infestations a threshold burden may be needed which stimulates the local immune response and the immune exclusion. This is supported by work by Smith et al (1984) that detected an output of IgA and blast cells in gastric lymph following challenge with 5,000 O. circumcincta larvae, but not with 1,000.

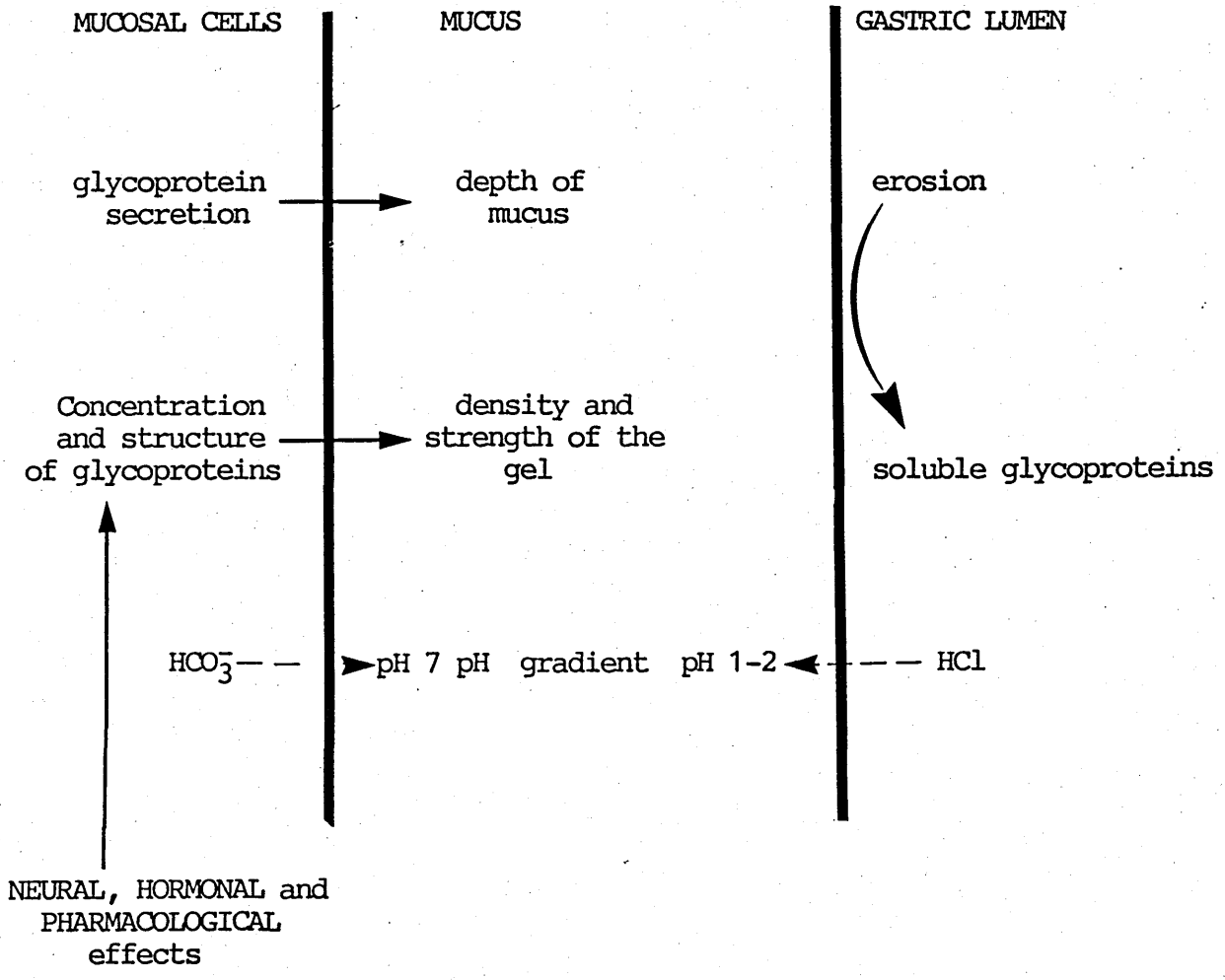
Increases in the numbers of mucus-producing cells have also been reported in O. columbianum infestations (Dobson, 1967) but found only in sheep on high protein diets. Poorly fed sheep did not show an increase (Dobson and Bawden, 1974). In Trichostrongylus colubriformis infestation in sheep there is also an increase in mucus-producing cell numbers. When this increase was related to the immunity of the animal and the anti-parasitic activity of the gastrointestinal mucus in preventing larval migration in vitro, it was found that there was a poor correlation. Globule leukocyte and eosinophil numbers appeared to be more closely related to immunity (Douch et al, 1986).

In contrast, Strongyloides ratti infestation and expulsion in rats is not associated with increased numbers of goblet cells (Mimori et al, 1982).

Therefore, it would seem that in a number of host-parasite relationships, gastrointestinal mucus plays an important role in host protection. The effects on mucus of the numerous changes that take place in immune animals after challenge, for example, increased mucosal permeability, release of non-specific mediators from globule leukocytes etc. also need to be elucidated.

FIGURE 19

Factors important in the maintenance of the gastric mucus layer


MUCOSAL CELLS
MUCUS
GASTRIC LUMEN
glycoprotein secretion
depth of mucus
erosion
Concentration and structure of glycoproteins
density and strength of the gel
soluble glycoproteins
HCO_3^-
pH 7 pH gradient pH 1-2
HCl
NEURAL, HORMONAL and PHARMACOLOGICAL effects

Bromhexine hydrochloride

Bromhexine hydrochloride (N-cyclohexyl-N-methyl-(2-amino -3, 5-dibromobenzyl)-ammonium chloride is a benzylamine compound derived from vasicine, which is an alkaloid of the Asian plant Adhatoda vasica (Amin and Mehta, 1959). The structure is shown in Figure 20. It is marketed in Britain by Boehringer Ingelheim for both human and veterinary use as a bronchial mucolytic (Bisolvon Injection, Powder, Tablets and Elixir). It is also available in combination with oxytetracycline (Bisolvomycin Injection). In Britain it is marketed for use in horses, cattle, pigs and small animals as well as man. Early workers (Burgi and Regli, 1967) studying the effect of bromhexine on bronchial mucus detected fragmentation of the acid mucopolysaccharide fibres (acid-staining glycoproteins) within the mucus, producing a decrease in the viscosity. The same workers showed a change in the goblet cell contents of the bronchi of guinea-pigs receiving bromhexine. There appeared to be more protein-bound carbohydrate contained within the cells and they concluded that the acid mucopolysaccharides were changed in structure as a result of treatment.

Other work using electron microscopic studies have suggested that there is an increase in lysosomal activity in the epithelial cells of the bronchial mucosa. The hydrolytic enzymes that they produce may explain the breakdown of glycoproteins within the mucus secretion (Caird et al, 1972; Takeda, Misawa and Yanaura, 1983).

Further studies into the effect of bromhexine on glycoprotein structure using isotopic labelling on hen tracheal

preparations, have demonstrated that there is not a general inhibition of glycoprotein synthesis. However, there does appear to be an effect on the rate of glycoprotein release from secreting cells that may be selective for certain glycoprotein components of mucus. Weakly acidic, major components are suppressed in favour of more acidic and highly sulphated fractions (Kent and Rogers [Boehringer Ingelheim Research Report], 1980). Marriott, Readman and Barrett-Bee (1983) studied the effect of bromhexine on pig tracheal mucus. It produced a general decrease in viscosity and also affected the mucus glycoproteins. The concentration of hexose rose, while the protein concentration fell, therefore the hexose/protein ratio exhibited a rise which continued after the termination of treatment suggesting a prolonged effect after drug withdrawal.

Attention has also been drawn to the fact that bromhexine treatment appears to increase certain protein fractions in the mucus, particularly immunoglobulins, with a simultaneous decrease in albumin and α-globulin fractions (Bürgi and Regli, 1967). These authors assumed that treatment with bromhexine activated the local humoral immune resistance to disease in addition to its mucolytic effect. Further studies by Götz (1970) have shown the increase to be due to a rise in secretory IgA concentration and to a lesser extent IgG concentration within the mucus. The author agrees that there may be stimulation of IgA-producing elements during bromhexine treatment but suggests a more plausible explanation. In the less viscous mucus produced by bromhexine treatment, IgA may be more effectively liberated from

the mucus. There may also be an increased permeability of the mucous membrane epithelia. Therefore, all the work shows that bromhexine does produce a less viscous bronchial mucus secretion with changes in the glycoprotein structure which may or may not contribute to increased amounts of IgA measured within the gel.

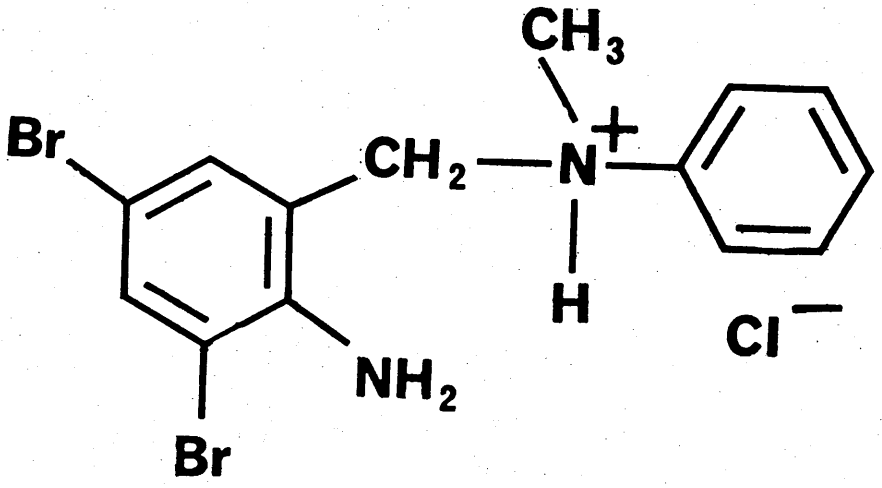
The drug is generally well tolerated but there have been largely unsubstantiated reports of gastric discomfort and nausea in human patients treated with the drug (Anon, 1971). Therefore, the effect of bromhexine treatment on gastrointestinal mucus is an important aspect of this research.

Guslandi (1982) suggests that treatment with mucolytics in patients with gastroduodenal disorders should be discouraged because of possible reduction in gastrointestinal mucus viscosity. Marriott (personal communication) also suggests that mucolytic drugs administered orally could affect gastrointestinal ulceration.

Studies with a bromhexine metabolite, ambroxol, which also acts as a bronchial mucolytic have shown no effect on gastric mucus in man (Guslandi and Zanoni, 1985). However, work by Szelenyi and Engler (1986) showed that ambroxol was cytoprotective in the gastrointestinal tract by increasing the surface active phospholipids below the mucus layer (a phospholipid layer equivalent to pulmonary surfactant in the lung which ambroxol is also known to promote). Bromhexine is also known to promote surfactant secretion in the lung and therefore it is possible that it shares this cytoprotective effect to some extent in the gastrointestinal tract.

FIGURE 20

Structure of bromexhine hydrochloride


CH3
Br
CH2
N+
H
Cl−
NH2
Br

The effects of bromhexine on gastrointestinal mucus are therefore not known.

The metabolism of bromhexine is known to show great differences between species. Humans and rabbits exhibit mainly urinary excretion, dogs and rats excrete about 50% in faeces (Kent and Rogers [Boehringer Ingelheim Research Report], 1980). Residue studies in calves, after oral administration of bromhexine for seven days, have shown that highest concentrations are found three hours after final administration in the liver at 220ug/kg, kidney at 79 ug/kg, muscle at 17 ug/kg and in the lung at 1 ug/kg. Seven days post-treatment, the concentrations in the tissues had reduced significantly, only the fat contained 68 ug/kg and kidney 12 ug/kg (Eichler and Kreuzer, 1975).

PRELIMINARY INVESTIGATION INTO THE EFFECTS OF BROMHEXINE HYDROCHLORIDE IN SHEEP

Materials and Methods

Animals

The animals used in this experiment were Scottish Blackface cross ewes aged greater than five years. Their weights ranged from 50 to 60 kg.

Experimental design

Group	Sheep number	Procedure
Control	1 58 121	No treatment
Bromhexine	64 65	Treated with bromhexine hydrochloride at a dose rate of 0.5 mg/kg daily for 3 days

Administration of bromhexine hydrochloride

Bromhexine hydrochloride was administered orally as a suspension in 20 ml of water. 0.5 mg/kg bodyweight was administered once daily to each sheep in the bromhexine group for three days. All the animals were killed on the third day of bromhexine administration.

Sampling techniques

(i) Mucus samples

Mucus was obtained from the abomasum and duodenum at necropsy. The samples were collected by carefully removing gross food contamination from the mucosal surface and then gently scraping with the edge of a glass microscope slide. An attempt was made to collect samples of bronchial mucus secretions but there was insufficient in both groups of sheep.

(ii) Histological sections

Sections were taken from the abomasal pylorus, anterior duodenum and anterior lung lobe. The samples were fixed in buffered 10% formalin and routinely processed, embedded, sectioned and stained with alcian blue/Periodic Acid Schiff pH 2.5 and pH 1.0 after digestion with diastase to remove glycogen.

Treatment of samples

It was found to be impossible to remove all food contamination without reducing markedly the amount of mucus collected. For this reason, a capillary viscometer (Coulter Electronics, Harpenden, Hertfordshire) was the only instrument available to measure the mucus viscosity and able to withstand

the presence of small food particles within the mucus. The mucus, however, had to be diluted 1 : 1 (v/v) with phosphate buffered saline (PBS) (pH 7) and homogenised using an Ultra-Turrax tissue homogeniser (Janke and Kunkel GmBH). The resultant suspension was centrifuged at 1,500 x g for 20 minutes and the supernatant stored at 4°C for less than 24 hours before the viscosity was measured. Insufficient abomasal mucus was collected to be measured in this manner.

Results

Mucus viscosity

There was no observable difference in the viscosity of the duodenal mucus between bromhexine-treated and untreated sheep.

The results of the viscosity measurements on the duodenal mucus samples are shown in Table 26.

Assuming a similar glycoprotein content of the samples there was little difference between the viscosity of those animals treated with bromhexine and the controls.

Histology

There was no obvious difference in mucus quantity, number of mucus-producing cells or staining characteristics of the mucus between treated animals and untreated controls. However, all the sections from the untreated controls showed paler staining than the treated animals. This was apparent at pH 1.0 and pH 2.5 of Alcian blue/PAS. Also, the submucosal duodenal glands of the untreated animal appeared less voluminous than those in bromhexine-treated animals.

Discussion

The histological evidence from this trial suggests that bromhexine hydrochloride did exert an effect on gastrointestinal mucus, possibly altering mucus character and secretion. Obviously, a reduction in the mucus viscosity would have proved its effect. However, it was impossible to measure viscosity accurately or detect small changes with the equipment available. A measurement of glycoprotein concentration may have determined viscosity changes indirectly, i.e. if the treated samples contained higher concentrations of glycoprotein than the untreated controls, the viscosity could have been reduced by the bromhexine treatment. Also, measuring the hexose/protein ratio may have detected changes in mucus structure.

Histology of the abomasum of the ruminant

The ruminant stomach consists of four parts: rumen, reticulum, omasum and abomasum. The forestomach consists of the first three chambers and is derived from the oesophageal region of the stomach. It is lined by an aglandular, stratified squamous epithelium.

The function of the forestomachs is to break down ingesta through mechanical and chemical activity. The rumen acts as a large fermentation vat in which bacteria and protozoa break down the ingested food and produce volatile fatty acids (VFAs), which are absorbed. The mechanical action of the reticulum and omasum converts the broken-down ingesta into quite fine, particulate

matter. The ingesta is then moved into the abomasum where enzymic digestion takes place.

The abomasum is the glandular stomach. Food delayed within the stomach is subjected to the hydrolytic and enzymatic action of the gastric secretions. The muscular wall of the organ induces mechanical mixing and breakup of the food. Peristaltic waves of contraction propel the partially digested foodstuffs into the duodenum.

Mucosa

The mucosa of the abomasum is folded, with the folds orientated parallel to the long axis. The epithelial surface is divided into small irregular units called gastric areas by numerous small grooves. The mucosal surface is marked with many small depressions called gastric pits. The gastric glands open into the bottom of the gastric pits.

The mucosal cells, including the gastric pits are composed of a simple columnar epithelium. The cells lining the abomasum are mucus-secreting.

Below the mucosa, the lamina propria contains lymphocytes, macrophages and plasma cells that impart a distinct hypercellularity. Scattered lymphatic follicles may also be present. Gastric glands penetrate various distances into the lamina propria.

A lamina muscularis mucosa is present consisting of two to four smooth muscle layers, orientated longitudinally and

circularly. Thin strands of smooth muscle extend into the lamina propria between the glands (Banks, 1986b).

Submucosa

Neuronal fibres and ganglion cell cytons form the submucosal (Meissner's) plexus.

Muscularis

Neurones and neuronal processes form the myenteric (Auerbach's) plexus between the inner and outer laminal of smooth muscle.

Glandular regions

The abomasum can be divided into three distinct regions:-

(i) Cardiac gland region

This is a very small area. The beginning is marked by the transition from the stratified squamous epithelium of the forestomachs, to columnar epithelium.

The cardiac glands are branched, tubular, coiled glands that can be divided into two regions.

The neck is the portion nearest the opening of the gastric pit. The body makes up the remainder of the gland. The neck and the upper part of body are lined by mucus-secreting cuboidal cells. The remaining cells are columnar, mucus-secreting cells.

Argentaffin (enterochromaffin) cells are small, pyramidal cells located between the glandular lining cells and the basement membrane. They are demonstrated by specific silver stains. As many as ten different cell types have been recognised as argentaffin cells. The products of these cells are secreted into the blood vessels of the lamina propria. They are hormone-

secreting cells, or gastrointestinal endocrine cells (enteroendocrine). Secretions of these cells include 5-hydroxytryptamine (5-HT), histamine and gastrin. These hormones exert some control on the secretory and muscular activities of the gastrointestinal tract.

(ii) Fundic gland region

The glands in this region are tubular glands that are longer but less branched than the cardiac glands. The fundic gland can be divided into four areas, base, body, neck and isthmus. The isthmus is the opening of gland continuous with the constricted neck. The body is the main portion of the gland that ends in a slightly dilated base.

Three cell types can be seen in this region, within the glands.

Mucus neck cells line the neck of the gland and are interspersed between the parietal cells. They may be capable of differentiating into surface lining and/or glandular lining cells.

Zymogen (chief) cells are the predominant cell type of the fundic gland. They are pyramidal-shaped cells with a round nucleus situated basally. Secretory (zymogen) granules are present in the apical portion of the cell. The basal portion of the cell contains rough endoplasmic reticulum (RER) and free ribosomes that produce a basophilia when stained. These cells are responsible for the synthesis and secretion of pepsinogen, rennin and gastric lipase.

Parietal (oxyntic) cells are easily distinguished by their characteristic staining with acidic dyes such as eosin, which is probably due to the large number of mitochondria (Ito, 1981). They are large cells scattered throughout the length of the gland. They are wedged between zymogen cells. Their basal borders are in contact with the basement membrane but not all of the cells reach the luminal surface of the gland.

When active, numerous canaliculi extend from the apical plasma membrane into the cytoplasm of the cell. Many microvilli project into the canaliculi. Parietal cells secrete hydrochloric acid.

(iii) Pyloric gland region (antrum)

The gastric pits are longer in this region, but the glands are short, simple or branched tubular glands. The predominant cell is the mucus-producing cell.

The parietal cell

The parietal cell is present within the fundic glands of the ovine abomasum. The ovine abomasum is normally maintained at pH 2-3.

Ultrastructure

The appearance of the parietal cell changes when active, secreting hydrochloric acid, compared to its resting state (Sedar and Friedman, 1961, Helander and Hirschowitz, 1972; Sachs and Berglindh, 1981; Berglindh, 1984).

The cells contain a large number of mitochondria (Helander and Hirschowitz, 1972) and are probably the cells richest in mitochondria in the body (Berglindh, 1984). At the apical area of the cell there is an infolding of the plasma membrane, forming

the intracellular canaliculus.

Electron microscopy of a resting cell shows that the cytoplasm is filled with round or elongated smooth membrane structures, termed tubulovesicles. During acid secretion there is a decrease in these structures, and an expansion of the intracellular canaliculus. The surface area of the canaliculus is further increased by numerous microvilli. Therefore the secretory surface of the cell increases greatly. This is the site of acid secretion from the cell.

Stimulation of acid secretion

Three different types of gastric acid stimulants have been discovered. These are, histamine, cholinergic compounds, for example, acetylcholine and peptides, for example, gastrin.

Workers have studied the effects of these stimulants and their specific antagonists on parietal cells both in vivo and in vitro. There is still a great deal of controversy as to the mode of action of these agents, mainly due to the different results obtained in vivo, when compared to isolated glands or parietal cells in vitro.

Associated with the stimulation of parietal cells, there is an increase in gastric blood flow, both in the total blood perfusing the stomach and a redistribution causing an increase of blood to the gastric glands (Jacobson, Swan and Grossman, 1967).

(i) Results obtained from in vivo studies

Histamine produced a potent and dose dependent stimulation of acid secretion. This effect is strongly inhibited by histamine H_2-receptor antagonists and prostaglandins. Atropine

only inhibits the response at very high concentrations. Cholinergic influence, from vagal stimulation or by the action of stable muscarinic agonists produce high rates of acid secretion. This response is inhibited by atropine, histamine H_2-receptor antagonists and prostaglandins.

Gastrin also causes a potent and dose dependent stimulation of the parietal cell. This effect is inhibited by histamine H_2-receptor antagonists and prostaglandins. Atropine is only effective at high concentrations (Sachs and Berglindh, 1981). In vivo interactions are also thought to occur between different agonists (Hirschowitz and Sachs, 1965). Therefore, in vivo, histamine H_2-receptor antagonists (for example, cimetidine) act to prevent the stimulation caused by all three types of stimulant. This has led some workers to believe that the response to histamine is the final, common pathway for parietal cell stimulation and histamine H_2 receptors, distinct from the H_1 receptors on smooth muscle etc., are present on the parietal cell (Black et al, 1972).

(i) Results obtained from in vitro studies

Studies in vitro using isolated cells do not show this wide effect of histamine H_2 antagonists (Soll, 1978a) and others regard the evidence as suggesting that there are specific cholinergic, muscarinic (Sachs and Berglindh, 1981) and gastrin receptors (Takeuchi, Speir and Johnson, 1979a, b) as well as histamine H_2 receptors.

Potentiating interactions between histamine and the other stimulants have been shown to occur in parietal cells *in vitro* (Soll, 1978b). It has been suggested that *in vivo*, the parietal cell, is constantly under the influence of histamine and acetylcholine which could explain the different results *in vivo* compared to *in vitro*.

The processes that occur within the cell after the stimulant-receptor coupling are not fully understood. From *in vitro* studies it would appear that histamine stimulation causes an increase in cyclic AMP (adenosine 3', 5' monophosphate) (cAMP) via adenylcyclase activation The cAMP would therefore act as a secondary messenger and promote acid secretion, possibly involving protein phosphorylation.

Cholinergic stimulation, however, appears to produce an increase in calcium ions within the cell, by an increased influx across the plasma membrane. The subsequent action of the increased calcium concentration is not known, but could involve binding to the protein calmodulin and effects on enzymes such as adenylate cyclase, phosphodiesterase and phospholipase A_2 (Cheung, 1980; Sachs and Berglindh, 1981).

Gastrin may also cause an increase in calcium ions within the cell.

Role of prostaglandins in parietal cell secretion

Prostaglandins have been shown to have strong inhibitory actions on gastric acid secretion. They inhibit the histamine induced rise in cAMP within the parietal cell (Wollin, Soll and Samloff, 1979; Soll, 1980).

Prostaglandins may also have a role in the calcium ion dependent pathway of acid secretion. Prostaglandins are derived from arachidonic acid present within phospholipids. The release of the precursor is dependent on the activation of the calcium-dependent enzyme, phospholipase A_2 (Marshall, Dixon and Hokin, 1980). Therefore prostaglandins could provide a regulatory system between the cAMP and calcium ion secondary messengers.

Enzyme systems present in parietal cells

(H^+K^+) ATPase has been isolated as the enzyme responsible for the secretion of hydrogen ions into the gastric lumen i.e. the proton pump. It is present almost exclusively in parietal cells, located on the microvilli of the secretory canaliculus (Saccomani et al, 1979; Berglindh, 1984).

It is thought that chloride ion secretion is coupled with potassium ion movements (Berglindh, 1984).

Carbonic anhydrase has been found in high concentrations within the parietal cell (Maren, 1967) in intimate association with the microvilli of the secretory canaliculus (Cross, 1970). Carbonic anhydrase is thought to play a protective role in maintaining cellular pH by buffering the hydroxide ions produced as a result of the proton (H^+) secretion. Inhibition of this enzyme with acetazolamide, leads to a decrease in the rate of acid secretion due to an increase in intracellular pH (Hersey and High, 1971).

Acid secretion from the ovine abomasum

The abomasum of the adult ruminant secretes acid and pepsinogen continuously (Masson and Phillipson, 1952; Hill, 1955; 1960 and Ash, 1961a, b).

The explanation offered for this is that the secretion has been stimulated by the entry of digesta with its fatty acid content from the forestomach into the abomasum (Hill, 1955; 1960; Ash, 1961a, b). Both chemical and mechanical stimulation of the abomasum contribute to the control of its secretion i.e. distension, pH of the contents and peptide concentration. This is mediated by vagal stimulation of endocrine cells to produce gastrin. The "gastric phase" of acid secretion. Low pH will inhibit secretion, probably by a reduction of gastrin release (Grossman, 1981). Distension can also inhibit gastric secretion. The volume and acidity of fundic abomasal pouches were both found to be higher when animals were feeding or had recently been fed than when they were during fasting (Masson and Phillipson, 1952; Hill, 1955; 1960; Ash, 1961a; McLeay and Titchen, 1970). Secretory responses to feeding or teasing with food were regularly obtained from fundic abomasal pouches (McLeay and Titchen, 1970). The rapidity of the response to teasing with food or immediately following ingestion suggested that the vagus nerve was involved. The vagus would also stimulate the release of gastrin. This is the 'cephalic phase' of gastric secretion (Grossman, 1981).

An increase in the acidity of abomasal contents leads to an inhibition of acid secretion. This is due to inhibition of gastrin release from the abomasal mucosa.

The presence of food in the small intestine can stimulate gastric secretion. It is thought that distension, peptide concentration and pH are important in this action. The "intestinal phase" of acid secretion. A low pH of the duodenal contents will inhibit gastric acid secretion in the sheep (Ash, 1961b). The mechanism for this is not known, but a part is played by a humoral agent, possibly gastrin or secretin (Grossman, 1981). A neural mechanism may also exist. Fat and hyperosmolar contents present in the duodenum have also been found to inhibit gastric acid secretion.

Influence of parasites on abomasal pH

O. circumcincta infestations in non-immune sheep cause the abomasal pH to rise. This was seen by eight days post challenge with 100,000 O. circumcincta larvae by Armour, Jarrett and Jennings (1966). The pH rose to six and remained at this level until day 35 post-challenge, but had not returned to normal by day 60. The rise in the abomasal pH was associated with larval development within the glands to the young adult stage (L_5) and the start of their emergence onto the abomasal mucosal surface. The glands became dilated and lined with a relatively undifferentiated epithelium, with no histological evidence of parietal cells. These changes do not solely affect parasitised glands. Neighbouring glands also become elongated and lined with a similar, undifferentiated epithelium. Ultrastructural studies by Murray, Jennings and Armour (1970) in the similar situation that occurs in O. ostertagi infestation in calves, showed that the parietal cells in surrounding glands contained structural

changes indicating reduced secretory activity before losing their structural identity.

As the abomasal pH returned towards normal levels there was evidence of a gradual regression of the lesions. The abomasal pH changes seen in calves with O. ostertagi are associated with similar structural changes but the larval development takes slightly longer and therefore the abomasal pH rise is later (Ritchie et al, 1966; Jennings et al, 1967; Murray, Jennings and Armour, 1970). When the infected animals are treated with anthelmintic, the pH falls but takes approximately five days to return to normal (Armour et al, 1967; Anderson, Blake and Titchen, 1976). The cause of the abomasal pH rise is obviously due to the loss of parietal cell mass within the abomasum. How this reduction comes about is not known.

Physical stretching of the abomasal glands and its surrounding area by the developing parasite has been suggested as the cause. However, as the lesions resolve, the previously-parasitised gland becomes lined with a mucus-secreting epithelium while the surrounding glands revert to their normal appearance. Therefore it was suggested that substances produced by the parasites, or the immune response by the host could be responsible (Armour, Jarrett and Jennings, 1966). Murray, Jennings and Armour (1970) added to this theory with the finding that the ultrastructural appearance of the parietal cells were similar to those found in rats when substances were administered to depress acid secretion.

Further work on this question (McLeay et al, 1973; Anderson, Blake and Titchen, 1976) using sheep surgically prepared with abomasal pouches which remain unparasitised when the sheep was infected, have implicated another change thought to be stimulated by the parasites, as a rise in plasma gastrin concentration. This gastrin rise promotes an increase in acid output in the abomasal pouch, while the remainder of the abomasum shows a decrease in secretion. The parietal cells in the abomasum which remained had the appearance of supressed secretion while those in the pouch appeared very active. The possible inhibitory actions on the parietal cells are suggested as being more important when adult parasites are present than when larvae are developing (McLeay et al, 1973).

Other results which argue against a purely physical cause for loss of acid secretion have come from the transfer of a mainly adult Ostertagia infection directly into the abomasi of previously worm-free sheep. A rise in abomasal pH occurred 5 to 7 days post-transfer. Therefore extensive invasion of the mucosa was not necessary for the pH rise. However, larval stages were present so some abomasal damage could have taken place (Anderson, Hansky and Titchen, 1985).

However, transfer of a purely adult population of O. ostertagi into calves did not produce a rise in abomasal pH (McKellar et al, 1986; 1987) but fewer adults were transferred and a threshold number of adult parasites may be needed to produce the pH rise (McKellar et al, 1987).

Work by Eiler et al (1981) using a homogenate of O. ostertagi adults showed that its intramuscular injection into

rats produced a rise in gastric pH. In vitro studies by the same authors suggested that adult parasites were unable to survive in an acidic pH and therefore it was necessary for the abomasal pH to rise for the survival of the parasite. The fact that Ostertagia survived in calves transplanted with adult parasites without a rise in pH would argue against this (McKellar et al, 1986; 1987).

However, a more important factor in parasite survival may be the presence of Ostertagia adults in close apposition to the mucosa within the mucus layer (Miller, 1984) where a pH gradient is known to occur (Williams and Turnberg, 1979; Takeuchi et al, 1983). This would serve to protect the parasites against the adverse effects of a low abomasal pH.

In contrast to the work by Eiler et al (1981), Hall and Oddy (1984) found that in sheep infected with adult O. circumcincta, raising the abomasal pH with cimetidine, a histamine H_2-receptor blocker, caused a removal of the parasites. However, the high dose rate of cimetidine used could have had a direct effect on the parasites.

Evidence that parasites can indirectly affect abomasal pH has come from work with the intestinal parasite Trichostrongylus colubriformis in sheep (Barker and Titchen, 1982). Lambs infected with this parasite showed a reduction in abomasal acid secretion even though less than 0.8% of the worm burden was established in the abomasum. Loss of prominence of parietal cells, with associated changes suggesting reduced activity, and encroachment of mucus cells was seen. These

changes were more pronounced than those induced solely by a reduction in food intake.

Therefore, it would seem probable that other mechanisms are acting to cause a rise in abomasal pH in a number of parasite infestations in addition to the response to mechanical damage. However, in animals that were previously infected with Ostertagia, there is only a transient rise in pH after challenge, even though their immunity is insufficient to prevent relatively large numbers of parasites establishing (Anderson, Blake and Titchen, 1976). Any affect of the parasite on the abomasal pH is therefore prevented.

In view of this, it was decided to assess the effects of a rise in abomasal pH at the time of challenge in adult immune sheep on the establishment of the parasites and the responses of the host. To raise the abomasal pH the affects of omeprazole, a substance that raises intragastric pH, was studied in sheep.

Omeprazole

Omeprazole (5-methoxy-2-[[(4-methoxy-3, 5-dimethyl-2-pyridinyl)-methyl]sulphinyl]-1H-benzimidazole) is a compound which acts to raise gastric pH by preventing the secretion of hydrochloric acid. It consists of a substituted benzimidazole ring and a substituted pyridine ring connected by a sulphoxide-containing chain (Brändstrom, Lindberg and Junggren, 1985).

The structure of omeprazole is shown in Figure 21.

It acts on an enzyme (H^+ K^+)ATPase which is found only at the secretory surface of the parietal cell in the membrane of the secretory canaliculi and prevents its catalyst of a one-to-one exchange of protons and potassium ions (Fellenius et al, 1981a;

1981b; Wallmark et al, 1983; Wallmark, Lorentzon and Larsson, 1985). There is one report of finding the enzyme elsewhere in the rabbit colon. The significance of this is unknown (Gustin and Goodman, 1981). Omeprazole is therefore acting at the end stage of acid production and prevents acid secretion in the basal, unstimulated, parietal cell and also the acid stimulated by, for example, histamine, pentagastrin and other substances that act intracellularly e.g. dibuthyl cAMP (Wallmark et al, 1983; Wallmark, Lorentzon and Larsson, 1985; Lind et al, 1983, Konturek et al, 1984). The inhibition of $(H^+ K^+)$ATPase has been found to be pH-dependent, the lower the pH, the greater the inhibition (Wallmark et al, 1983; Wallmark, Lorentzon and Larsson, 1985). This may be because, to act, omeprazole has to be protonated, or that a group within the $(H^+ K^+)$ATPase enzyme requires protonation to react with the omeprazole. Also, omeprazole, being a lipid permeable weak base, would accumulate within the low pH compartments within parietal cells (Fellenius et al, 1981a; 1981b).

All these properties therefore combine to produce a very specific action within active parietal cells.

Omeprazole has been found to inhibit gastric acid secretion in a wide-variety of species. It can be administered orally or by intravenous injection.

Oral administration results in a dose-dependent inhibition of acid secretion which correlates with the area under the plasma concentration time curve. The duration of action of a single dose is not dependent on a sustained plasma concentration (Lind

et al, 1983). In man, one dose produces an effect for 2-3 days (Cederberg et al, 1985) and also in the dog (Larsson et al, 1985) which could be explained by accumulation within the acid-producing cells. There also appears to be an accumulative effect when administered daily with the degree of acid inhibition stabilising after five days (Cederberg et al, 1985; Larsson et al, 1985).

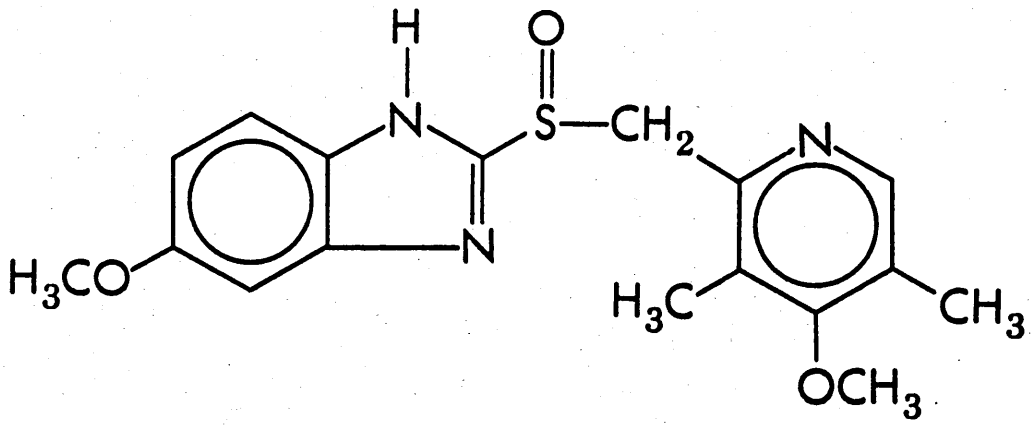
Intravenous administration of omeprazole is not as effective in its inhibition of acid secretion. Although a rapid rise in gastric pH is achieved, higher doses and more frequent administration are required. This could suggest that the drug has a local effect with the gastric compartment before absorption when given orally, or that an active metabolite is formed within the gastrointestinal tract (Walt et al, 1985). Further evidence for this comes from Konturek et al (1984), who found that omeprazole infused into the Heidenhain pouch of a dog produced inhibition of histamine stimulated acid secretion without any significant change in the response of the main stomach or the plasma concentration of the drug.

The effects of omeprazole on the abomasal pH in ruminants do not appear to have been investigated.

Work to study the effect of omeprazole on other gastric secretions have produced various results. Using a perfused mouse stomach in vitro Fimmel and Blum (1983) found that high doses of omepraole which inhibited acid secretion caused an increase in pepsinogen secretion, while work on pepsinogen-producing cells from the rabbit gastric mucosa showed that omeprazole had no effect on pepsinogen release (Fryklund et al, 1984).

FIGURE 21

Structure of omeprazole



Omeprazole

Toxicological studies on omeprazole have shown very low acute toxicity with LD_{50} values not able to be reached (greater than 4 g/kg in rodents). The major features of repeated administration are hyperplasia of parietal mucosal cells, increase in stomach weight, mucosal thickness and mucosal folding, seen in the dog, rat and mouse. These effects were reversible upon withdrawal of the drug. Gastric carcinoids have also been observed in rats.

The hyperplasia is due to the pronounced hypergastrinaemia that occurs due to the profound effects on gastric acid secretion and can be prevented by antrectomy to prevent the increased gastrin concentrations (Ekman et al, 1985).

Therefore, omeprazole represents a high selective and efficient method of raising gastric pH, and was therefore the drug chosen to alter abomasal pH and assess its effect on gastrointestinal immunity.

PRELIMINARY INVESTIGATION INTO THE EFFECTS OF OMEPRAZOLE IN SHEEP

Materials and methods

Animals

The animal used in this preliminary study was an adult Dorset cross ewe aged three years.

Cannulation of the abomasum

The animal had been prepared with a permanent abomasal cannula four months prior to the start of the experiment. The cannula and surgical techniques were as described previously.

Experimental design

Animal number	Procedure
5	Study 1. 40 mg omeprazole administered twice daily at intervals of 8 and 16 hours.
	Study 2. 40 mg omeprazole administered twice daily at 12 hour intervals
	Study 3. 40 mg omeprazole administered as a single injection to closely monitor changes in abomasal pH

Administration of omeprazole

Initially, oral administration of omeprazole was investigated. A 30 mg capsule of omeprazole was administered to the sheep. This did not produce any rise in abomasal pH. Therefore the intravenous route was used.

Omeprazole was obtained as a powder kindly supplied by Astra Chemicals which was dissolved in polyethylene glycol 400 (PEG_{400}) (BDH Chemicals Ltd.) and stored in aliquots at -20°C for not more than one week prior to use, as omeprazole is known to be unstable in aqueous solution.

Before administration, it was allowed to warm to room temperature and diluted with sterile water. A final solution of 4 mg/ml omeprazole in 40% PEG_{400} was used. The omeprazole was injected intravenously into the jugular vein via a 19 gauge 1" needle over a two minute period. A dose of 40 mg omeprazole was given to the animal at each administration, a dose rate of 1.2 mg/kg bodyweight.

Sampling techniques

(i) Abomasal fluid

Abomasal fluid samples were taken into a plastic beaker via

the cannula. The animal was restrained in the standing position. The sample was collected by allowing the normal passage of ingesta to pass the fluid through the cannula. The first portion was discarded as unrepresentative of the abomasal contents. The pH of the abomasal fluid was measured immediately using a upH sensor (Whatman Labsales Ltd.).

(ii) Blood samples

Blood samples were collected into heparinised syringes (Monovettes, Sarstedt) using a 20 g 1" needle. The blood was centrifuged at 1,000 x g for ten minutes. The plasma supernatant was removed and stored at -20°C for estimation of plasma pepsinogen activity.

Results

Abomasal pH

The results are shown in Table 27 and Figure 23.

(i) Study 1

It was hoped to maintain an abomasal pH greater than pH 4 over a 24 hour period. The most convenient interval between injections was eight hours and 16 hours. This was the first regime investigated. The abomasal pH rose from 2.8 to pH 6 two hours after administration of omeprazole. The pH then slowly fell to reach pH 5 at eight hours.

A second administration of omeprazole was carried out at this time. Four hours after this second injection the abomasal pH was 5.9 but 16 hours after the second administration the pH had returned to 3.1.

This administration regime therefore did not maintain an abomasal pH above pH 4 over a 24 hour period.

(ii) Study 2

An injection regime of 40 mg of omeprazole every 12 hours was then studied.

Prior to omeprazole administration, the abomasal pH was 3.1. This rose to pH 5 four hours after injection but by 12 hours had fallen to pH 2.7, therefore 12 hourly intervals between omeprazole injections would not maintain an abomasal pH above pH 4.

(iii) Study 3

A single injection of omeprazole was administered and the abomasal pH monitored to assess the optimal time interval between injections. After administration of 40 mg of omeprazole, the pH rose to a maximum of pH 6 two hours later. The abomasal pH gradually fell. At eight hours a pH of 4.2 was reached which had fallen to pH 2.9 at 12 hours.

Therefore it was necessary to inject 40 mg of omeprazole every eight hours to maintain the abomasal pH above pH 4.

Plasma pepsinogen

The results of plasma pepsinogen activity during the administration of omeprazole are shown in Table 27. The plasma pepsinogen activity fluctuated between 0.324 I.U. and 0.514 I.U. but it was not clearly associated with the rise in abomasal pH.

Therefore, from this experiment, it was obvious that omeprazole produces a rise in abomasal pH when administered intravenously at eight hour intervals.

Corticosteroids

Two classes of corticosteroids are synthesised and released from the adrenal cortex under the control of adrenocorticotropic hormone (ACTH). ACTH is released from the adenohypophysis (anterior pituitary) and stimulates the synthesis of glucocorticoids (cortisol and corticosterone) and to a much lesser extent, a mineralocorticoid, aldosterone. ACTH release is under the influence of the nervous system which employs the neurohumoral substance, corticotrophin-releasing-factor (CRF), which is transported from the hypothalamus, and also a negative feedback exerted by the glucocorticoids.

The corticosteroids produced by the body have wide and numerous effects. Carbohydrate, protein and fat metabolism, electrolyte and water balance, functions of the cardiovascular system, kidneys, skeletal muscle and the nervous system can all be affected.

Cholesterol is an obligatory intermediate in the biosynthesis of corticosteroids.

The plasma concentrations of glucocorticoids display diurnal variation as does ACTH concentration. Their release is also increased during stress.

Synthetic corticosteroids are divided into two categories of sodium retaining (mineralocorticoids) and anti-inflammatory and gluconeogenic (glucocorticoids) according to their relative potency (Haynes and Murad, 1985). Newly developed drugs have little overlap between these categories.

Action of glucocorticoids

Glucocorticoids are thought to act by controlling the synthesis of proteins. A steroid-receptor complex is formed on the cell surface. This complex becomes modified and passes into the cell, through the cytoplasm to the nucleus. It binds there and directs the production of RNA and therefore, ultimately, proteins. It may inhibit production, as in lymphoid cells, or stimulate it, as in the liver (Parrillo and Fauci, 1979).

Effects on blood formed elements

Glucocorticoid administration leads to an increase in the number of polymorphonuclear leukocytes in the blood, due to increased entrance and decreased removal from the circulation (Bishop et al, 1968; Parrillo and Fauci, 1979). Lymphocytes, eosinophils, monocytes and basophil numbers decrease. In man, the maximum effect on lymphocytes and monocytes is seen four to six hours after hormone administration. The effect is seen later for eosinophils and basophils. The decrease in cell numbers is transient, with recovery 24 to 72 hours later (Claman, 1975; Parrillo and Fauci, 1979; Cupps and Fauci, 1982).

Effects on inflammation

Cortisol and its synthetic analogues prevent or supress the heat, redness, swelling and tenderness of inflammation. The early events of oedema, capillary dilatation, migration of leukocytes, phagocytosis and fibrin deposition are all inhibited by glucocorticoids as well as capillary proliferation, fibroblast proliferation, deposition of collagen and scar formation. These effects depend on the direct local action of the steroids.

The most important effect maybe their ability to inhibit the migration of neutrophils and monocytes-macrophages into the inflamed area (Parrillo and Fauci, 1979). They may also affect the ability of neutrophils to adhere to capillary endothelial cells (MacGregor, 1977).

Glucocorticoids also have effects on the response of macrophages in delayed-type hypersensitivity. In these reactions the lymphocytes have been previously sensitised to the antigen. When they encounter the antigen they become activated and produce factors called lymphokines that control the cellular response.

Macrophage migration inhibitory factor (MIF) is one of the lymphokines produced which promotes macrophage accumulation in the area (Bloom and Bennett, 1966). Glucocorticoids inhibit the effect of MIF on macrophages so that they do not accumulate (Balow and Rosenthal, 1973).

Also, the formation of plasminogen activator, produced by neutrophils is inhibited. This enzyme facilitates fibrinolysin which is thought to aid the entrance of leukocytes into inflamed areas.

Another action, which probably plays a major role in their anti-inflammatory effects, is their inhibition of arachidonic release from phospholipids by action on phospholipase A_2. This action is thought to be mediated by an inhibitory protein produced or released as a result of glucocorticoid administration (Danon and Assailine, 1978; Flower and Blackwell, 1979; Blackwell et al, 1980; Blackwell and Flower, 1983). This protein has been isolated from guinea-pig lung and called macrocortin. A similar protein has been isolated from rabbit neutrophils and called lipomodulin (Heiman and Crews, 1984). This action therefore reduces the formation of prostaglandins, leukotrienes, thromboxanes and prostaglandin endoperoxidase.

Glucocorticoids are known to prevent immunoglobulin-dependent release of arachidonic acid from rat mast cells (Heiman and Crews, 1984) and they also prevent the release of histamine from isolated rat and murine mast cells (Daëron et al, 1982; King et al, 1985). 5-hydroxytryptamine (5-HT) release from rat mast cells is also reduced (Marquardt and Wasserman, 1983).

The number of rat globule leukocytes is also markedly reduced by glucocorticoid treatment (Kent et al, 1954).

Effect on lymphoid tissue and immune responses

Glucocorticoids have different effects on different species. Rabbits, rats and mice are termed 'steroid sensitive' and in these species there is a rapid lysis of lymphoid tissue and inhibition of antibody production. In contrast, man, monkeys and guinea-pigs do not show the above effects. Metabolism of complement is probably also not altered (Claman, 1975). However, circulating monocytes do show an impaired ability to kill

micro-organisms but phagocytosis is not affected. There is also evidence from in vitro studies that the function of naturally occurring supressor lymphocytes is inhibited by glucocorticoids in man (Haynes and Fauci, 1979).

Effects on ovine lymphoid tissue

Studies into the effect of a glucocorticoid, methylprednisolone in sheep, showed that they are similar in their responses to man i.e they could be termed 'steroid resistant' (Zukoski and Montgomery, 1985).

In agreement with the studies in man, sheep exhibit a marked peripheral blood granulocytosis and lymphopaenia which reached a maximum four hour post-injection but had returned to normal by 24 hours. Also, there is a marked decrease in the cell numbers in lymph. However, by 24 hours post-glucocorticoid administration cell numbers had increased to above pre-injection numbers. Zukoski and Montgomery (1985) also found that with continuous dosing of methylprednisolone, the above changes were noted, but all parameters returned to normal levels by 56 hours even though the glucocorticoid was still being administered.

There was no evidence of glucocorticoid-induced lympholysis.

Protein and IgG concentrations measured in lymph decreased after drug administration. This could reflect a decrease in protein efflux into the lymph as there were no comparable changes in serum protein or IgG concentration.

It was found, in these studies, that the changes in white blood cells and lymphatic protein concentrations were not dose-dependent. Maximal responses occurred at the lowest dose of

methylprednisolone tested (31 ug/kg). The effects on lymph cell concentration were, however, highly dose-dependent.

Absorption of glucocorticoids

Cortisol and its numerous synthetic analogues are effective when given orally in simple-stomached species. Water soluble esters can also be administered intravenously to produce high concentrations in body fluids rapidly. More prolonged effects are obtained by intramuscular injection of water insoluble suspensions.

Transport

In plasma, 90% of cortisol is reversibly bound to protein. There are two proteins capable of binding corticosteroids.

(i) corticosteroid-binding globulin, a glycoprotein.

(ii) albumin.

The globulin has a high affinity for corticosteroids but a low total binding capacity. In contrast, the albumin has a low affinity but a relatively large binding capacity. At normal concentrations, corticosteroids are bound to the globulin. Increased concentrations produce more albumin bound corticosteroid and also increases the free, unbound concentration. The unbound drug is active.

Structure of dexamethasone

The basic structure common to all corticosteroids is a 17 carbon skeleton. For glucocorticoid and anti-inflammatory activity it is necessary to have certain structural features. There must be a double bond between C_4 and C_5, a carbonyl group at C_3, a hydroxyl group on C_{11} and a two carbon side chain on C_{17}.

The presence of a fluoride atom on C_9 enhances the activity of dexamethasone. Dexamethasone is 25 times more potent as an anti-inflammatory drug when compared to cortisol. It also has an increased plasma and biological half-life.

Dexamethasone acetate was used in this experiment. This is a water-insoluble suspension which further extends the time of activity of the dexamethasone by acting as a depot at the site of injection. The anti-inflammatory activity persists for up to eight days. The structure of dexamethasone is shown in Figure 22.

Effects of glucocorticoids on host-parasite relationships

Glucocorticoids have been widely used to study a large number of gastrointestinal parasite infestations and the processes responsible for their expulsion.

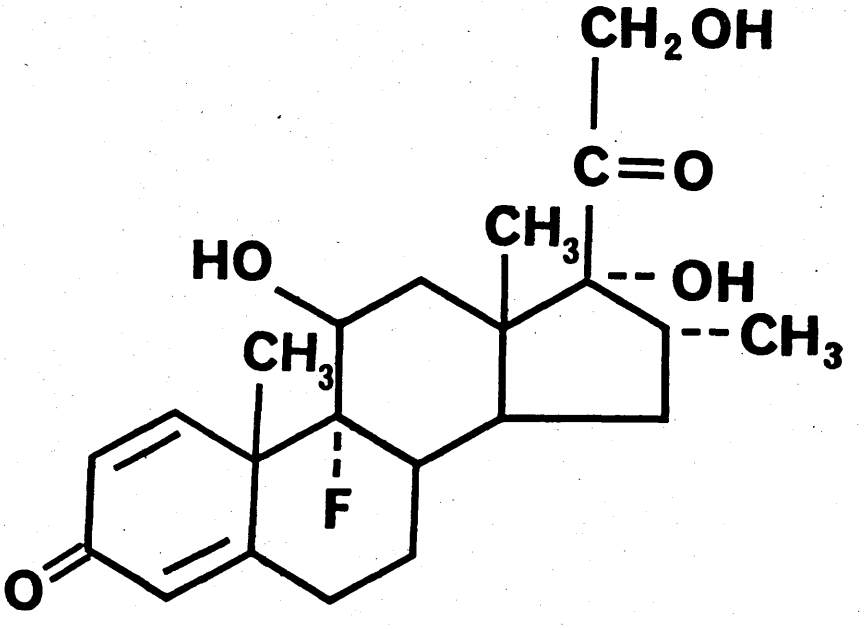
Generally, the administration of glucocorticoids to animals, previously rendered immune to a parasite, abrogates resistance. This is associated with changes in cell numbers within the gastrointestinal wall.

Studies in rats and mice, the "steroid sensitive" species, have shown that resistance to Nippostrongylus brasiliensis (Ogilvie, 1965; Murray, Jarrett and Jennings, 1971; Miller and Nawa, 1979a), Trichinella spiralis (Sukhdeo and Croll, 1981; Bell, McGregor and Adams, 1982), Strongyloides ratti (Olson and Schiller, 1978; Moqbel, 1980) and Trichuris muris (Wakelin, 1975) can be abolished by glucocorticoid treatment.

Work on N. brasiliensis infestation in rats has shown that the rise in the numbers of mast cells and goblet cells associated

FIGURE 22

Structure of dexamethasone


CH2OH
C=O
CH3
HO
OH
CH3
CH3
F
O

with parasite expulsion was inhibited. Also the associated increase in mucosal permeability was abolished (Murray, Jarrett and Jennings, 1971).

More recent work has shown that glucocorticoids prevent the exclusion of N.brasiliensis larvae from the intestinal mucosa of rats and the mucus trapping that occurs in immune animals (Miller and Huntley, 1982a).

Glucocorticoids also prevented the protection produced by antibody transfer to naive animals (Miller and Nawa, 1979a).

In immune animals, when N. brasiliensis antigen is injected intravenously, an anaphylactic shock syndrome associated with the intestine is produced. Local anaphylaxis is thought to be important in bringing about parasite expulsion in naturally infected rats. This results in increased mucosal permeability and mucus secretion. There is also hyperaemia and epithelial shedding. A similar reaction occurs at the time of self cure (Barth, Jarrett and Urquhart, 1966). The development of these lesions is associated with release of a protease, rat mast cell protease II (RMCPII) into the blood and gut lumen from mucosal mast cells. RMCPII has been implicated as the cause of the increased mucosal permeability. Glucocorticoid treatment prevented the anaphylaxis, decreased the release of RMCPII and prevented the increase in mucosal mast cell numbers (Urquhart et al, 1965; King et al, 1985). Neither connective tissue mast cells or parasite specific IgE was affected by the drug treatment.

Studies with Strongyloides ratti infestation have shown that glucocorticoids cause a decrease in the numbers of eosinophils

and degranulating mast cells in a primary infection (Moqbel, 1980). The immune expulsion in both primary and secondary infections was also prevented (Olson and Schiller, 1978).

A decrease in the number of eosinophils in T. spiralis infestation in mice was also noted with steroid treatment (Sukhdeo and Croll, 1981).

Interestingly, work by Farmer (1982) has shown that betamethasone has an effect on the smooth muscle of isolated rat intestine from animals infected with N. brasiliensis. In infected animals there is an increase in the maximum responses to 5-hydroxytryptamine and acetylcholine. Betamethasone prevented this increase.

Work with gastrointestinal parasites of ruminants has yielded similar changes in cell populations.

Treatment of sheep immune to Haemonchus contortus and Trichostrongylus colubriformis is associated with a decrease in the numbers of mast cells, globule leukocytes and eosinophils (Miller et al, 1985; Douch et al, 1986). There was also an increase in the number of H. contortus parasites established in steroid-treated non-immune sheep compared to untreated controls (Jackson, personal communication).

The prevention of immune exclusion and mucus trapping in a number of species by corticosteroid treatment could be due to a direct effect on mucus secretion, or, a prevention of formation of leukotrienes thought to have a role in the mucus trapping (Douch et al, 1986). Prevention could also be due to a decrease in mucosal permeability and therefore prevention of translocation

of immunoglobulins etc. into the gastrointestinal lumen (Murray, 1972).

However, studies by Adams (1982; 1983) and Adams and Davies (1982) suggested that glucocorticoids given at the same time as a challenge of H. contortus larvae in non-immune, immune or in sheep of undefined immune status, reduced the numbers of parasites established compared to untreated immune sheep, possibly implicating some suppressor mechanism acting which was prevented by glucocorticoid treatment.

Steroid administration to cattle with Ostertagia ostertagi infestation has shown that there is a slower loss of parasites from treated animals (Michel, 1969). Higher faecal egg counts and fewer signs of immunological damage to the parasites were also noted. Treated animals harboured longer parasites and there was a slower decrease in worm length towards the end of the infection compared to controls. There were also fewer female O. ostertagi with poorly developed vulval flaps (Michel and Sinclair, 1969).

Similar results of increased parasite burdens and faecal egg counts are seen in glucocorticoid treated sheep infested with Ostertagia circumcincta and other parasites e.g. H. contortus, Nematodirus, Cooperia and Trichostrongyle spp. This was associated with a decrease in the number of circulating lymphocytes and a slight decrease in the antibody response to a bacterial vaccine (Matthews, Brunsdon and Vlassoff, 1979).

Other work in these species has centred on the effects of glucocorticoids on arrested larvae. Dunsmore (1961) showed that steroid treated sheep allowed fewer O. circumcincta larvae to

arrest than in untreated controls and treatment of calves with an existing population of arrested O. ostertagi did not initiate redevelopment of the larvae, but inhibited the natural rejection of the infestation (Prichard, Donald and Hennessy, 1974).

EXPERIMENT TO ASSESS THE EFFECTS OF BROMHEXINE, OMEPRAZOLE AND DEXAMETHASONE ON THE IMMUNITY OF SHEEP TO *Ostertagia circumcincta*

Materials and methods

Animals

Twenty adult Blackface ewes were obtained aged greater than five years, which had been reared and maintained extensively on a hill farm since birth. This farm was known to be infected with *O. circumcincta*. These animals were considered to be immune to *O. circumcincta*.

Experimental design

Group	Sheep number	Procedure
Control	74 104 110 113 114 122	Challenged with 200,000 *O.circumcinta* L_3 on day 0.
Bromhexine	73 103 105 109 111	Bromhexine hydrochloride administered orally at a dose rate of 0.5 mg/kg once daily starting 5 days prior to challenge and continuing until day 4 of the experiment. Challenged with 200,000 *O. circumcincta* L_3 on day 0.
Corticosteroid	52 101 102 112 121	Dexamethasone acetate injected intramuscularly once every 7 days at a dose rate of 0.08 mg/kg starting 8 days prior to challenge and continued throughout the experiment. Challenged with 200,000 *O. circumcincta* L_3 on day 0.
Omeprazole	8 72 107 108	Omeprazole administered at 8 hour intervals for 3 days beginning on day 0, 2 hours prior to challenge with 200,000 *O. circumcincta* L_3.

Administration of infective stage larvae (L_3) of O. circumcincta

Prior to the start of this experiment, all the sheep were dosed twice weekly with 2,000 O. circumcincta L_3 to ensure immunity to the parasite. This was continued for four weeks.

At the end of this period the animals were weighed, allocated into four groups and dosed with an anthelmintic, oxfendazole (Synanthic Suspension, Syntex) at 5 mg/kg to remove any gastrointestinal nematodes.

The start of the experiment for the omeprazole group was delayed due to difficulties in obtaining the drug. Therefore this group together with sheep number 104 of the control group underwent another period of dosing with 4,000 O. circumcincta L_3 eight weeks later in order to maintain a strong immunity. These animals received 4000 O. circumcincta larvae (L_3) once daily for four days. The anthelmintic treatment was then repeated.

All of the sheep were challenged with 200,000 O. circumcincta L_3 as a single dose one week after anthelmintic treatment. The groups which were subjected to bromhexine, corticosteroid and omeprazole treatment were challenged during the period of administration of the drug.

Drug administration

(i) Bromhexine

Bromhexine was given orally as described previously and was administered daily for ten days around the time of challenge. The sheep were challenged with 200,000 O. circumcincta L_3 on the sixth day of bromhexine treatment.

(ii) Corticosteroid

Dexamethasone acetate (Duphacort LA, Duphavet Ltd.) was injected intramuscularly at the recommended dose rate of 0.08 mg/kg bodyweight. Injections were carried out weekly from eight days prior to challenge until slaughter.

(iii) Omeprazole

The omeprazole was administered intravenously as described previously, at eight hour intervals for three days around the time of challenge. The sheep were challenged with 200,000 O. circumcincta L_3 on the first day of omeprazole administration, two hours after the first injection. From the previous studies the abomasal pH was expected to be above pH 4 at this time and to remain so during the three days of omeprazole treatment.

Sampling techniques

(i) Blood samples

Blood was taken as previously described and the plasma used for estimation of pepsinogen activity.

(ii) Faecal samples

Faecal samples were obtained per rectum and parasite egg numbers estimated by a modified McMaster technique.

Samples of faeces were also collected daily for three days after challenge and subjected to both a Baermanisation technique and a salt flotation technique to detect any larvae which had not established, survived digestion and had passed through the animal.

Procedure at necropsy

The animals were killed by stunning with a captive bolt and

exsanguination. The abomasum together with omasum and 3 to 4 feet of duodenum were removed. The ends were secured to prevent escape of their contents.

Sampling techniques at necropsy

(i) Abomasal contents

The abomasum was separated from the omasum and incised along its greater curvature. The abomasal contents were collected. Abomasal pH was measured using a upH sensor. The contents of the abomasum were processed for estimation of parasite numbers as described previously.

(ii) Abomasal mucosa

The abomasum was halved lengthwise. One half of the abomasum was processed for pepsin/hydrochloric acid digest and estimation of parasite numbers as described previously.

(iii) Abomasal mucus

The remaining half of the abomasal mucosa was gently cleaned of food particles manually, placed on a cooled board and removed to a cold environment (5^{o}C). The mucus layer was carefully removed as described previously using cold microscope slides and collected into cold, preweighed universal bottles. The weight of mucus collected was calculated.

(iv) Duodenal mucus

The duodenum was cut into small 15 cm sections and the contents allowed to run out. Each section was opened lengthwise and the mucus collected as described above in the same cold conditions.

(v) Isolated smooth muscle

Two animals from each group were randomly chosen. Small sections of the abomasum and duodenum were removed as soon as possible post mortem and transferred to a beaker of Krebs solution at room temperature. The responses of the smooth muscle of these sections were investigated using tissue baths.

Processing of mucus samples

To each mucus sample, 10 ml of cold, HPLC grade methanol was added. This was then homogenised using a tissue homogeniser (Ultraturrex). The sample was kept in a large beaker of ice in order to maintain a low temperature during this process.

The homogenate was centrifuged at 10,000 x g for 15 minutes and the supernatant collected and stored at -20^{o}C for estimation of leukotriene content. The concentration of leukotriene C_4 was measured using a radioimmune assay by Dr. Moqbel and colleagues at Brompton Hospital, London.

Results

Plasma pepsinogen activity

The results of the measurement of plasma pepsinogen activity are shown in Tables 28, 29, 30 and 31 and the mean of each group in Figure 24.

From Figure 24, it can be seen that the mean plasma pepsinogen activity of each group rose after challenge on day 0 to reach maximum at day 4 to 6 and then fell.

From day 10 of the experiment the mean values of each group differed.

The mean plasma pepsinogen activity of the control group

rose to reach a maximum of 4.361 I.U. by day 22 of the experiment, falling slightly by day 24.

The mean plasma pepsinogen activity of the bromhexine group remained below 1.5 I.U. to the end of the experiment.

The mean plasma pepsinogen activity of the corticosteroid group rose more steeply than that of the control group. It reached a maximum of 5.934 I.U. by day 20 of the experiment after which it declined to 4.297 I.U. and then rose to 5.088 I.U. by the end of the experiment.

The omeprazole group showed the steepest rise in plasma pepsinogen after day 10 of the experiment. The mean value reached a maximum of 4.558 I.U. at day 16 and thereafter declining until day 24.

Figure 25 shows the mean plasma pepsinogen activity of the omeprazole group and the pepsinogen activity of sheep number 104 which acted as the untreated control for this group. Sheep 104 did not show any significant rise in plasma pepsinogen during the experiment, activities remaining below 1.3 I.U. In contrast, the mean pepsinogen values, for the omeprazole group showed two peaks of activity, the first at day 6 of the experiment to 1.795 I.U. and the second, larger rise, reaching a maximum at day 16, to 4.558 I.U.

Therefore, when comparing the omeprazole group to the one animal that acted as the group's control there would appear to be distinct differences in the plasma pepsinogen activities, which are not seen when the group is compared to the control group for the whole experiment. In Figure 26 the individual plasma

pepsinogen activities for each member of the omeprazole group are shown together with number 104, the control. Although there is a range in activities in the animals treated with omeprazole, three of the four sheep show two distinct rises in plasma pepsinogen activities.

Faecal egg counts

The results of the examination of faeces for parasite egg numbers are shown in Table 32 and Figures 27, 28 and 29. Eggs were detected in all of the sheep of the control group except number 104 from day 17 post-challenge.

The appearance of parasite eggs in the faeces of the bromhexine group occurred later, from day 23 post-challenge.

All of the sheep in the corticosteroid group expelled parasite eggs in their faeces except sheep number 121. Eggs were first detected on day 17 post-challenge.

Only in one sheep, number 107, in the omeprazole group were eggs detected in very low numbers, despite very high plasma pepsinogen activities seen in this animal.

Detection of third stage larvae

Very small numbers of larvae were detected in the faeces for the first three days of the experiment. The results are shown in Table 33.

Abomasal pH

The abomasal pH at necropsy of the majority of the sheep in this experiment was higher than might be expected. The average abomasal pH for each group ranged between pH 4 and 5 (Table 34).

Abomasal parasite counts

The results of these are shown in Table 35 and the

percentage establishment of the challenge of 200,000 O. circumcincta (L_3) in Table 36.

In general the highest numbers of parasites were found in the sheep of the corticosteroid group, an average of 30,580 parasites being present. The omeprazole and bromhexine groups possessed lower numbers of parasites at slaughter, mean values of 4,300 and 6,140 parasites respectively.

It is interesting to note that low numbers of parasites were found in all the animals of the omeprazole group, a mean percentage establishment of 2.15%, despite high plasma pepsinogen activites seen towards the end of the experiment.

The control group contained an average of 15,370 larvae.

In vitro studies

These were carried out as described elsewhere to assess the effects of bromhexine hydrochloride and omeprazole on the motility of O. circumcincta L_3 in vitro. The results are shown in Tables 37 and 38. Neither drug affected parasite motility during the experiment. It is therefore unlikely that these drugs are able to exert direct toxic effects on these parasites.

Leukotriene C_4 concentration in mucus

The concentration of LTC_4 in the mucus of the four groups of sheep are shown in Table 39.

Control Group: The mean values for this group were 1,735 pg/g of abomasal mucus, and 2,301 pg/g of duodenal mucus, however there was a great deal of individual variation. Abomasal mucus concentrations ranged from 168 pg/g from sheep number 114 to 3,342 pg/g from sheep number 110. The concentrations of LTC_4 in

the duodenal mucus showed less variation from 1,746 pg/g for sheep number 114 to 3,141 pg/g for sheep number 104.

Corticosteroid group: The mean concentrations of LTC_4 in the abomasal and duodenal mucus samples were lower than the value for the control group. The abomasal concentrations ranged from 1,029 pg/g for sheep number 101 to 1,933 pg/g for sheep number 121. The duodenal LTC_4 concentration varied from 1,118 pg/g for sheep number 102 to 2,235 pg/g for sheep number 101.

Bromhexine group: In this group there was a higher mean LTC_4 concentration in the abomasal mucus than the value for the control group, but the LTC_4 concentration in the duodenal mucus was slightly lower. The lowest concentration of LTC_4 in abomasal mucus was 1,748 pg/g from sheep number 111 and the highest, from sheep number 109 of 2,914 pg/g. The concentrations of LTC_4 in duodenal mucus varied from 932 pg/g for sheep number 73 to 3,811 pg/g for sheep number 103.

Omeprazole group: The mean values were similar for both mucus types to those obtained for the bromhexine group. Abomasal concentrations varied from 1,145 pg/g (sheep 108) to 3,728 pg/g (sheep 72) while duodenal concentrations ranged from 1,072 (sheep 8) to 3,645 (sheep number 108).

Generally, the concentrations of LTC_4 in the mucus samples were similar for the control, bromhexine and omeprazole groups. The values for the corticosteroid group were lower. Concentrations of LTC_4 were not identical in mucus samples taken from the abomasum and duodenum of the same animal but the variation was not large.

Isolated smooth muscle responses

The smooth muscle from the abomasum and duodenum of sheep number 113 of the control group contracted markedly to acetylcholine.

Prostaglandins E_1 and E_2 produced contractions of the longitudinal smooth muscle of the duodenal preparation only. Prostaglandin E_2 produced a greater contraction than prostaglandin E_1.

Similar responses were observed in tissue obtained from sheep number 103 of the bromhexine group. Tissue from both sheep numbers 121 and 101 of the corticosteroid group proved to be unsatisfactory for testing although they were prepared in the same way as the tissues above. Little response was elicited with acetylcholine in the longitudinal muscle of the duodenum of both sheep. The abomasal tissues exhibited large spontaneous, irregular contractions which did not reduce upon cooling the tissue.

The muscle preparations obtained from sheep numbers 107 and 72 of the omeprazole group failed to contract to acetylcholine and did not show any spontaneous activity even though the tissues had been prepared in the same way as those from the control and bromhexine groups.

Discussion

The most obvious conclusion from this experiment is that despite the immunising schedule, the animals of the control group allowed large numbers of parasites to establish and high plasma pepsinogen activities were recorded. Obviously, the immunising

regime carried out was insufficient to stimulate immunity. Therefore it is difficult to ascertain the effects of the drugs on the immunity and responses of the animals to a challenge of O. circumcincta L_3.

However, the animals of the control (excluding animal number 104), bromhexine and corticosteroid groups were all treated similarly and therefore it is possible that bromhexine treatment was acting to reduce parasite establishment and plasma pepsinogen activities. There could be a number of reasons for this effect.

(i) Mucus glycoproteins were altered sufficiently by bromhexine treatment that the incoming parasites were unable to recognise their environmental niche and failed to establish. Few larvae were detected in the faeces following challenge of the bromhexine group and it would be expected that more larvae would be found if this had occurred.

(ii) The altered structure made it impossible for the parasites to be maintained within this layer after development to adult stages, which would subsequently by lost.

One animal produced high numbers of parasite eggs, indicating that adult parasites were established, the rest of the animals passed few parasite eggs. It is interesting to note that the animals in this group with the highest number of parasites at necropsy, passed few parasite eggs. This underlines the unreliability of faecal egg counts as a measure of parasitism.

(iii) The altered structure allowed the increased liberation of immunoglobulins and a more effective response against the

incoming larvae.

Alternatively, it is possible that the differences observed between the bromhexine and control groups are due to differences in their immunity to O. circumcincta and this was unaffected by bromhexine treatment. If the bromhexine group is compared to sheep number 104 of the controls that underwent a more prolonged immunisation period prior to the experiment, there is little difference between plasma pepsinogen response and parasites established.

The responses of the steroid group show that even at a low dose of dexamethasone, higher plasma pepsinogen activities, faecal egg counts and numbers of parasites established were recorded. The number of parasites at necropsy was significantly higher statistically than those of the bromhexine and omeprazole groups. This lends further support to the idea that the control group was unable to prevent parasite establishment.

When the plasma pepsinogen activities of the animals treated with omeprazole are compared to the control group there is little difference, the pepsinogen reaching a maximum slightly earlier in the omeprazole treated animals compared to the controls. There were also fewer parasites established at necropsy compared to the control group.

However, when the plasma pepsinogen activity of the omeprazole treated group is compared to that of sheep 104 which acted as the group control, differences are seen. There was no obvious peak in plasma pepsinogen activities in sheep number 104 compared to the high activities recorded after day 12 in the omeprazole group. There was little difference in the numbers of

parasites established.

Omeprazole was administered only for the first three days of the experiment, but during this time the abomasal pH was unlikely to fall below pH 4. Therefore there would be a reduction in the formation of pepsin from pepsinogen within the abomasal glands. Concomitant with this, there would be a rise in gastrin concentration due to the acid suppression.

The rise in plasma pepsinogen activity in the omeprazole group could be due to any one or a combination of reasons.

(i) A higher concentration of pepsinogen in the abomasal lumen would be present during omeprazole treatment and therefore more would be available to pass into the plasma. But omeprazole was only administered for a short time at the start of the experiment and its effects on abomasal pH were shown to last less than 12 hours. The high plasma pepsinogen activities were recorded 12 days later.

(ii) The challenge caused a hypersensitive response which caused the mucosal permeability to increase causing a rise in plasma pepsinogen early on in the experiment, seen in the rise in pepsinogen activity reaching a maximum at day 6. It is possible that the hypersensitive increase in permeability and the higher concentration of pepsinogen acted together to cause the elevation, but this is unlikely considering the hypersensitive response occurred around day 6 and the pepsinogen activity fell until day 12.

(iii) The treatment with omeprazole would have caused gastrin levels to rise. The effect of gastrin on acid and pepsinogen

production could also contribute to the pepsinogen rise.

(iv) The plasma pepsinogen activity increase could have been caused by parasite damage due to emergence of larvae. However, there was little evidence of parasite establishment at necropsy and no parasite eggs were detected in the faeces. The treatment with omeprazole over the time of challenge could have allowed the parasites to establish and cause the damage to produce the high pepsinogen activities but as the young adults emerged they were removed by the host's immune response.

The fact that parasite eggs were not detected in the faeces may also be explained by this. It would be expected that animals showing high pepsinogen activities as three animals of this group did, would be due to parasite establishment and parasite eggs would be detected. Only one parasite egg was detected on one occasion in one animal (107).

The possibility of omeprazole allowing the parasites to establish in previously immune sheep is not understood. It is possible that altering abomasal pH allowed a less effective specific immune response against the incoming larvae, or that non-specific mediators were less effective.

Alternatively, the rise in gastrin concentrations caused by omeprazole treatment could have an adverse effect on host immunity.

The results obtained from leukotriene C_4 measurement in the various mucus samples were interesting. It must be remembered that the animals were killed 24 days post-challenge (26 days for the bromhexine group) and the concentration of leukotriene C_4 may have changed markedly in this time.

It is thought that the leukotrienes are released from mucosal mast cells, eosinophils and possibly other cells that increase in the gastrointestinal tract at parasite challenge (Moqbel et al, 1987).

Globule leukocyte numbers correlate well with the amount of anti-parasitic activity of gastrointestinal mucus (Douch et al, 1986) and these are thought by many to be derived from mast cells (Miller, 1984; 1987) and by others to be a distinct cell type (Douch et al, 1986). It has been reported that LTC_4 concentrations in gut tissue and the mucus were significantly higher in immune sheep compared to non-immune animals challenged with O. circumcincta (Moqbel, Smith, Newlands and MacDonald, cited Moqbel et al, 1987) This would suggest a possible protective role of these mediators incorporated into mucus. It was not stated if these immune sheep had been recently challenged. Raised leukotriene mucus concentrations have also been recorded in rats undergoing parasite challenge (Moqbel et al, 1986; 1987).

Two, non-immune, unchallenged lambs were used for gastrointestinal mucus collection in the same way as described above, and LTC_4 concentration determined. The values for both abomasal and duodenal mucus were higher than those recorded in the adult sheep. Abomasal mucus concentrations of 8,150 pg/g and 12,245 pg/g, and duodenal concentrations of 2,081 and 5,720 pg/g were recorded. The reason for this is unknown as it was expected that lower LTC_4 concentrations would be obtained. The high abomasal concentrations may be explained by the difficulty in

obtaining sufficient mucus from the small abomasa of these lambs. More vigorous scraping of the mucosal surface may have resulted in the generation of inflammatory mediators from the mucosal cells even in the cooled conditions.

Ideally these controls for LTC_4 concentrations would have been the same age etc. as the adult sheep used in this experiment, but this was not possible.

Inflammatory gastrointestinal conditions could have altered the leukotriene concentration of the mucus in these young animals, associated with change of diet or infection but there was no evidence of this at the time of slaughter.

There was no obvious correlation between leukotriene concentration in the mucus and numbers of parasites established after challenge, but the group treated with a low dose of corticosteroid had a lower concentration of leukotriene C_4 in the gastrointestinal mucus than the other groups. This would add weight to the idea that the origin of the leukotriene was from mast cells, known to be depleted by corticosteroid treatment. However, other cells e.g. macrophages would also be affected. It is known that corticosteroids inhibit leukotriene synthesis in alveolar macrophages (Peters-Golden and Thebert, 1987).

Obviously, further studies need to be done on the possible effects of bromhexine and omeprazole on host-parasite relationships before conclusions can be drawn.

TABLE 26

Viscosity of duodenal mucus samples obtained from five adult sheep, two sheep were treated with bromhexine, three sheep were untreated controls

Group	Sheep number	Viscosity measurement (Centipoise)
Control	121	2.456, 2.502
	1	1.902, 1.904
	58	2.508, 2.484
	Mean ± SEM	2.293 ± 0.123
Bromhexine	64	2.276, 2.270
	65	2.368, 2.484
	Mean ± SEM	2.350 ± 0.050

TABLE 27

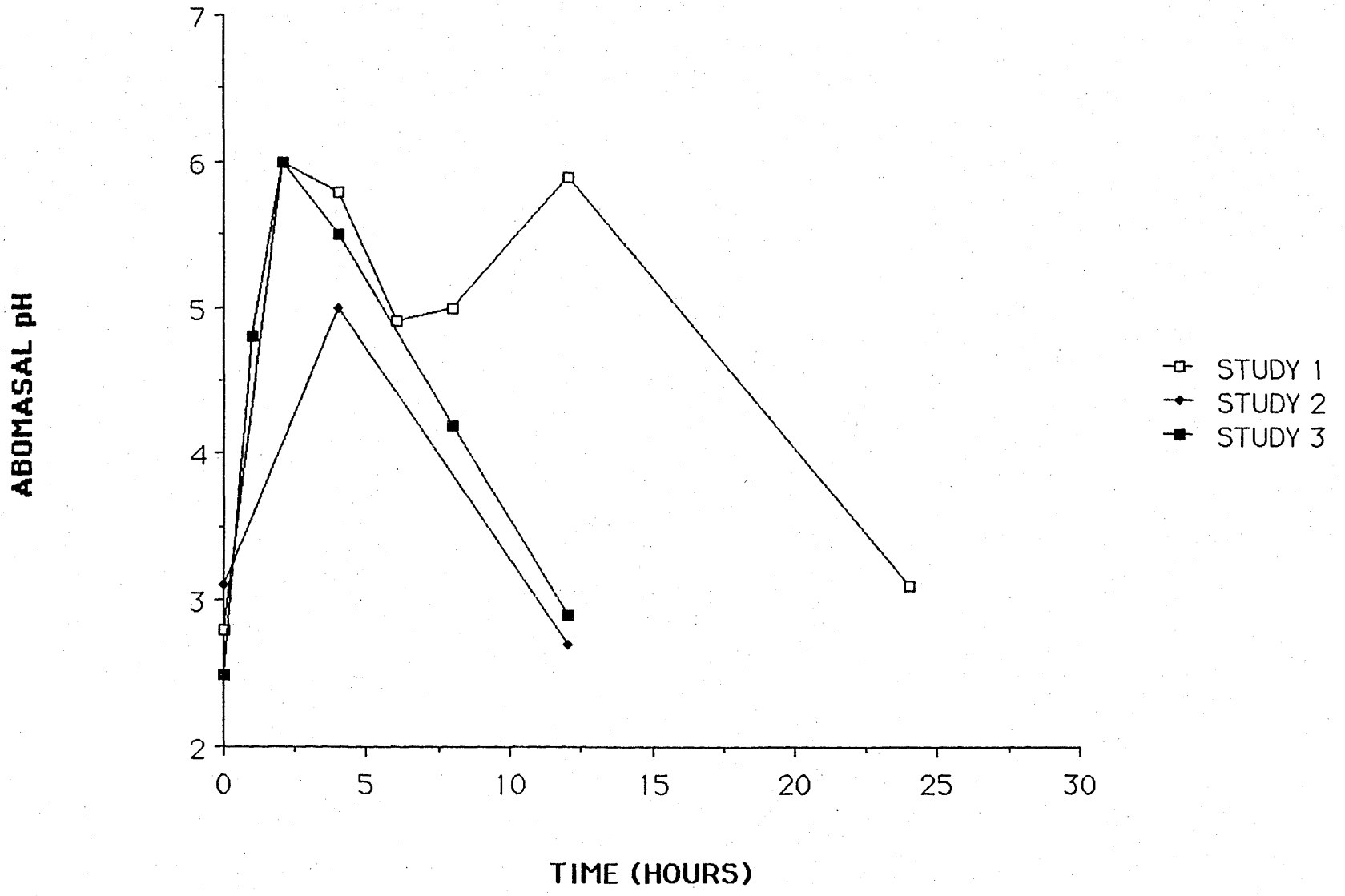
Abomasal pH and plasma pepsinogen activity in a sheep treated with omeprazole

Time (hours)	Abomasal pH	Plasma pepsinogen activity (I.U.)
Study 1.		
+0	2.8	0.324
2	6.0	-
4	5.8	-
6	4.9	-
+8	5.0	0.398
12	5.9	-
24	3.1	0.504
Study 2.		
+0	3.1	0.504
4	5.0	-
12	2.7	0.355
Study 3.		
+0	2.5	0.366
1	4.8	-
2	6.0	0.514
4	5.5	-
8	4.2	-
12	2.9	-

+ Time of intravenous injection of 40 mg omeprazole.

FIGURE 23

Abomasal pH after treatment of an adult sheep with omeprazole, a substance that raises intragastric pH


ABOMASAL pH
7
6
5
4
3
2
0
5
10
15
20
25
30
TIME (HOURS)
STUDY 1
STUDY 2
STUDY 3

The control group was untreated.

The bromhexine group received bromhexine hydrochloride orally at a dose rate of 0.5 mg/kg for ten days around the time of challenge.

The corticosteroid group received dexamethasone acetate by intramuscular injection, once weekly, at a dose rate of 0.08 mg/kg throughout the experiment.

The omeprazole group received 40 mg omeprazole by intravenous injection every eight hours for three days around the time of challenge.

TABLE 28

Plasma pepsinogen activity (I.U.) in four groups of adult sheep. All animals were challenged with 200,000 *O. circumcincta* L_3 on day 0 as a single dose

Control Group

Time (days)	Animal number 74	104	110	113	114	122	Mean ± SEM
Pre 0	0.801	0.870	1.188	1.709	1.326	0.745	1.107 ± 0.149
2	1.701	1.112	1.397	1.746	2.042	1.153	1.525 ± 0.147
4	2.478	1.163	2.317	2.523	3.202	1.921	2.267 ± 0.273
6	2.182	0.788	1.478	3.313	2.893	1.141	1.966 ± 0.401
8	1.714	0.748	0.920	2.523	2.005	0.582	1.415 ± 0.312
10	1.566	1.223	0.733	1.672	1.919	0.454	1.261 ± 0.228
12	1.561	0.880	0.722	1.413	2.362	0.431	1.228 ± 0.280
14	1.800	1.102	0.839	1.252	3.498	0.443	1.489 ± 0.433
16	2.429	1.031	1.223	1.388	4.078	0.396	1.758 ± 0.526
18	5.226	1.122	2.992	1.795	3.954	0.361	2.575 ± 0.732
20	8.937	0.708	5.216	2.437	4.460	0.361	3.687 ± 1.290
22	9.146	1.274	6.194	3.535	5.669	0.349	4.361 ± 1.318
24	8.616	1.031	5.938	4.016	5.348	0.303	4.209 ± 1.254

TABLE 29

Plasma pepsinogen activity (I.U.) in four groups of adult sheep.

All animals were challenged with 200,000 *O. circumcincta* L_3

on day 0 as a single dose.

Bromhexine Group

Time (days)	Animal number 73	103	105	109	111	Mean ± SEM
0	0.706	0.767	0.515	0.821	1.427	0.847 ± 0.154
2	1.023	1.188	1.023	0.700	1.899	1.167 ± 0.200
4	2.231	1.595	2.816	0.862	2.540	2.009 ± 0.351
6	1.379	0.808	2.879	0.862	2.105	1.607 ± 0.394
8	0.604	0.523	2.078	0.713	1.947	1.173 ± 0.345
10	0.477	0.482	1.443	0.795	1.790	0.997 ± 0.265
12	0.489	0.550	1.290	0.835	1.524	0.938 ± 0.204
14	0.375	0.374	1.328	1.148	2.350	1.115 ± 0.365
16	0.604	0.509	1.087	1.392	2.648	1.248 ± 0.385
18	0.680	0.495	0.947	1.908	2.443	1.295 ± 0.376
20	0.756	0.550	0.947	2.057	2.370	1.336 ± 0.367
22	0.845	0.537	0.884	1.840	2.612	1.344 ± 0.385
24	0.858	0.563	0.820	1.582	2.540	1.273 ± 0.359
26	0.743	0.469	0.718	1.378	2.261	1.114 ± 0.324

TABLE 30

Plasma pepsinogen activity (I.U.) in four groups of adult sheep. All animals were challenged with 200,000 *O. circumcincta* L_3 on day 0 as a single dose.

Corticosteroid Group

Time (days)	Animal number 52	101	102	112	121	Mean ± SEM
0	1.450	1.485	1.473	0.441	0.847	1.139 ± 0.212
2	1.255	2.063	1.160	0.539	1.258	1.255 ± 0.242
4	0.980	2.508	2.339	1.313	1.476	1.723 ± 0.298
6	0.882	2.435	1.858	1.108	1.331	1.523 ± 0.280
8	0.990	2.014	1.004	0.412	0.931	1.070 ± 0.260
10	0.931	2.027	0.848	0.912	0.895	1.123 ± 0.227
12	2.039	3.806	0.968	0.470	1.173	1.691 ± 0.586
14	2.676	4.528	0.800	0.441	1.222	1.933 ± 0.752
16	5.244	8.112	0.692	1.000	1.887	3.387 ± 1.432
18	6.538	13.968	1.570	3.274	3.193	5.709 ± 2.217
20	6.078	11.744	2.929	4.117	4.801	5.934 ± 1.539
22	4.048	8.448	2.231	1.520	5.236	4.297 ± 1.228
24	4.401	10.637	2.676	1.569	6.156	5.088 ± 1.591

TABLE 31

Plasma pepsinogen activity (I.U.) in four groups of adult sheep. All animals were challenged with 200,000 *O. circumcincta* L_3 on day 0 as a single dose.

Omeprazole Group

Time (days)	Animal number 8	72	107	108	Mean ± SEM
0	0.653	1.160	0.768	0.495	0.769 ± 0.142
2	1.239	2.309	1.314	1.526	1.597 ± 0.245
4	1.611	2.568	1.658	0.930	1.692 ± 0.336
6	1.650	2.771	1.961	0.798	1.795 ± 0.408
8	0.743	1.453	1.870	0.940	1.252 ± 0.255
10	0.574	1.036	1.334	1.647	1.148 ± 0.228
12	0.743	1.036	2.032	1.677	1.372 ± 0.294
14	0.484	1.014	6.924	2.818	2.810 ± 1.460
16	0.417	1.521	10.877	5.415	4.558 ± 2.364
18	0.372	1.936	11.109	4.092	4.377 ± 2.370
20	0.439	2.962	10.563	3.445	4.352 ± 2.173
22	0.484	2.444	5.984	2.334	2.812 ± 1.149
24	0.450	1.972	3.285	1.889	1.899 ± 0.579

FIGURE 24

Mean plasma pepsinogen activities (I.U.) of three groups of sheep treated with various substances and the untreated control group. All animals were challenged with 200,000 *O. circumcincta* L_3 on day 0

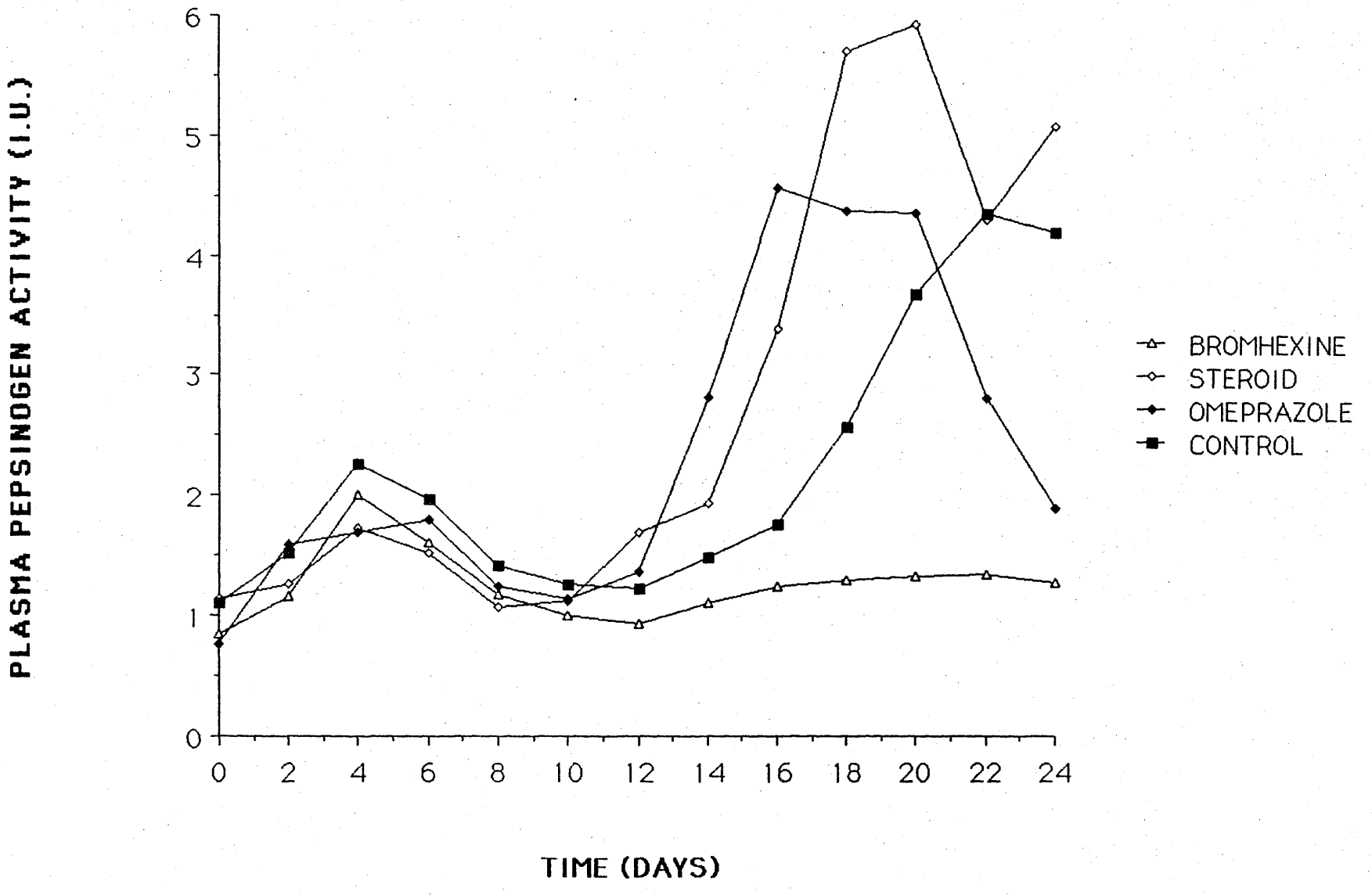

PLASMA PEPSINOGEN ACTIVITY (I.U.)
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (DAYS)
BROMHEXINE
STEROID
OMEPRAZOLE
CONTROL

FIGURE 25

Mean plasma pepsinogen activity (I.U.) of the group treated with omeprazole and the plasma pepsinogen activity of sheep number 104 that acted as an untreated control. All animals were challenged with 200,000 *O. circumcincta* L_3 on day 0

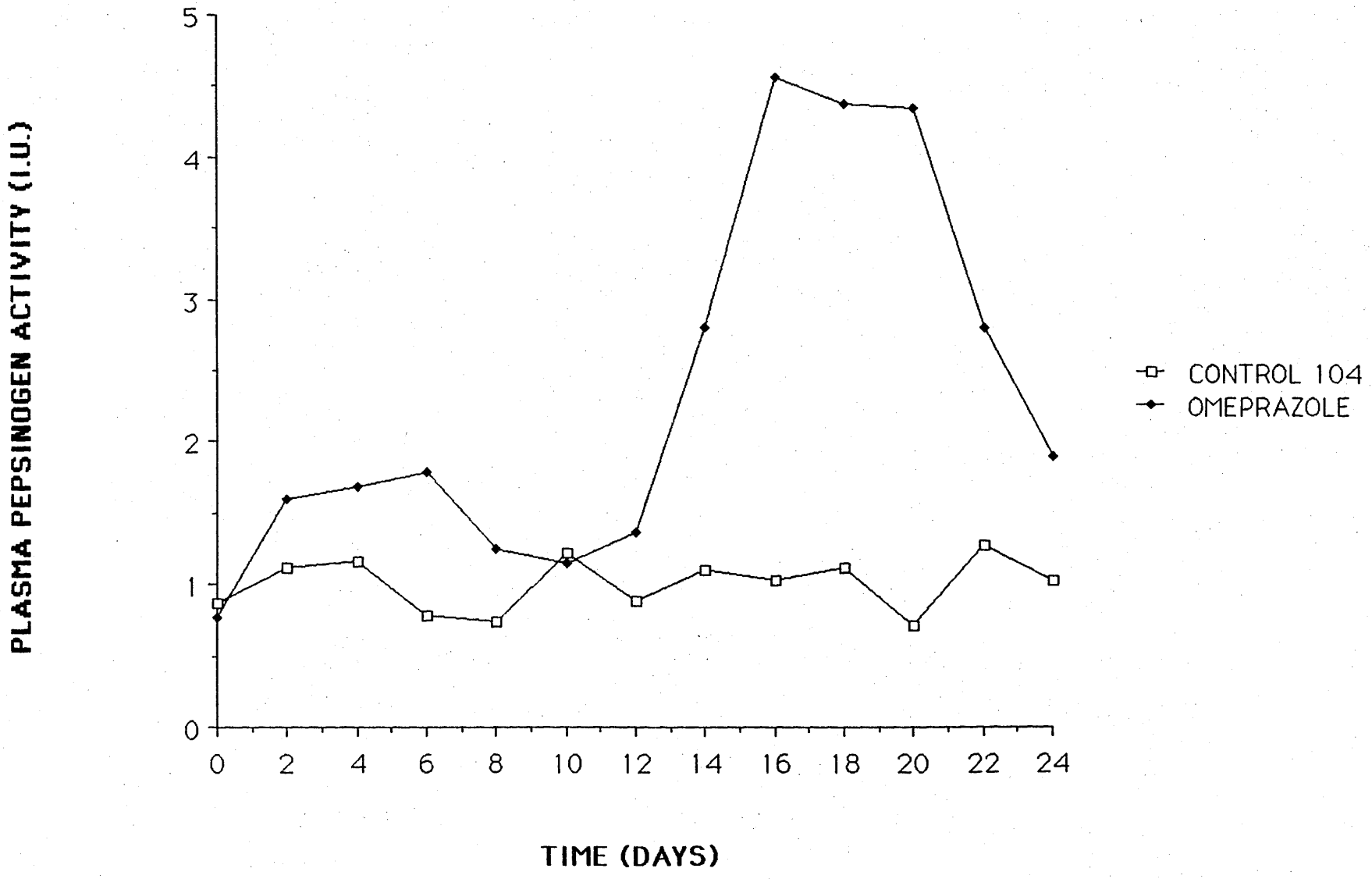

PLASMA PEPSINOGEN ACTIVITY (I.U.)
5
4
3
2
1
0
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (DAYS)
CONTROL 104
OMEPRAZOLE

FIGURE 26

Plasma pepsinogen activities (I.U.) of the sheep treated with omeprazole and sheep number 104 that acted as the untreated control. All animals were challenged with 200,000 *O. circumcincta* L_3 on day 0

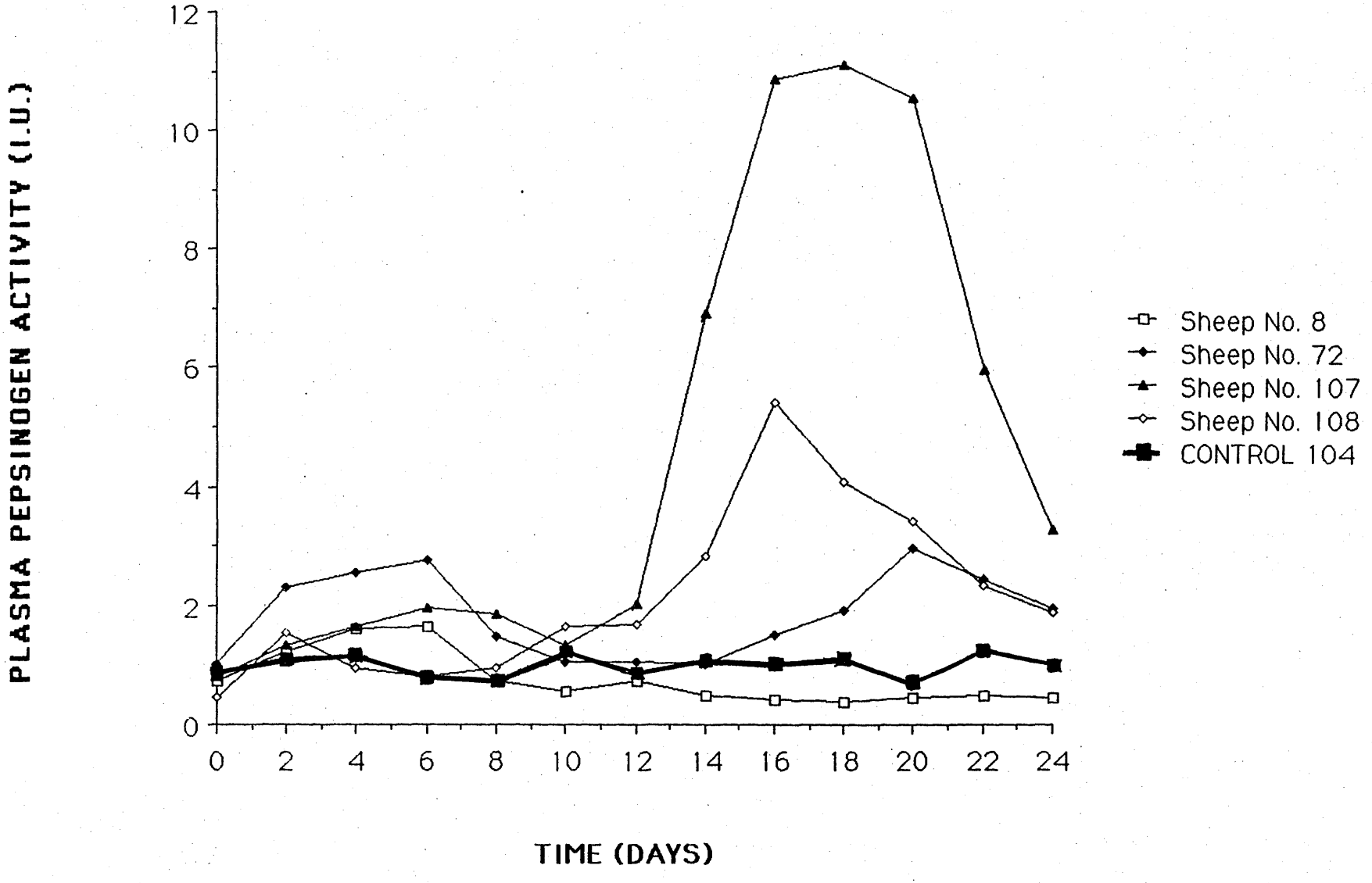

PLASMA PEPSINOGEN ACTIVITY (I.U.)
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (DAYS)
Sheep No. 8
Sheep No. 72
Sheep No. 107
Sheep No. 108
CONTROL 104

TABLE 32

Number of parasite eggs per gram of faeces in four groups of adult sheep. All animals received 200,000 *O. circumcincta* L_3 on day 0 as a single dose

	Time (days)							
	0	12	17	19	21	23	25	27
Control Group								
74	0	0	100	50	400	50	0	-
104	0	0	0	0	0	0	0	-
110	0	0	0	100	250	150	50	-
113	0	0	0	0	0	0	100	-
114	0	0	0	0	50	0	50	-
122	0	0	0	0	0	600	500	-
Bromhexine Group								
73	0	0	0	0	0	50	200	550
103	0	0	0	0	0	100	100	300
105	0	0	0	0	0	0	0	50
109	0	0	0	0	0	0	0	0
111	0	0	0	0	0	0	0	50
Corticosteroid Group								
52	0	0	1,200	600	100	50	250	-
101	0	0	200	150	0	150	150	-
102	0	0	0	0	50	0	50	-
112	0	0	0	0	0	50	350	-
121	0	0	0	0	0	0	0	-
Omeprazole Group								
8	0	0	0	0	0	0	0	-
72	0	0	0	0	0	0	0	-
107	0	50	50	0	0	0	0	-
108	0	0	0	0	0	0	0	-

FIGURE 27

Number of parasite eggs detected in the faeces of sheep of the control group. No parasite eggs were detected in the faeces of sheep number 104

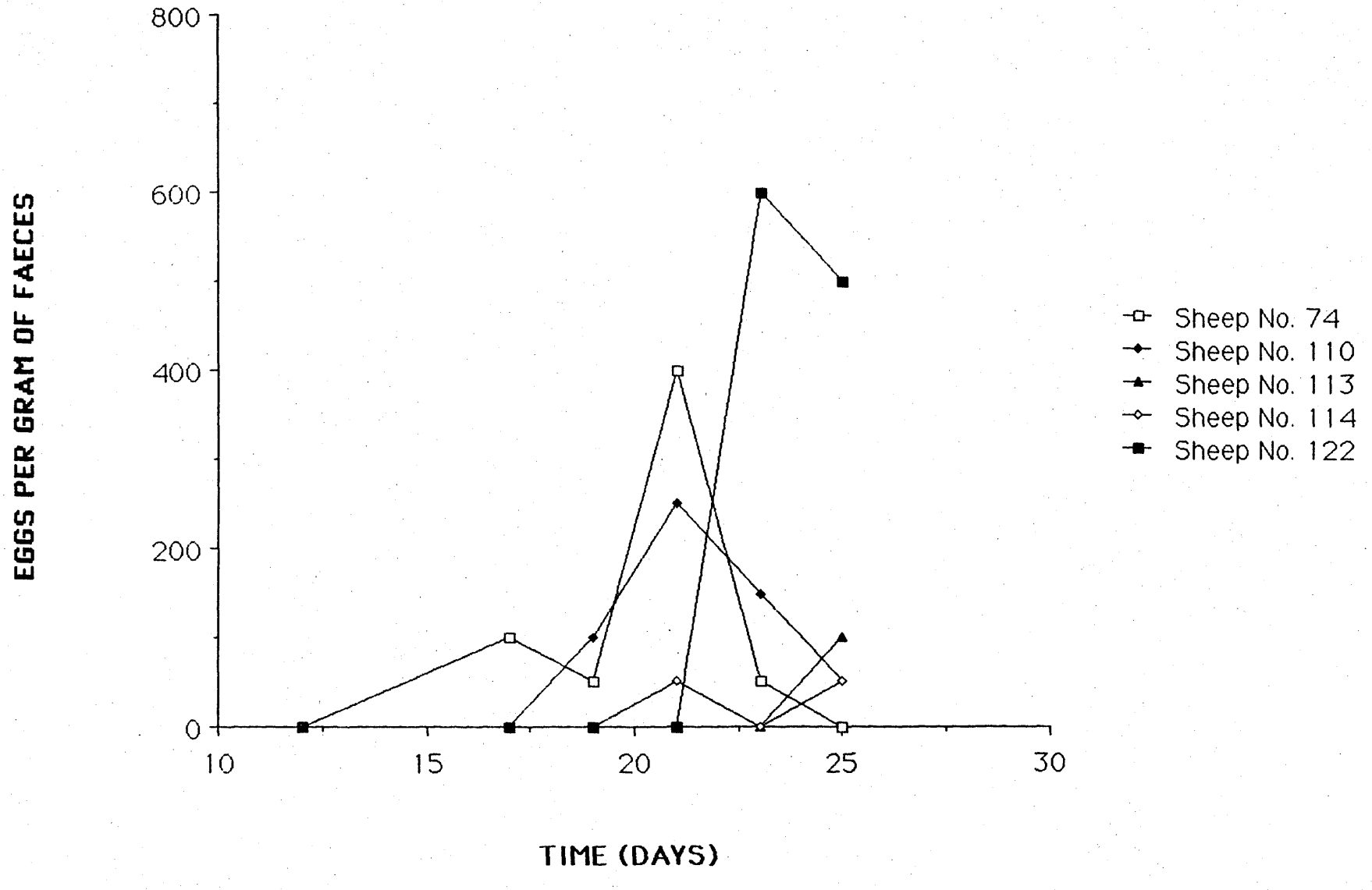

800
600
400
200
0
10
15
20
25
30
EGGS PER GRAM OF FAECES
TIME (DAYS)
Sheep No. 74
Sheep No. 110
Sheep No. 113
Sheep No. 114
Sheep No. 122

FIGURE 28

Number of parasite eggs detected in the faeces of sheep treated with bromhexine. No parasite eggs were detected in the faeces of sheep number 109

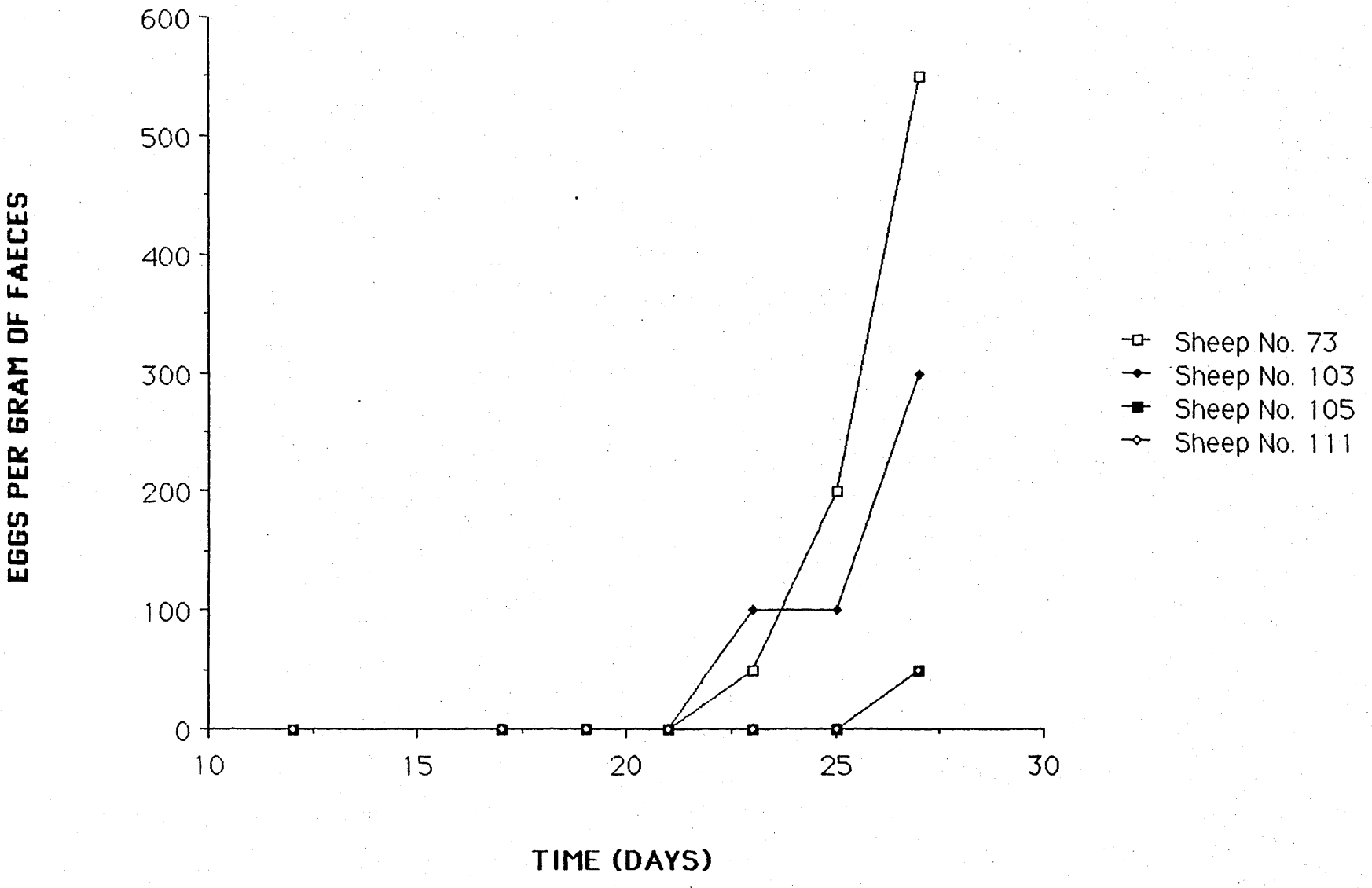

EGGS PER GRAM OF FAECES
600
500
400
300
200
100
0
10
15
20
25
30
TIME (DAYS)
Sheep No. 73
Sheep No. 103
Sheep No. 105
Sheep No. 111

FIGURE 29

Number of parasite eggs detected in the faeces of sheep treated with a corticosteroid. No parasite eggs were detected in the faeces of sheep number 121

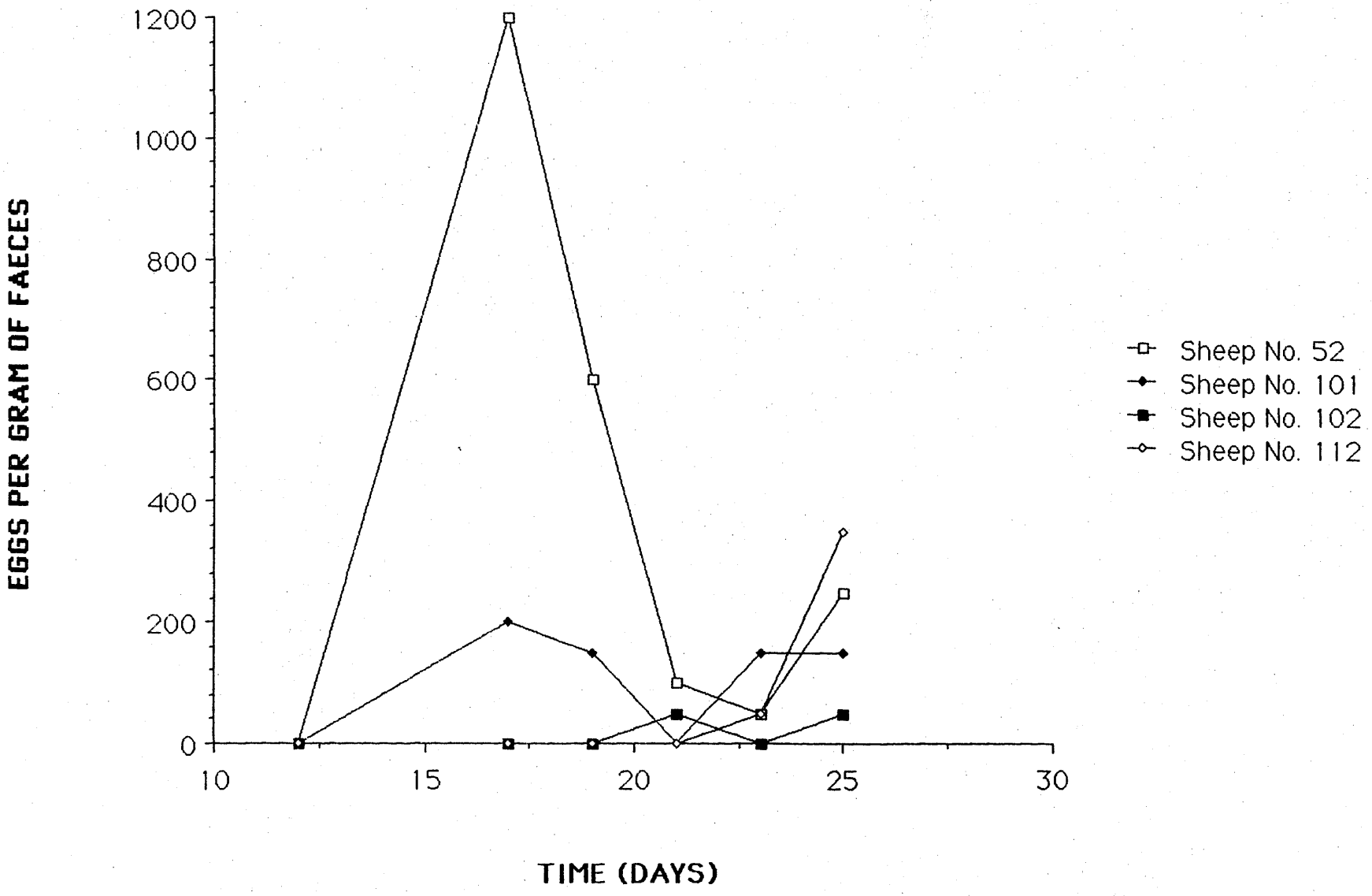

EGGS PER GRAM OF FAECES
1200
1000
800
600
400
200
0
10
15
20
25
30
TIME (DAYS)
Sheep No. 52
Sheep No. 101
Sheep No. 102
Sheep No. 112

TABLE 33

Number of larvae detected in the faeces of four groups of adult sheep after challenge with 200,000 *O. circumcincta* L_3. Larvae were detected by Baermanisation and salt flotation techniques

Time (days)	Control Group											
	Animal number											
	74		104		110		113		114		122	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0	0	0	0	0	7	0	4	0	0	1	0
2	0	0	0	0	0	0	0	0	0	0	0	2
3	0	0	0	0	0	1	0	0	0	0	0	0
4	0	0	-	-	0	0	0	0	0	0	0	0

Time (days)	Bromhexine Group									
	Animal number									
	73		103		105		109		111	
	A	B	A	B	A	B	A	B	A	B
1	0	1	0	0	0	0	0	0	0	1
2	0	0	0	0	0	0	0	0	0	0
3	0	1	0	0	0	0	0	0	0	0
4	-	-	-	-	-	-	-	-	-	-

Time (days)	Corticosteroid Group									
	Animal number									
	52		101		102		112		121	
	A	B	A	B	A	B	A	B	A	B
1	0	0	0	0	0	1	0	0	0	2
2	0	0	0	8	0	1	0	0	0	0
3	0	2	0	2	0	0	0	0	0	0
4	-	-	-	-	-	-	-	-	-	-

Time (days)	Omeprazole Group							
	Animal number							
	8		72		107		108	
	A	B	A	B	A	B	A	B
1	0	0	0	1	0	0	0	0
2	0	0	1	1	0	0	0	0
3	0	0	0	0	0	0	0	0
4	-	-	-	-	-	-	-	-

A - results from salt flotation technique.
B - results from Baermanisation technique.

TABLE 34

The pH of abomasal contents at necropsy of four groups of adult sheep. All animals received 200,000 *O. circumcincta* L_3 on day 0 as a single dose

Group	Animal number	Abomasal pH
Control	74	6.7
	104	5.6
	110	4.2
	113	4.2
	114	4.4
	122	2.7
	Mean ± SEM	4.63 ± 0.56
Bromhexine	73	4.3
	103	3.5
	105	3.9
	109	3.8
	111	5.8
	Mean ± SEM	4.26 ± 0.41
Corticosteroid	52	5.9
	101	5.5
	102	6.0
	112	4.6
	121	6.8
	Mean ± SEM	5.76 ± 0.36
Omeprazole	8	4.1
	72	6.1(b)
	107	4.2
	108	3.5(a)
	Mean ± SEM	4.48 ± 0.56

(a) tumour infiltration down the gastrointestinal tract in the form of discrete nodules.

(b) very little sample present.

TABLE 35

Numbers of abomasal parasites at necropsy in four groups of adult sheep treated with various agents. All animals were challenged with 200,000 *O. circumcincta* L_3 on day 0 as a single dose

		Abomasal contents					Abomasal digest					
				Adults					Adults			Total
Group	Animal number	L_4	L_5	♂	♀	Total number	L_4	L_5	♂	♀	Total number	in abomasum
Control	74	100	200	14,600	22,400	37,300	0	0	100	400	500	37,800
	104	50	0	0	150	200	3,000	0	0	0	3,000	3,200
	110	0	400	9,100	9,100	18,600	200	100	700	600	1,600	20,200
	113	2,800	3,900	5,400	6,000	18,100	1,800	200	0	0	2,000	20,100
	114	200	1,000	3,300	5,300	9,800	400	600	400	200	1,600	11,400
	122	0	0	400	0	400	0	0	700	600	1,300	1,700
											Mean	15,733
											± SEM	5.482.3
Bromhexine	73	0	0	700	200	900	0	0	0	0	0	900
	103	0	0	500	500	1,000	0	0	0	0	0	1,000
	105	0	400	100	100	600	0	0	0	0	0	600
	109	1,900	500	4,600	4,400	11,400	400	0	100	100	600	12,000
	111	11,600	1,200	1,500	700	15,000	1,200	0	0	0	1,200	16,200
											Mean	6,140
											± SEM	3,111.7
Steroid	52	400	200	4,100	6,200	10,900	200	100	300	300	900	11,800
	101	4,700	800	19,200	20,800	45,500	1,000	0	400	800	2,200	47,700
	102	3,750	1,900	4,400	3,450	13,500	2,900	0	100	100	3,100	14,600
	112	6,100	1,100	11,100	10,600	28,900	8,800	200	1,400	800	11,200	40,100
	121	2,800	0	17,600	16,500	36,900	1,800	0	0	0	1,800	38,700
											Mean	30,580
											± SEM	7,259.5
Omeprazole	8	0	0	2,700	4,500	7,200	0	0	100	100	200	7,400
	72	0	0	700	600	1,300	0	0	0	0	0	1,300
	107	0	0	800	2,400	3,200	0	0	200	200	400	3,600
	108	0	0	2,500	2,200	4,700	0	0	100	100	200	4,900
											Mean	4,300
											± SEM	1.273.5

TABLE 36

Establishment of parasites after a single challenge of 200,000 *O. circumcincta* L_3 in four groups of adult sheep treated with various agents. Establishment expressed as a percentage

Group	Sheep number	Total number of parasites	Establishment (%)
Control	74	37,800	18.90
	104	3,200	1.60
	110	20,200	10.10
	113	20,100	10.05
	114	11,400	5.70
	122	1,700	0.85
		Mean ± SEM	7.87 ± 2.74
Bromhexine	73	900	0.45
	103	1,000	0.50
	105	600	0.30
	109	12,000	6.00
	111	16,200	8.10
		Mean ± SEM	3.07 ± 1.66
Corticosteroid	52	11,800	5.90
	101	47,700	23.85
	102	14,600	7.30
	112	40,100	20.05
	121	38,700	19.35
		Mean ± SEM	15.29 ± 3.63
Omeprazole	8	7,400	3.70
	72	1,300	0.65
	107	3,600	1.80
	108	4,900	2.45
		Mean ± SEM	2.15 ± 0.64

TABLE 37

Motility of *O. circumcincta* L_3 incubated *in vitro* with various concentrations of bromhexine hydrochloride. Motility expressed as percentage of control motility

Concentration bromhexine hydrochloride (ug/ml)	Time (hours) 1	3	6	24
30	128.35	137.23	96.17	113.32
3	104.62	104.22	103.83	107.29
0.3	110.21	82.28	100.87	102.61
0.03	113.36	105.98	105.60	99.29
0.003	138.88	122.00	101.42	134.39

TABLE 38

The motility of *O. circumcincta* L_3 incubated *in vitro* with various concentrations of omeprazole and known anthelmintic drugs. Motility expressed as percentage of control motility

Concentration of test substance	Time (hours) 17	24	48	72
0.1 mg/ml omeprazole	104.73	123.44	114.74	103.48
0.04 mg/ml omeprazole	105.80	119.62	106.52	106.77
0.01 mg/ml omeprazole	121.67	110.78	104.17	112.87
0.005 mg/ml omeprazole	122.05	85.61	93.01	102.35
Albendazole sulphoxide 10 ug/ml	28.57*	31.87*	18.82**	28.73*
Thiabendazole 20 ug/ml	39.38*	10.20*	11.89**	9.51*
Levamisole 30 ug/ml	23.87*	17.81**	11.66*	11.81*

* $p < 0.05$ ** $p < 0.01$

indicating levels of significant difference from control values

TABLE 39

Concentration (pg/g mucus) of leukotriene C_4 in the gastrointestinal tract of four groups of adult sheep

Control Group	Animal number						
Mucus sample	74	104	110	113	114	122	Mean ± SEM
Abomasal	2,906	2,764	3,342	965	168	263	1,735 ± 584
Duodenal	2,748	3,141	1,788	1,928	1,746	2,457	2,301 ± 234

Corticosteroid Group	Animal number					
Mucus sample	52	101	102	112	121	Mean ± SEM
Abomasal	1,345	1,029	1,468	1,775	1,933	1,510 ± 159
Duodenal	1,413	2,235	1,118	1,247	1,822	1,567* ± 205

Bromhexine Group	Animal number					
Mucus sample	73	103	105	109	111	Mean ± SEM
Abomasal	2,760	2,826	1,927	2,914	1,748	2,435 ± 246
Duodenal	932	3,811	2,735	1,502	2,199	2,236 ± 498

Omeprazole Group	Animal number				
Mucus sample	8	72	107	108	Mean ± SEM
Abomasal	2,402	3,728	1,256	1,145	2,133 ± 603
Duodenal	1,072	3,020	1,390	3,645	2,282 ± 623

* $p < 0.05$ significantly different from control values

In vitro STUDIES ON ISOLATED GASTROINTESTINAL SMOOTH MUSCLE PREPARATIONS

INTRODUCTION

Smooth muscle of the gastrointestinal tract

Many reviews of the structure and function of intestinal smooth muscle have been published recently (Bolton, 1979; Bülbring, 1979; Horton, 1979; Gabella, 1981; Hartshorne, 1981). A general, brief summary on the subject is presented here.

Smooth muscle constitutes the muscular layers of the gastrointestinal tract. It is also present in other sites of the body. It is associated with the gastrointestinal tract as two outer layers. The inner layer consists of muscle fibres orientated circularly around the tubular tract, the circular layer, and the outer layer where the muscle fibres run longitudinally, the longitudinal layer. There is also present, a very small layer, the muscularis mucosa, which separates the mucosa and submucosa. The circular and longitudinal muscle layers of the gastrointestinal tract are important in the propulsion and mixing of the tract contents. Contained within each muscle cell are proteins, actin and myosin, present as filaments, which constitute part of the contractile apparatus of the cell. In isotonic contraction the cells shorten to a similar degree as the whole muscle. It is thought this is due to the actin and myosin filaments sliding past each other as in skeletal muscle, but less is known about the process of contraction in smooth muscle. Tension would be generated as a consequence of cyclic cross bridge-actin interactions which is associated with

the hydrolysis of adenosine triphosphate (ATP). As in skeletal muscle the concentration of intracellular calcium plays an important role in the process of contraction.

The innervation of the gastrointestinal tract is both intrinsic and extrinsic.

The most prominant plexus containing intrinsic neurones is the myenteric (Auerbach's) plexus which is present between the circular and longitudinal layers of the smooth muscle. Axons from this plexus penetrate both smooth muscle layers. The neurones also receive input from receptors in the mucosa and the smooth muscle. Therefore local reflexes within the gastrointestinal tract wall can be set up. These are important in peristalsis. Another plexus present is the submucosal (Meissner's) plexus. This is thought to be more important in the control of secretion and receiving sensory stimuli from the mucosa and submucosa.

In addition to the intrinsic control involving the myenteric plexus, extrinsic control is also exerted on the gastrointestinal tract. Axons from neurones of the parasympathetic and sympathetic nervous systems enter the plexuses. Parasympathetic control is mainly via the vagus nerve. Sympathetic control is via different ganglia and usually exerts an inhibitory action on neurones within the nerve plexuses.

Studies into the different neurotransmitters present in the wall of the gut have shown a majority of neurones contain cholinesterase; indicating acetylcholine as the neurotransmitter involved. Large amounts of acetylcholine are stored and released by the myenteric plexus (Dikshit, 1938; Welsh and Hyde, 1944).

A catecholamine, nor-adenaline, is present in axons entering from the sympathetic nervous system. As stated previously, one of the important effects of sympathetic stimulation or exogenously applied nor-adrenaline is to inhibit release of acetylcholine from cholinergic nerve endings (Paton and Vizi, 1969; Kosterlitz, Lydon and Watt, 1970).

ATP or related purines may be present in some inhibitory neurones that are non-adrenergic (Burnstock et al, 1970). Enkephalins are also present in neurones of the gut. These are pentapeptides that have high affinity for opiate receptors. They also appear to be involved in the inhibition of acetylcholine particularly within the myenteric plexus (North and Williams, 1976).

Gastrins, cholecystokinin, substance P and somatostatin have all been localised within gastrointestinal neurones.

The contractions of the upper gastrointestinal tract are controlled by a number of different mechanisms.

1. Activity of extrinsic nerves.
2. Activity of intrinsic nerves.
3. Inherent, spontaneous smooth muscle cell activity.
4. Circulating or locally released chemical mediators e.g. prostaglandins, 5-hydroxytryptamine and adrenaline.

Spontaneous smooth muscle cell activity in the gastrointestinal tract is produced as the muscle cells have an unstable resting membrane potential. Periodic depolarisations occur at regular intervals due to regular fluctuations of ionic permeability of the muscle cell membrane (slow waves). These

transmembrane potential changes are synchronised for several cells to produce the basal electrical rhythm (BER). These are always present. Contractions are not associated with each of these periodic depolarisations. Action potentials (spike potentials) represent fast depolarisations of smooth muscle cells and cause contraction of the smooth muscle. The greater the frequency of action potentials in a burst, the greater the contraction. Muscle cells of both the longitudinal and circular muscle layers generate these. Spontaneous contractions probably arise as a result of simultaneous depolarisation of many cells (Creed, 1979).

In the intestine, the spike potentials occur in a phase called, the 'phase of regular spiking activity' (RSA) and is preceded by a long period during which the spike potentials are irregularly superimposed on the slow waves 'phase of irregular spiking activity' (ISA) and followed by a period of no spiking activity. These two cyclic phases ISA and RSA, are propagated from the duodenum where they originate, to the ileum as a 'migrating myoelectric complex' (MMC). The slow waves and the action potentials can be modified by nervous and humoral factors.

Peristalsis

This is an intrinsic neural reflex which is stimulated by radial stretch of the intestine. It is the main form of propulsion in the intestine and the control is very complex (for a detailed description see Hirst, 1979). Basically it consists of contraction of a ring of circular smooth muscle that passes down the tract. It is preceded to a greater or lesser degree by relaxation of the muscle directly in front of the ring of

contraction. The longitudinal muscle may contract prior to the contraction of the circular muscle which is then accompanied by the simultaneous relaxation of the longitudinal muscle.

Other motility patterns of the small intestine have been described and include, pendular movements, segmentation contractions, tonus changes and villi movement. Their main function is to mix the contents of the lumen.

Ovine abomasal-intestinal motility

In most animals, the change in volume of gastric contents is important in changing motility present during fasting to that of a feeding pattern. In ruminants, however, the long periods of slow ingestion and the presence of the forestomachs produce an almost constant abomasal flow (Bueno and Fioramonti, 1979).

In adult animals, the abomasal inflow is largely dependent on reticulo-omasal motility and the mean pressure of the abomasum. Abomasal emptying is largely dependent on antral motility i.e. that of the pyloric area, and duodenal coordination.

Rhythmic slow peristaltic contractions of the pyloric area of the abomasum occur. It is thought that the pylorus opens and shuts in phase with the peristaltic waves.

Slow waves and action potentials occur in the abomasum as described above.

Small intestinal activity is different from that described previously in that slow waves are absent from the first 8 cm of the duodenum of the sheep. Instead there are bursts of action potentials. RSA and ISA occur and migrate along the intestine as

MMCs.

In ruminants this pattern is present independent of food ingestion and feeding behaviour.

Emptying of the abomasum is not followed by a duodenal propulsive wave. Distension of the duodenal bulb occurs first which then empties, caused by a burst of action potentials propagated along the intestine. Most of the intestinal contents flow intermittently through the tract at the same frequency as the MMC.

Therefore there is a continuous presence of MMCs in the ruminant compared to their disruption before and after feeding in other species. Limited access to concentrate increases the flow of digesta and the duration of spiking activity (Thompson and Lamming, 1972).

It would seem that secretin and cholecystokinin are able to reduce reticulo-omasal flow of digesta, as can gastrin which also inhibits rumino-reticular contractions and therefore alters abomasal filling (Ruckebusch, 1971; Wilson, Goetsch and Huber, 1976). It has been found that secretin in sheep produced a different result compared to that seen in dogs. In sheep there is an increase in the number of duodenal MMCs and decreased antral motility. A review of the control of abomasal-duodenal junction is given by Ruckebusch and Merritt (1985).

Role of gut motility in intestinal parasite infestations

Abnormalities in gut motility have been recorded in a number of animals infected with different parasites, either in association with their expulsion or with their presence in the gastrointestinal tract.

Stewart (1953) reported on the 'self-cure' phenomenon in sheep, whereby ingestion of infective larvae of Haemonchus contortus by sheep already infected with the parasite caused the expulsion of adult parasites present, not only H. contortus but also O. circumcincta and Trichostrongylus spp. Further investigations by Stewart (1955) observed that injection of H. contortus directly into the abomasum in infected sheep caused an increase in peristalsis and segmentation in the organ within ten minutes, indicating that the altered peristalsis was associated with the loss of adult parasites and this was triggered by incoming infective larvae. Bueno, Dakkak and Fioramonti (1982) recorded gastro-duodenal motor and transit disturbances associated with H. contortus infection in sheep. There were increases in the frequency of duodenal MMCs and in duodenal flow of ingesta but a shortening of the 80 - 90 minute cycles of antral contractions i.e. contractions of the pyloric area of the abomasum. These were all related to other consequences of parasite infection by the authors i.e. the alteration in duodenal MMCs was thought to be due to the altered gastric acid secretion that takes place during larval development of the parasite. (This has been disputed by Gregory, Rayner and Wenham (1984) who showed that duodenal MMCs can be initiated in sheep by duodenal acidification, while duodenal alkalinisation had no influence on MMC frequency). The increased duodenal flow was thought to be a consequence of the ionic permeability changes of the mucosa. Intestinal disturbances, mainly detected as an increase in transit time have also been reported in

O. circumcincta infections in sheep. This was also related to alterations in permeability and secretory activities of the abomasum (Dakkak and Khallaayoune, personal communication).

Studies into the effect of Trichostrongylus colubriformis infection in sheep also showed alterations in motility. Horak, Clark and Gray (1968) and Roseby (1977) showed a slowing of intestinal transit rate associated with a large single challenge of this intestinal parasite. However, no account was made of the decrease in food intake that also occurred which could alter transit times (Coombe and Kay, 1965). Gregory, Miller and Brewer (1985) have shown that abomasal volume and rate of digesta flow from the abomasum along the small intestine are linearly related to the level of food intake i.e., the smaller the amount of food ingested the longer the small intestinal transit time.

Work by Gregory and Wenham (1984) and Gregory et al (1985) looked at the effect of a chronic subclinical infection of T. colubriformis. Again there was a slowing of the small intestinal transit rate. Abomasal volume increased and abomasal outflow decreased. These changes occurred in absolute terms and also when compared with values predicted for the level of food intake. There was an increase in the frequency of MMCs and a progressive inhibition of abomasal and small intestinal motility. The changes resolved as the animals became resistant and also when given anthelmintic. There was no significant change in abomasal pH throughout the study. It was suggested by these authors that altered secretion of gastrointestinal hormones due to the presence of parasites could account for some or all of these motility changes and the reduction in food intake could

also be due to changes in hormones e.g. secretin and/or cholecystokinin (CCK).

Trichinella spiralis infection in rats and dogs has been studied by a number of workers for its effects on intestinal motility. Castro et al (1976) and Castro, Post and Roy (1977) reported an increase in intestinal transit associated with the intestinal phase of T. spiralis infection during a primary infection. No changes in motility were observed when immune rats were challenged with T. spiralis larvae. Schanbacher et al (1978) recorded a similar result in dogs and abnormal electrical activity of the intestinal smooth muscle associated with T. spiralis infection. Hookworm infestation in dogs also appeared to alter intestinal myoelectric activity (Weisbrodt and Castro, 1977).

Nippostrongylus brasiliensis infection in rats has been shown to slow the transit of food in the upper small intestine (Symons, 1966). Farmer (1981b) reported an increase in intestinal propulsive activity eight days post infection in rats, but it appeared that propulsion in the upper small intestine may have decreased on day 6 post infection. The increase recorded in intestinal propulsive activity did not seem to be related to parasite expulsion, which begins at approximately day 10. Work with isolated rat intestine and addition of adult N. brasiliensis into the lumen has also recorded a decrease in smooth muscle contractions (Biggin et al, 1983). Farmer (1981a), Farmer, Brown and Pollock (1983) and Farmer and Laniyonu (1984) reported that during N. brasiliensis infection in rats there is an alteration

in the maximum response of isolated intestine to acetylcholine and 5-hydroxytryptamine (5-HT). There is also a specific subsensitivity to 5-HT during the infection and although the maximum responses are increased above controls, the increase is not as large as occurs with acetylcholine. The cause of the subsensitivity to 5-HT may be due to increased concentrations during infection as prolonged exposure to 5-HT renders rat intestine unresponsive to the contractile action of this amine (Gillan and Pollock, 1980) and depletion of the gut of 5-HT by parachlorophenylalanine prevented the subsensitivity (Farmer and Laniyonu, 1984). The increase in maximum responses may be partly due to hypertrophy of the smooth muscle, but there was also an increase in contractile capacity and Farmer (1982) postulated this could be the result of the immune response, as betamethasone inhibited this increase.

One factor which may result in altered smooth muscle motility, at least locally, is the evidence that a number of parasites secrete acetylcholinesterase. These are:- Ascaris lumbricoides (Lee, 1962); Necator americanus (McLaren, 1974), Nippostrongylus brasiliensis (Sanderson, 1969; Sanderson and Ogilvie, 1971; Jones and Ogilvie, 1972), Oesophagostomum radiatum (Bremner et al, 1973; Ogilvie et al, 1973), Trichostrongyle spp (Rothwell, Ogilvie and Love, 1973; Ogilvie et al, 1973), Haemonchus contortus and Ostertagia circumcincta (Ogilvie et al, 1973). It has been suggested that secretion of acetylcholine could reduce activity in the muscles in the area of the parasites and aid the parasite's maintenance in that area (Lee, 1970).

Philipp (1984) offered an alternative suggestion, that the primary site of action of the acetylcholinesterase would be on the parasympathetic control of mucus cells. This is discussed elsewhere.

The origin of the enzyme appears to be commonly, the oesophageal and subventral excretory glands of the parasite (Lee, 1962; 1970; Ogilvie et al, 1973, Rothwell, Ogilvie and Love, 1973; McLaren, Burt and Ogilvie, 1974) and is also associated with the parasite's nervous system. It would seem probable that the acetylcholinesterase from the oesophageal glands passes into the oesophageal lumen and buccal capsule and out of the parasite (McLaren, Burt and Ogilvie, 1974).

Studies into acetylcholinesterase presence and secretion from the parasite have indicated that the amount increases as the parasite becomes adult i.e. third stage larvae contain and secrete less than adults (Sanderson, 1969; Sanderson and Ogilvie, 1971). Changes have also been detected in the secretion rate. As the host becomes immune to the parasite, the acetylcholine secretion increases (Edwards, Burt and Ogilvie, 1971; Sanderson and Ogilvie, 1971; Jones and Ogilvie, 1972).

Antibodies to acetylcholinesterase have also been detected in a number of animals infected with different parasites, including Ostertagia and Trichostrongylus spp in cattle and sheep (Rothwell et al, 1976) as well as N. brasiliensis infection of rats (Jones and Ogilvie, 1972).

Isolated tissue preparations

Isolated tissue preparations are used widely in the field of pharmacology to examine the activity of substances and also to assay that activity (Livingstone, 1968). Criticism of this approach is aimed at the unphysiological nature of these experiments, the tissue being suspended in salt solutions, and therefore the difficulty of extrapolating results to the in vivo situation.

However, many preparations do provide a relatively simple and reliable measurement of drug activity and understanding of different receptors that would be difficult to study using other systems.

Indeed, many of the major advances in pharmacology in the last twenty years, the classification of histamine, B-adrenergic and opioid receptors have all been made using isolated tissue preparations for differentiation of the many types of receptor.

The gastrointestinal smooth muscle preparation is widely used and the one studied most frequently is the guinea-pig ileum.

Generally, acetylcholine is able to cause contraction in a wide variety of smooth muscle preparations. However, the circular muscle of the guinea-pig ileum, when separated from the longitudinal muscle and the myenteric plexus is relatively or completely insensitive to acetylcholine (Harry, 1963). The reason for this may be due to high concentrations of specific cholinesterase within the circular muscle (Koelle, Koelle and Friedenwald, 1950). It was shown that if the cholinesterase was inhibited in the circular muscle of guinea-pig ileum then there was a response to acetylcholine (Harry, 1963).

Histamine also causes contraction of the longitudinal muscle of the guinea-pig ileum, but that of the rat intestine does not respond (Livingstone, 1968; Sakai et al, 1979).

5-hydroxytryptamine (5-HT) also causes contraction of isolated smooth muscle of the gastrointestinal tract in commonly used preparations (Livingstone, 1968; Sakai et al, 1979; Kitchen, 1984).

Prostaglandins produce differing responses. Generally prostaglandin E_2 (PGE_2) relaxes circular muscle, but contracts longitudinal muscle, while prostaglandin $F_{2\alpha}$ causes contraction in both (Bennett and Fleshler, 1970).

In a number of preparations, prostaglandins have been produced by the tissues and detected in the bathing salt solution (Posner, 1970; Ferreira, Herman and Vane, 1972). It has been suggested that prostaglandins help maintain the resting tone of the tissue, as their inhibition by non-steroidal anti-inflammatory drugs, causes a loss of the resting tone and abolishes prostaglandin production (Bennett and Posner, 1971; Ferreira, Herman and Vane, 1972). But the production of prostaglandins could be due to the damage caused to the tissue in setting up the preparation. Prostaglandin PGEs have been found to inhibit nor-adrenaline release in intestinal smooth muscle and it has been suggested that prostaglandins have a role in controlling nor-adrenaline release from adrenergic nerves (Hedqvist, 1977). This may account in part for their contractile response on smooth muscle. Prostaglandins E_1 and E_2 have also been found to enhance the contractile response to stimulation by

cholinergic nerves (Harry, 1968; Kadlec, Masek and Seferna, 1974) but there was no potentiation of the response to exogenous substances (Hall, O'Neill and Sheenan, 1975). Bennett, Eley and Stockley (1976) found that inhibition of prostaglandin synthesis by indomethacin or aspirin on guinea-pig isolated ileum and colon greatly decreased all aspects of peristalsis. Therefore it would appear that prostaglandins also stimulate cholinergic nerves at prejunctional sites, but they have been also found to have a direct effect on the smooth muscle itself (Bennett and Fleshler, 1970; Kadlec, Masek and Seferna, 1974). The reaction of smooth muscle to 5-hydroxytryptamine may also be mediated by stimulation of prostaglandin synthesis and release (Famaey et al, 1977).

Little work has been carried out on the responses of ruminant gastrointestinal smooth muscle in vitro. Duncan (1954) studied the responses of the gastric musculature in sheep both in vivo and in vitro. The results obtained in vitro were 'not satisfactory'. Acetylcholine produced contractions of strips from all chambers of the stomach. Histamine gave inconclusive results, but strong contractions were not observed even with concentrations of 60 ug/ml. Sanford (1961) carried out a similar study in vitro. Similar results were obtained with acetylcholine as those found by Duncan, but different results were obtained with histamine. Contractions were not observed in any strips from the various compartments of the ruminant stomach. Instead, relaxation of muscle strips of rumen and abomasum were obtained, but only in those preparations exhibiting spontaneous contractions. Mepyramine (a H_1 receptor blocker) failed consistently to antagonise the action completely.

Histamine was found to cause three different types of response in longitudinal and circular smooth muscle preparations of bovine rumen and reticulum by other workers. A contraction, a contraction followed by relaxation and a relaxation. From work with H_1 and H_2-receptor antagonists, it was concluded that histamine H_1 receptors mediate the contraction and histamine H_2 receptors the relaxation (Ohga and Taneika, 1978). Work by Vergara and Ballesteros (1985) showed inhibition of spontaneous activity of ovine omasum both longitudinal and circular muscles, by histamine, but they recorded contractions of ovine ruminal muscle in response to histamine. Contractions in response to 5-hydroxytryptamine were obtained in muscle from reticulum and abomasum at concentrations of 0.05 - 10 ug/ml, but repeated doses showed marked tachyphylaxis.

The Schultz-Dale Reaction

This is defined as the contraction of smooth muscle in in vitro preparations from sensitised animals in response to a specific antigen.

It has become a standard technique for demonstrating anaphylactic hypersensitivity and has been demonstrated in a wide variety of species and smooth muscle preparations, including calf jejunum (Aitken, Deline and Eyre, 1975). In contrast to this, a unique relaxation response has been obtained in ovine pulmonary vein (Eyre, 1975; Eyre and Deline, 1976).

It was first observed in 1910, when Schultz recorded contraction of guinea-pig ileum to a specific antigen. Dale (1913) reported a similar response in sensitised guinea-pig uterus.

Chand and Eyre (1978) record several characteristics of this response.

(i) The response is specific for the antigen and sensitive to small doses.

(ii) A latent period of variable length occurs before the response.

(iii) Tachyphylaxis can occur readily after two to three doses of antigen, without altering its response to other substances.

(iv) The tissue can recover after the tachyphylaxis if rested for a couple of hours.

(v) The antigen does not cause any direct action on the smooth muscle itself.

(vi) There is a slow relaxation of the tissue in vitro after contraction.

Studies using specific antagonists of possible mediators of the Schultz-Dale reaction would appear to point to histamine as an important mediator of anaphylaxis in the gastrointestinal smooth muscle of the guinea-pig (Geiger, Hill and Thompson, 1956; Califano and Scapagnini, 1968; Dale and Zilletti, 1970; Joiner et al, 1974). Work by Herman, Laekeman and Van Beek (1979) would indicate that SRS-A is also involved in the Schultz-Dale response seen in guinea-pig small intestine.

In the chicken ileum preparations, the anaphylactic contractions may involve prostaglandins and leukotrienes (Chand and Eyre, 1976).

Therefore, different mediators have been implicated in different preparations of smooth muscle.

There are several theories as to the mechanism of action of this response.

(i) Physical theory of mechanism of response

Early workers suggested that the antigen had a direct action on the smooth muscle and the muscle cells were themselves sensitised (Dale, 1920).

(ii) Chemical theory of mechanism of response

This theory is that the release of pharmacologically active mediators, e.g. histamine, from tissue mast cells is the main initiation in the response. This is supported by the partial or complete inhibition by histamine H_1 antagonists (Califano and Scapagnini, 1968; Joiner et al, 1974).

Although the presence of antihistamines will block many reactions, as will the prevention of histamine release from mast cells by prior treatment with mast cell stabilisers, the presence of mast cells is not necessary for the Schultz-Dale reaction to take place (Guschin, 1975).

As some responses are not affected by histamine H_1 antagonists and preparations that do not contract to histamine can produce a Shultz-Dale response, other mediators, such as prostaglandins, may be important in some tissues.

(iii) Neuronal theory of mechanism of response

Some agents which prevent nerve transmission block the Schultz-Dale contraction of guinea-pig ileum without affecting its response to histamine and acetylcholine (Geiger, Hill and Thompson, 1956).

(iv) Lymphocyte theory of the mechanism of response

It has been suggested that the tissues that do not exhibit Schultz-Dale e.g. guinea-pig vasa deferentia and seminal vesicles are devoid of lymphocytes and plasma cells (Katsh, 1958) and that production of some factor by these cells by antigen causes the response.

Therefore it is possible that a combination of these theories and other mechanisms may explain the Schultz-Dale response in sensitised smooth muscle. The tachyphylaxis observed in this response and its partial recovery similarly have a number of theories as to its mechanism. In summary these are:-

(i) The antigen-antibody complexes formed or already present are active for only a short time and may interfere with new complexes being formed.

(ii) The antigen-binding sites on antibody become occupied leading to de-sensitisation of the preparation. These may become dissociated later.

(iii) There is exhaustion of the mediators e.g. histamine that may produce the response, this could be restored leading to recovery.

(iv) Enzyme systems that may be activated by antigen challenge become inactive, which may be able to be reactivated later.

(v) Inhibitory factors may be acting.

In vitro STUDIES

Materials and methods

Animals

The animals used as sources of material were generally part of other experiments and were slaughtered as part of that experimental regime. The animals had mainly been parasitised experimentally and treated with anthelmintic prior to slaughter. The small number of animals that had been treated differently are detailed.

The animals were all killed by stunning and exsanguination. The material used in these experiments was obtained as soon as possible after death, usually within five minutes. The animals classed as having low immunity were either parasite-naive or had been indoors for greater than three months. Those non-parasitised were either naive or had been treated with anthelmintic. The animals considered to be immune were adult animals which had been grazing pasture known to be infected with Ostertagia spp and other trichostrongyles for a number of years.

Preparation of the isolated tissue

At necropsy, sections of abomasal and duodenal tissue were taken which were larger than the amount finally used in the experiment. The abomasal smooth muscle was taken from the pyloric region, the longitudinal axis being parallel to the longitudinal axis of the abomasum. A cross-section of duodenum was obtained approximately 5 cm from the pyloris. The material was placed in a beaker containing physiological salt solution (Kreb's) and transported to the laboratory. During this time the tissue was attaining room temperature.

In the laboratory, the tissues in the Kreb's solution were allowed to warm gently to 37°C by placing the beakers in a water bath maintained at that temperature. The Kreb's solution was gassed during this period with a mixture containing 95% O_2, 5% CO_2 (carbogen).

The tissue remained in the beaker for 30 minutes. The sections for the experiment were carefully cut with scissors to an approximate size of 3/4 cm by 1/2 cm. Sections of the longitudinal muscle of both abomasum and duodenum were used. The mucosa and circular muscles were not removed from the preparation as it was considered the less trauma the tissue received, the more likely it would be to respond to different drugs.

Tissue bath apparatus

The tissue sections were each suspended in 50 ml glass vessels containing Kreb's solution. This chamber allowed the salt solution to enter at the side and to be removed at the bottom of the vessel. The Kreb's solution filled the vessel via rubber and glass tubing from a reservoir situated above the apparatus. The tissue was anchored in the chamber by a loop to a glass rod hook at the bottom of the chamber and to a lever at the top of the vessel balanced with a small weight.

The glass rod was also connected to a gas cylinder and served to aerate the vessel with carbogen. This inner chamber was surrounded by a water bath, the temperature of which was controlled (Bioscience heat controller). The Kreb's solution passed through the water bath via glass coils before entering the inner vessel to ensure a consistent temperature for the tissue.

The tissue was washed every ten minutes by two complete changes of Kreb's solution.

Kreb's solution

This was the physiological salt solution used. The composition for five litres is shown below.

NaCl	27.7 g
KCl	1.75 g
$MgSO_4.7H_20$	1.45 g
$CaCl_2.2H_20$	1.85 g
KH_2PO_4	0.80 g
$NaHCO_3$	10.50 g
Glucose	10.50 g

The buffering capacity of the Kreb's solution is completed by 5% CO_2 in the carbogen aeration, bringing the solution to pH 7.4.

Temperature

The tissues were warmed to 37°C, but a number exhibited irregular spontaneous contractions which were reduced or abolished by cooling the tissue to 30°C.

Recording tissue responses

Isotonic recording was used in these experiments i.e. the tissue was allowed to change length against a constant tension and the movement of the muscle was recorded. The tissue was connected to a lever as described previously. The movement of the tissue and therefore the lever was converted to an electrical potential by a transducer which was amplified and recorded as a pen movement on a flat bed potentiometric chart recorder.

Resting tension

The first experiments were conducted on bovine tissue and a load of 6 g was used on the lever. This was later reduced as it was thought that a lighter weight might allow the tissue to respond more readily to drugs. Duodenal muscle was loaded with a weight of 0.7 g and abomasal muscle was loaded with 1 g.

Spontaneous contractions

Although some duodenal preparations exhibited spontaneous contractions, the majority of the abomasal tissues showed large, irregular and continuous spontaneous contractions. Cooling could sometimes reduce them so that responses to drugs could be measured. However many preparations were abandoned because the irregular nature of their activity made it impossible to analyse responses of the tissue to different drugs.

Substances tested

All solutions were made up after the tissue had been placed in the apparatus.

(i) Acetylcholine

Acetylcholine chloride (Sigma Chemicals) was made into solution using deionised water and dilutions made from 0.5 M solution of acetylcholine. Its structure is shown in Figure 30.

(ii) Histamine

This was obtained as histamine diphosphate (Sigma Chemicals) and made into solution using deionised water. Dilutions were made from 0.1 M solution of histamine. Its structure is shown in Figure 31.

(iii) 5-hydroxytryptamine (5-HT)

This was obtained as the creatinine phosphate complex (Sigma

Chemicals), dissolved in deionised water and dilutions were made from 0.05 M solution of 5-HT. Its structure is shown in Figure 32.

(iv) Sodium meclofenamate

This was obtained as an aqueous solution (Parke-Davis) at a concentration of 100 mg/ml.

(v) Prostaglandins

Prostaglandins E_1 and E_2 were obtained (Sigma Chemicals) and freshly made up to 1 mg/ml solutions with redistilled methanol. Initially the diluent used was absolute alcohol but there were problems with the stability of the prostaglandins which were producing reduced responses and therefore the diluent was changed. Their structures are shown in Figure 33.

(vi) Leukotrienes

The leukotrienes B_4, C_4 and D_4 were obtained as solutions of the potassium salt of the free acids (ICN Biomedicals). LTB_4 was supplied in phosphate buffered saline (PBS) pH 6.9, LTC_4 and LTD_4 were supplied in 20% ethanol/PBS pH 6.9. They were diluted with PBS to obtain 10 ug/ml solutions immediately prior to testing. Their structures are shown in Figure 34.

(vii) O.circumcincta homogenates

Homogenates of O. circumcincta adults and L_3 were used.

The adult parasites were obtained from the abomasal contents of a lamb experimentally infected with O. circumcincta L_3 24 days earlier. The contents were placed in gauze bags and suspended in beakers of warm water. The adult parasites migrated into the water and tended to clump together. These were collected,

homogenised using a hand-held glass homogeniser and stored at -30°C until use.

The larvae were collected as described previously and homogenised as above. These were added to the tissue without being stored.

Addition of substances to the tissue

The volume of test substance added did not exceed 5% of the tissue bath volume. If a response was obtained, the process was repeated and a mean response calculated. Acetylcholine was allowed 30 seconds contact with the tissue generally. The other substances were allowed longer contact to ascertain any response by the tissue.

The tissue was washed twice with Kreb's solution before further drug addition and allowed to return to its unstimulated length.

Results

Bovine abomasal and duodenal smooth muscle

(i) Acetylcholine

The dose response curves of bovine duodenum to acetylcholine is shown in Table 40 and Figure 35. All of the tissues tested showed very similar dose response curves. All the animals were of low immunity and were not parasitised. Table 42 and Figure 37 show the response of bovine abomasum to acetylcholine. This animal was also considered to have low immunity and was not parasitised.

(ii) Prostaglandins

Seven preparations were tested, of these two failed to respond to either prostaglandin. Both of these preparations were

duodenal tissue of animals having low immunity. One animal was parasitised with trichostrongyles, the other was not parasitised.

Duodenal preparations of parasitised animals showed responses of 1 to 4% of maximum contraction when compared to the dose-response curve of acetylcholine for concentrations 9.5×10^{-9} M to 1.9×10^{-7} M PGE_1 and PGE_2. A duodenal preparation from a non-parasitised animal showed a contraction 24.5% of the maximum for 9.5×10^{-7} M PGE_2 but this could not be repeated and the tissue did not contract in response to prostaglandins again despite continuing to contract to acetylcholine.

Duodenal preparations of immune animals showed responses 2% to 5% of maximum in response to 1.4×10^{-6} M to 2.8×10^{-6} M PGE_1.

The responses of the tissues to prostaglandins were very inconsistent. Often second, larger doses would evoke a poorer response. Stability of the prostaglandins was thought to be a problem and the diluent changed but the response was still erratic and dose-response curves could not be obtained.

(iii) Histamine

Three preparations were tested. Two responded in producing contractions. A circular muscle preparation of the duodenum of a parasitised animal showed no response up to 0.02×10^{-4} M histamine. This preparation also did not respond to acetylcholine.

There was a tendency for spontaneous contractions to start after the addition of histamine. The significance is unknown,

but was also reported by Duncan (1954).

Duodenal preparations of non-parasitised animals of low immunity showed responses 26 to 64% of maximum contraction for a dose range of 5.6×10^{-4} M to 4.5×10^{-3} M but the responses were not consistent.

(iv) 5-hydroxytryptamine (5-HT)

Two preparations were tested, only one preparation responded producing small contractions.

A duodenal preparation of a parasitised animal contracted to less than 1% of maximum for concentrations 1.6×10^{-6} to 3.2×10^{-6} M 5-HT.

A duodenal preparation of an immune animal showed no response.

(v) Sodium meclofenamate

Concentrations of 1 mg/ml to 4 mg/ml tended to cause relaxation of the tissue and also reduce or abolish any spontaneous activity.

(vi) Leukotriene B_4

One preparation of duodenal tissue of an immune animal was tested and failed to respond. Concentrations of up to 0.08 ug/ml (2.07×10^{-7} M) were used.

Ovine abomasal and duodenal smooth muscle

(i) Acetylcholine

The dose response curves of ovine duodenal tissue are shown in Table 41 and Figure 36. All the animals were considered to have low immunity but were not parasitised. Numbers 1 and 2 were adult ewes that had been housed for a number of months. Numbers 3 and 4 were parasite naive lambs. It can be seen that

the dose response curves for the lambs were slightly further to the left of the curves for the adult sheep.

The dose response curve of ovine abomasal tissue is shown in Table 42 and Figure 37. This animal was also of low immunity and was not parasitised.

(ii) Prostaglandins

Nine preparations were tested, five failed to respond to either prostaglandin E_1 or E_2.

There was no response in abomasal tissue from:-

(i) adult immune sheep.

(ii) meclofenamic acid treated adult immune sheep.

(iii) adult, low immunity, parasitised sheep.

(iv) bromhexine treated adult immune sheep.

The details of the treated sheep are described elsewhere. One duodenal preparation from a parasitised animal also did not respond.

The tissues that did respond were:-

(i) Duodenal preparation from an adult immune animal

2.8×10^{-6} M to 1.1×10^{15} M PGE_1 produced contractions less than 1% of maximum.

5.7×10^{-6} M to 1.4×10^{-5} M PGE_2 also produced contractions less than 1% of maximum.

(ii) Duodenal preparation from adult immune animal, treated with bromhexine.

1.1×10^{-5} M PGE_2 produced contraction 9% of maximum.

PGE_1 produced no response.

(iii) Duodenal preparation from adult, low immunity, parasitised animal.

5.7×10^{-7} M to 2.3×10^{-5} M PGE_2 produced contractions 2 to 6% of the maximum contraction.

PGE_1 produced no response.

(iv) Duodenal preparation from adult immune sheep, treated with meclofenamic acid.

PGE_1 1.4×10^{-6} to 1.1×10^{-5} M produced contractions less than 1% of maximum.

PGE_2 5.7×10^{-6} to 3.4×10^{-5} M produced contractions less than 1% of maximum.

In most preparations, repeated administration of prostaglandins caused a reduction in the response of the tissue. The diluent of the prostaglandin solution was changed but did not alter the situation.

(iii) 5-hydroxytryptamine (5-HT)

Seven preparations were tested, only one responded to produce small contractions at high concentrations.

There was no response in the abomasal preparations obtained from:-

(i) adult immune animal, treated with meclofenamic acid.

(ii) adult, low immunity, parasitised.

(iii) adult immune animal, treated with bromhexine.

There was no response to 5-HT in the following duodenal preparations:-

(i) non-parasitised, low immunity animal.

(ii) adult, parasitised, low immunity.

(iii) adult, immune animal, treated with bromhexine.

The contraction obtained was in a duodenal preparation of an adult, immune animal, treated with meclofenamic acid.

1×10^{-2} M to 2×10^{-2} M produced contraction of less than 1% of maximum.

(iv) Histamine

Six preparations were tested, only one preparation responded with small contractions.

There was no response in abomasal preparations from:-

(i) adult, immune animal treated with meclofenamic acid.

(ii) adult, low-immunity, parasitised animal.

(iii) adult immune animal, treated with bromhexine.

There was no response from duodenal preparations of:-

(i) adult, low immunity, parasitised animal.

(ii) adult, immune animal, treated with bromhexine.

Contractions were obtained in a duodenal preparation of adult immune animal treated with meclofenamic acid.

2×10^{-4} M to 4×10^{-4} M produced contractions less than 1% of maximum.

(v) Sodium meclofenamate

There was no response in the four tissues tested, up to 4 mg/ml.

There was no response in the abomasal preparations from:-

(i) adult immune.

(ii) adult immune animal, treated with meclofenamic acid.

There was no response in the duodenal preparations from:-

(i) adult immune.

(ii) adult immune animal, treated with meclofenamic acid.

(vi) Leukotrienes (LTs)

LTB_4, C_4 and D_4.

There was no response in the five tissues tested.

Abomasal preparations from:-

(i) adult immune animal, treated with meclofenamic acid.

(ii) adult immune animal, treated with bromhexine.

Duodenal preparations from:-

(i) adult immune animal, treated with meclofenamic acid.

(ii) adult, low immunity, parasitised animal.

(iii) adult immune animal, treated with bromhexine.

Concentrations up to 0.14 ug/ml were used i.e.

3.6×10^{-7} M LTB_4, 2.2×10^{-7} M LTC_4, 2.8×10^{-7} M LTD_4.

(vii) O.circumcincta homogenate

Homogenates of O. circumcincta adults and larvae (L_3) did not produce a response in the three tissues tested.

(i) Abomasal preparation of immune, adult animal.

(ii) Duodenal preparation of immune, adult animal.

These tissues were tested with an homogenate of O. circumcincta adults. A homogenate of approximately 4,500 adults was added to each tissue.

(iii) Duodenal preparation of an immune adult animal.

This was tested with an homogenate of O. circumcincta larvae (L_3). A homogenate of approximately 1,000 larvae was added to the tissue.

Discussion

The results obtained with these experiments were generally

disappointing as the ovine and bovine tissue appeared to be very unresponsive to different substances known to elicit responses in other isolated tissue preparations and no obvious differences between immune and non-immune, parasitised and non-parasitised animals were found.

The dose response curves obtained to acetylcholine from the bovine duodenal preparations were all very similar as were the animals used as sources of tissue for these experiments. All were young, of low immunity that had been parasitised but had been treated with anthelmintic before slaughter.

The results obtained by acetylcholine in ovine duodenal tissue were more varied. It is interesting that the two lambs produced similar responses as did the two adults.

It would appear that the animals would have to be more closely matched for age and perhaps sex to obtain similar dose response curves.

The responses of abomasal tissue were more varied but only one preparation each of ovine and bovine tissue could be tested. The results obtained for each were similar to those obtained for the duodenal preparations. The difference between the responses of bovine and ovine tissue might be explained by the heavier load on the bovine preparations when compared to the ovine.

The response to histamine was in broad agreement with that found by Duncan (1954) in both ovine and bovine tissue. Relaxation of the ovine tissue was never observed in response to histamine in contrary to results described by Sanford (1958; 1961). It would seem unlikely that the apparatus would not detect relaxation as this was observed when sodium meclofenamate

was added to the bovine tissues. However, ovine tissue did not appear to relax to sodium meclofenamate and it is possible that the preparations were fully relaxed and further relaxation was impossible. It is interesting that histamine was able to produce contractions in bovine preparations compared to the general lack of response in ovine tissue. The reason for this difference is unknown.

These experiments also failed to produce contractions to 5-hydroxytryptamine (5-HT) which were also reported by Sanford (1958; 1961). The concentrations of 5-HT used here were within the range used by Sanford. Only small contractions were obtained at high concentrations in both tissues. Sanford used abomasal preparations from the fundus of the abomasum and reported that the circular muscle was more sensitive which may account for the different results obtained.

The difficulties with the stability of the prostaglandins make the results obtained difficult to interpret. All abomasal preparations of ovine tissue that were tested failed to respond to the prostaglandins and those duodenal preparations of both ovine and bovine tissue only produced small contractions, except one bovine duodenal preparation which produced a stronger contraction on one occasion to prostaglandin E_2.

The responses obtained to sodium meclofenamate were in agreement with results obtained in other tissues (Bennett and Posner, 1971; Ferreira, Herman and Vane, 1972).

It was interesting that the leukotrienes did not cause any detectable response in the ovine tissue and the one bovine

preparation tested. There has been a great deal of work centred on the role of leukotrienes in parasite infection (Moqbel et al, 1987) and it was possible that they might affect the smooth muscle of the gastrointestinal tract in some way. There was no evidence of this in these experiments.

The reason for failure of a response to O. circumcincta homogenate is not known. It is possible that storage of adult parasites may have abolished the Schultz-Dale response, or perhaps the adult parasites would not stimulate the response. The third stage larvae might be considered more likely to produce a response and these were not stored prior to their addition to the tissue. The mediators thought to be involved in producing the Schultz-Dale response, histamine, prostaglandins and leukotrienes did not cause a dramatic response in these tissue preparations and this might explain the lack of response. Alternatively, it could be that immune responses caused by sensitisation of the animal by parasites are not of the type to cause a Schultz-Dale response.

FIGURE 30

Structure of acetylcholine

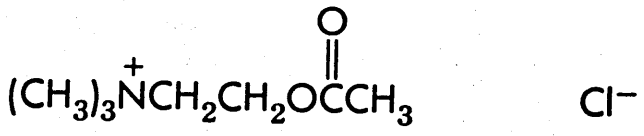
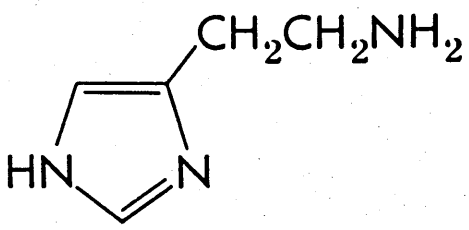


FIGURE 31

Structure of histamine



Histamine

FIGURE 32

Structure of 5-hydroxytryptamine

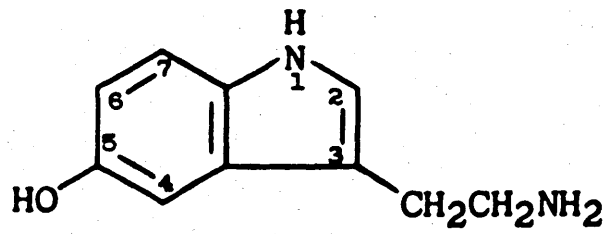
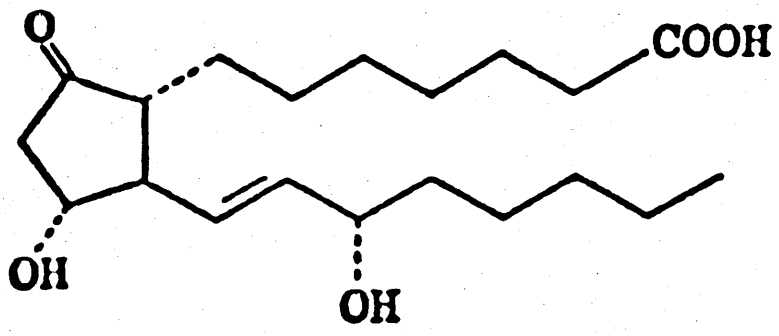
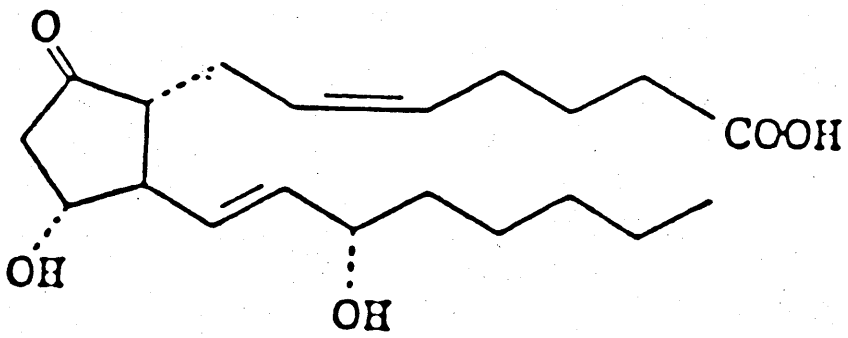

H
N
1
2
3
4
5
6
7
HO
CH2CH2NH2

FIGURE 33

Structures of prostaglandins E_1 and E_2



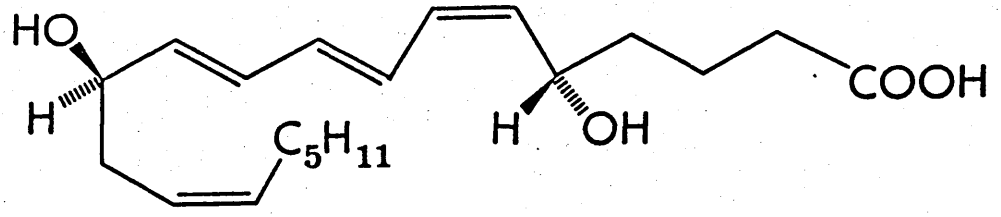
Prostaglandin E_1



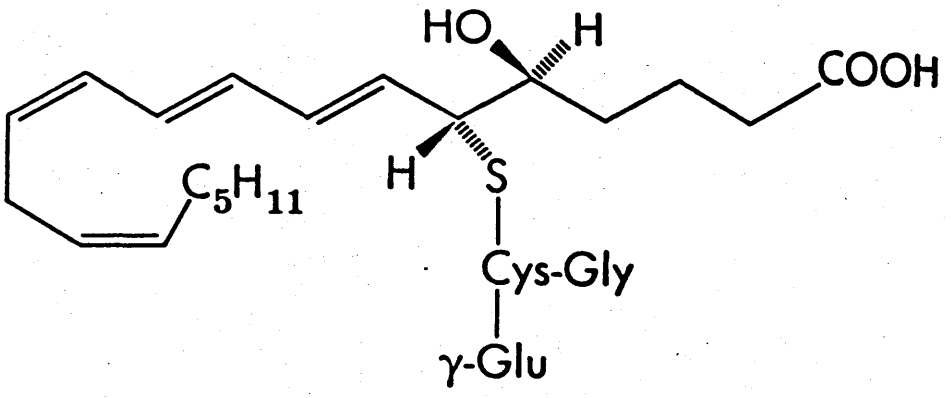
Prostaglandin E_2

FIGURE 34

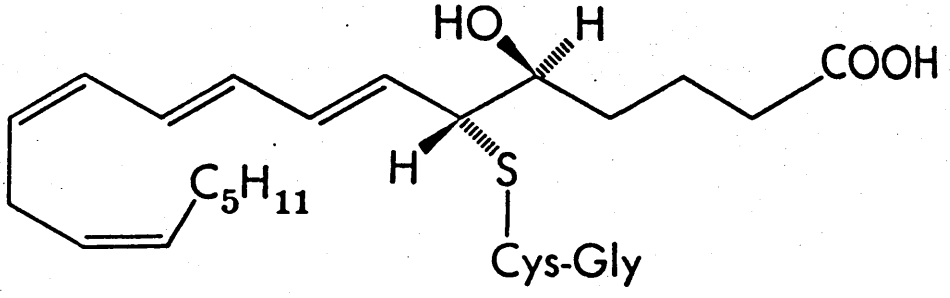
Structures of leukotrienes B_4, C_4 and D_4



Leukotriene B_4



Leukotriene C_4



Leukotriene D_4

TABLE 40

Contraction, as a percentage of the maximum, of bovine duodenal isolated tissue preparations to various concentrations of acetylcholine *in vitro*. The tissues were obtained from low immunity, non-parasitised animals

Concentration of acetylcholine ($\times 10^{-3}$ M)	Animal 1	Animal 2	Animal 3	Animal 4
0.0008	0	0	3	0
0.0016	0	4	5	0
0.01	3	12	-	8
0.02	5	17	18	10
0.04	8	27	23	12
0.08	13	32	26	13
0.16	21	37	35	-
1.0	42	54	49	38
2.0	44	69	62	48
4.0	60	76	77	65
8.0	84	79	89	83
16.0	91	100	100	100
20.0	100	93	95	100
24.0	98	-	-	-
25.0	-	-	82	83

FIGURE 35

Mean dose response curves of bovine duodenal tissue preparations to acetylcholine

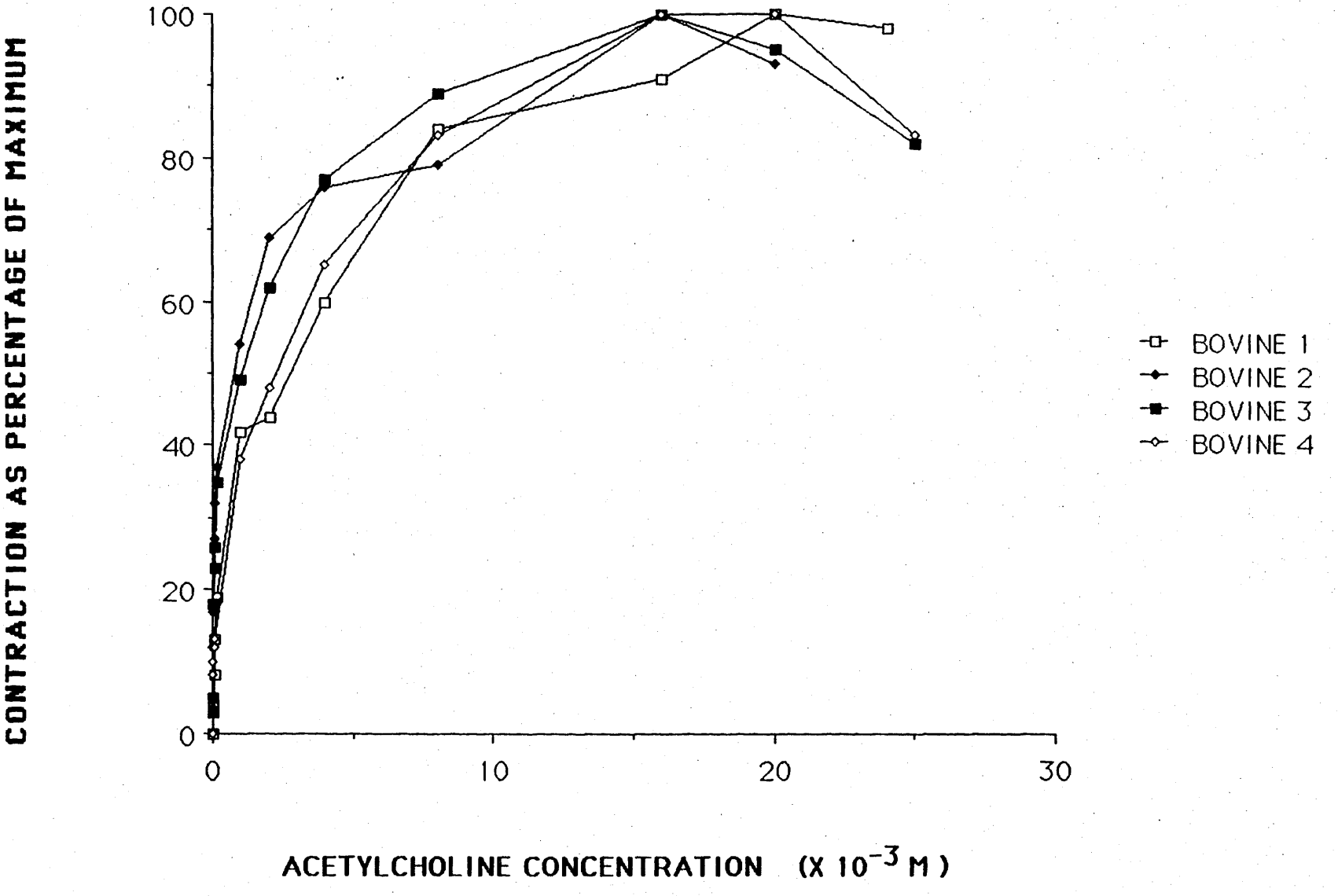

CONTRACTION AS PERCENTAGE OF MAXIMUM
100
80
60
40
20
0
0
10
20
30
ACETYLCHOLINE CONCENTRATION (X 10^{-3} M)
BOVINE 1
BOVINE 2
BOVINE 3
BOVINE 4

TABLE 41

Contraction, as a percentage of the maximum, of ovine duodenal isolated tissue preparations to various concentrations of acetylcholine *in vitro*. The tissues were obtained from low immunity, non-parasitised animals

Concentration of acetylcholine ($\times 10^{-3}$ M)	Animal 1	Animal 2	Animal 3	Animal 4
0.0004	0	0	2	0
0.0008	0	0	4	0
0.0016	7.5	-	9	0
0.01	-	-	19	4
0.02	-	-	19	5
0.04	-	-	28	7
0.08	-	29	39	10
0.16	17	29	41	16
1.00	-	-	78	45
2.00	-	-	100	62
4.00	56	57	90	74
8.00	73	57	95	80
10.00	-	-	74	97
16.00	100	100	-	-
20.00	64	79	-	99
30.00	-	-	-	100
40.00	-	-	-	98

FIGURE 36

Mean dose response curves of ovine duodenal tissue preparations to acetylcholine

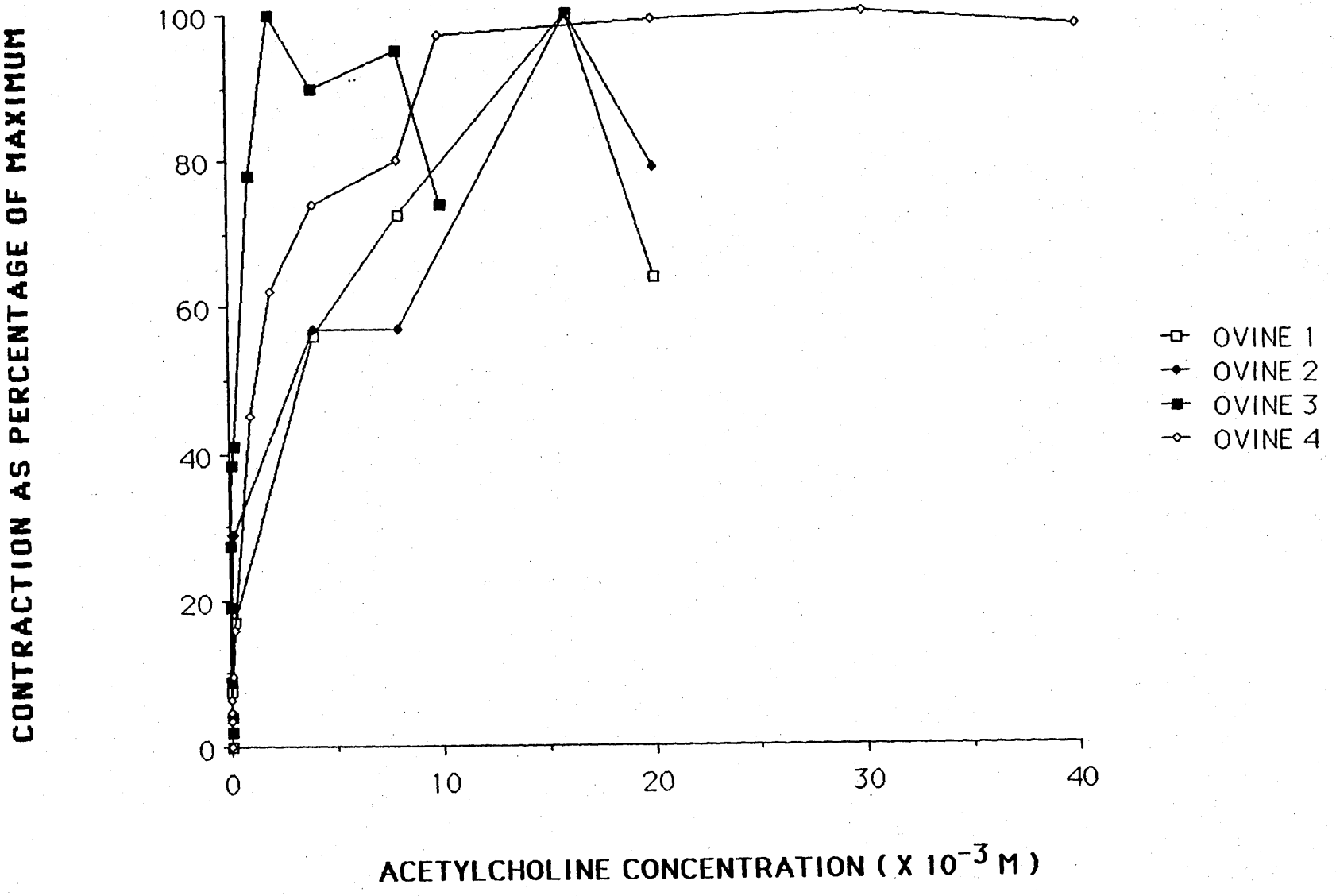

CONTRACTION AS PERCENTAGE OF MAXIMUM
100
80
60
40
20
0
0
10
20
30
40
ACETYLCHOLINE CONCENTRATION (X 10⁻³ M)
OVINE 1
OVINE 2
OVINE 3
OVINE 4

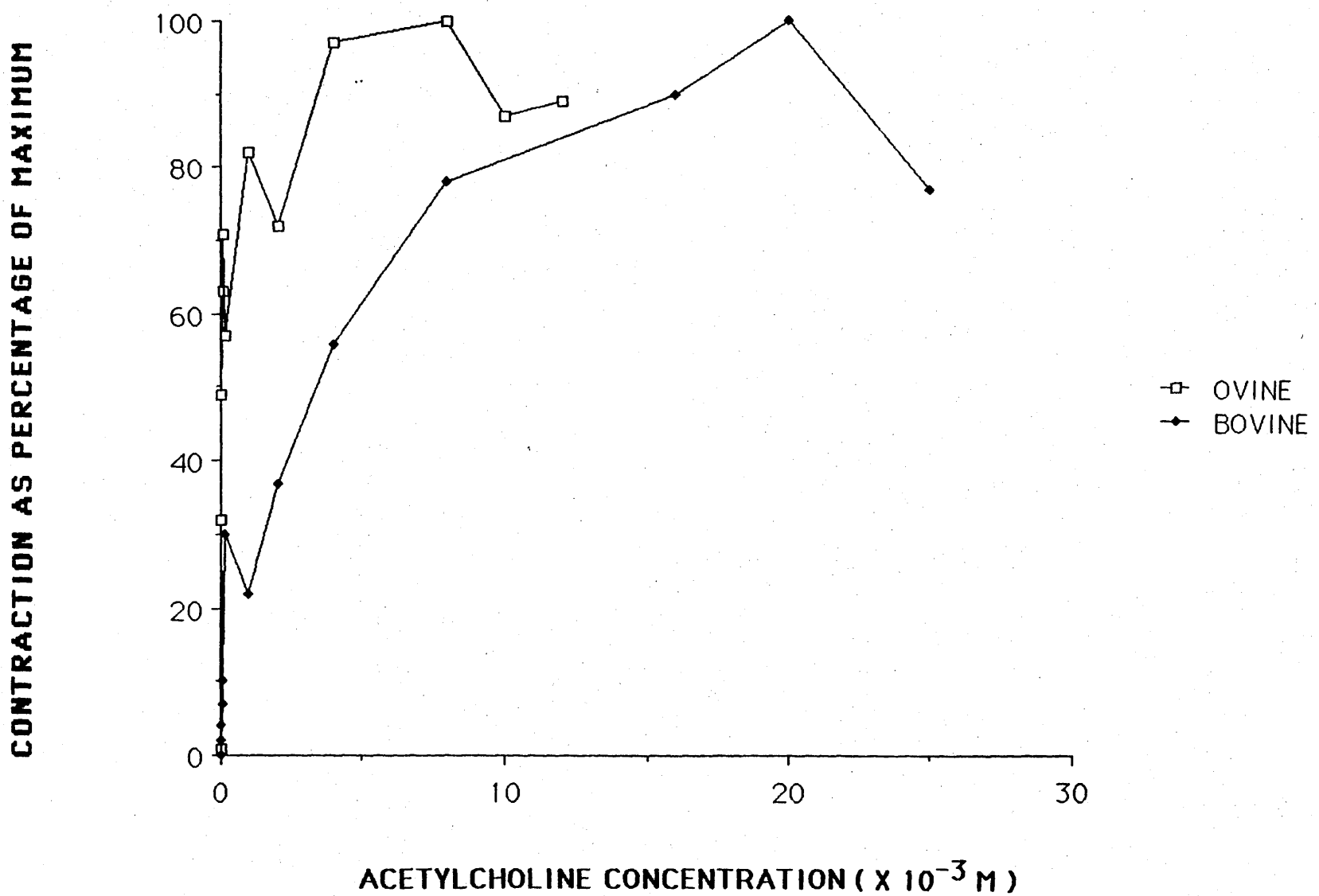
TABLE 42

Contraction, as a percentage of the maximum, of bovine and ovine abomasal isolated tissue preparations to various concentrations of acetylcholine *in vitro*. The tissues were obtained from low immunity, non-parasitised animals

Concentration of acetylcholine ($\times 10^{-3}$ M)	Ovine	Bovine
0.0016	1	-
0.01	32	4
0.02	49	2
0.04	63	7
0.08	71	10
0.16	57	30
1.0	82	22
2.0	72	37
4.0	97	56
8.0	100	78
10	87	-
12	89	-
16	-	90
20	-	100
25	-	77

FIGURE 37

Mean dose response curves of bovine and ovine abomasal tissue preparations to acetylcholine


CONTRACTION AS PERCENTAGE OF MAXIMUM
100
80
60
40
20
0
0
10
20
30
ACETYLCHOLINE CONCENTRATION (X 10^{-3} M)
OVINE
BOVINE

In vitro STUDIES WITH *Ostertagia circumcincta*

ANTHELMINTICS ACTIVE AGAINST GASTROINTESTINAL NEMATODES IN RUMINANTS

Since the introduction of thiabendazole as the first broad-spectrum anthelmintic in 1961, a large number of drugs have been developed to control parasitism in animals.

There are many recent reviews of anthelmintic action and efficacy (for example Behm and Bryant, 1985; Bogan and Armour, 1987; Prichard, 1987).

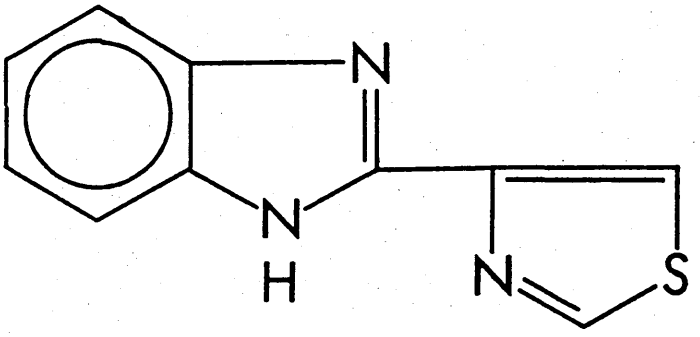
The anthelmintics currently available for controlling gastrointestinal nematodes in ruminants can be divided into groups containing drugs of similar chemical structure and mode of action.

Benzimidazoles

There are a large number of members of this group available commercially. Thiabendazole was the first member marketed. All have the same basic benzimidazole structure, with substituents on positions 2 and 5. The structure of thiabendazole is shown in Figure 38. This group is characterised by low toxicity and it has been impossible to obtain an LD_{50} (lethal dose of drug required to kill 50% of individuals treated) for thiabendazole and fenbendazole. However, some members of this group have been found to be teratogenic. Cambendazole, parbendazole, oxfendazole and fenbendazole have been implicated in this respect. Thiabendazole given orally is rapidly absorbed. In cattle, the drug is extensively and rapidly metabolised, such that only the inactive metabolite hydroxythiabendazole is found in plasma with

FIGURE 38

Structure of thiabendazole


N
N
H
N
S

maximum concentrations occurring at four hours (Prichard, 1978), whereas in sheep, the parent drug predominates, also reaching a maximum concentration at four hours (Weir and Bogan, 1985). The newer benzimidazoles have structures that have a slower rate of metabolism and decreased solubility making them effective at lower dose rates and having a broader spectrum of activity. These bezimidazoles are fenbendazole, oxfendazole and albendazole, in which the thiazole ring at position 2 of thiabendazole has been replaced by methylcarbamate group. These drugs are sometimes termed the benzimidazole carbamates (Lacey, 1985). The structure of fenbendazole is shown in Figure 39. In sheep, maximum plasma concentrations are achieved 24 to 48 hours after dosing with fenbendazole or oxfendazole (Marriner and Bogan, 1981a, b). They are all metabolised to a sulphoxide, which is anthelmintically active and a sulphone. They are active against adult, immature and arrested larval stages of all the important gastrointestinal nematodes of ruminants. They are all ovicidal.

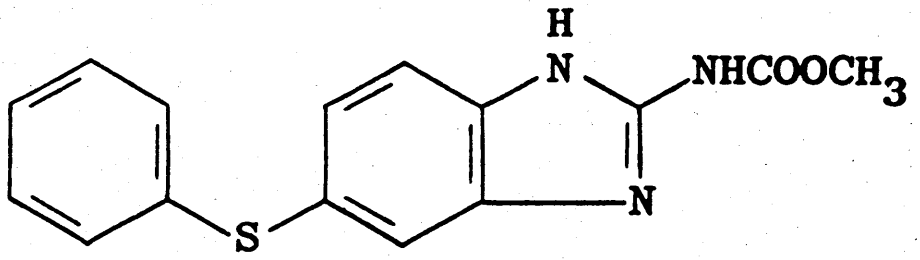
There is a great variation in withdrawal periods for members of the benzimidazole group, from zero for meat and milk for thiabendazole up to five days for milk and 21 to 28 days for meat for others.

Mode of action

Initially it was thought that benzimidazoles acted by inhibiting the fumarate reductase system of anaerobic respiration in parasites. It was also found that in benzimidazole resistant strains of Haemonchus contortus this system was inhibited less by the drugs than susceptible strains (Prichard, 1973).

FIGURE 39

Structure of fenbendazole


H
N
NHCOOCH3
N
S

Other workers reported decreased uptake of glucose and induction of glycogen depletion by these anthelmintics and also reduction in acetylcholinesterase secretion.

Borgers et al (1975) observed derangement of microtubule function in absorptive cells of parasites caused by benzimidazole treatment and this led to further study in this area.

Microtubules are composed of, and exist in equilibrium with tubulin, a soluble protein existing as a dimer. Microtubules form the mitotic spindle which is responsible for chromosome separation in dividing cells and have other functions.

Benzimidazole carbamates in vitro are known to bind to tubulin and to be potent inhibitors of the rate of polymerisation of tubulin to microtubules (Barrowman, Marriner and Bogan, 1984; Lacey, 1985; Sangster, Prichard and Lacey, 1985). This is now thought to be the primary site of action of the benzimidazoles. Disruption of these structures can also explain the other observed effects of the benzimidazoles (Lacey, 1985).

Pro-benzimidazoles

The anthelmintic activity of febantel, thiophanate and the newer netobimin is thought to be due primarily to the formation of benzimidazoles by ruminal and hepatic metabolism in vivo. They are therefore referred to as pro-benzimidazoles (Bogan and Armour, 1987).

Imidothiazoles

Tetramisole and levamisole are members of this group. Tetramisole is the racemic mixture of D and L-forms. The L-form is levamisole which was found to be the anthelmintically active

portion. The D-form contributed to toxicity. Levamisole is now marketed, at a lower dose rate than that used for tetramisole and with a greater therapeutic index.

Levamisole has a broad spectrum of activity against adult and immature gastrointestinal nematodes and in sheep, arrested larvae. It is available as oral, injectable, pour-on and in-feed preparations.

It is not ovicidal or teratogenic in comparison to the benzimidaoles.

In sheep and cattle, peak blood concentrations are achieved within 30 minutes of parenteral administration and are very low by six hours (Prichard, 1978).

It is more toxic than the benzimidazoles, producing ganglion stimulation associated with parasympathetic effects of bradycardia, salivation, muscle tremors and respiratory distress.

Mode of action

Levamisole produces a reversible ganglion stimulatory effect, causing neuromuscular paralysis of the parasites (Van Nueten, 1972).

The similarity in tertiary structure of levamisole to acetylcholine suggests that it may act as a cholinergic agonist (Hsu, 1980).

Levamisole also has other, possibly less important effects on the energy metabolism of the host.

Tetrahydropyrimidines

Pyrantel and its analogue morantel, are members of this group. Morantel is more potent than pyrantel and is available in the UK as a slow release device for cattle containing morantel

tartrate.

Morantel has good activity against adult parasites, less against larvae but the continuous release device prevents establishment. It has no activity against arrested stages.

It is generally of low toxicity and non-teratogenic.

Mode of action

They act as depolarising muscle relaxants producing paralysis of the parasites. The phenomenon of side-resistance between levamisole and morantel in some strains of parasites suggest similar sites of action for the two anthelmintics.

Avermectins

Ivermectin is the only drug of this group available. Its efficacy and safety have been recently reviewed (Campbell and Benz, 1983).

Ivermectin has an extremely broad spectrum of activity against adult, immature and arrested larvae. It is also active against many ectoparasites when administered parenterally. It is very potent, with very small doses necessary to exert an effect.

Ivermectin is persistent in the tissues and protects against establishment of some parasitic larvae for up to two weeks (Barth, 1983; Armour et al, 1985). It has a long withdrawal of 28 days for meat and is not recommended for milking cattle. It is of low toxicity and is safe in pregnant animals. A subcutaneous injection formulation is available for cattle, while in sheep, ivermectin is in the form of an oral drench. Subcutaneous administration shows greater potency than oral administration, particularly against ectoparasites, which is

thought to be due to avoidance of rumen metabolism which would reduce the bioavailability (Prichard, 1987).

Mode of action

Ivermectin acts on the nervous system of the parasite producing paralysis. It acts by potentiating the action of γ - aminobutyric acid (GABA) at the synapses of GABA-ergic nerves. In nematodes and arthropods GABA-ergic nerves are present in the peripheral nervous system; in mammals these nerves are located principally in the central nervous system (CNS) protected by the blood-brain barrier.

In Ascaris suum ivermectin appears to act at two sites:-

(i) at inhibitory neuromuscular synapses.

(ii) at a site in the ventral nerve cord, producing a block of interneuronal stimulation of excitatory motorneurons.

Both sites are thought to be GABA-ergic (Kass, Stretton and Wang, 1984). From studies in rat brain preparations, it is thought that ivermectin stimulates pre-synaptic release of GABA and increases binding to the post-synaptic GABA receptor (Pong, Wang and Fritz, 1980; Pong and Wang, 1982).

In vitro ASSESSMENT OF THE EFFECT OF VARIOUS SUBSTANCES ON PARASITIC LARVAE

Introduction

The aim of this work was to develop a technique which could detect effects caused by substances that may be important in the immunity of sheep to gastrointestinal parasites. A further aim was to use this technique to detect anthelmintic activity in test substances, a system that could have a use as a preliminary

screen for substances that may be anthelmintically active.

As discussed elsewhere, the mucus layer lining the gastrointestinal tract is closely associated with parasites in this region, at least for part of their life cycle, and is usually the first host barrier met. It has been suggested that mucus plays a role in trapping larval parasites before they reach the mucosa in immune animals (Miller, Huntley and Wallace, 1981; Miller, 1984; 1987). Work by Douch et al (1983) has shown that the mucus from immune animals inhibits larval migration in vitro. This was found not to be specifically directed against the parasite to which resistance was expressed, but to inhibit migration of other parasite species. The activity of mucus from immune animals was thought to be associated with slow reacting substance of anaphylaxis (SRS-A) in the mucus which is known to consist of leukotrienes C_4, D_4 and E_4 (Lewis and Austen, 1981; 1984). Other possible mediators, e.g. histamine, prostaglandins and 5-hydroxytryptamine were tested for their possible effect on larval migration but their role appeared to be negligible.

The method used in this work was based on the principle of larval migration from agar gels which had been used previously, for isolation of infective Dictyocaulus larvae from herbage (Jorgensen, 1975) and of nematodes from gastrointestinal ingesta and mucosa (Van Wyk and Gerber, 1978; Van Wyk, Gerber and Groeneveld, 1980).

Other workers have used parasitic larvae to develop in vitro screening techniques for possible anthelmintic drugs rather than using totally free-living nematodes which had been used

previously or have used *in vivo* techniques. These relied on drug-induced effects on the development of larval stages, motility, viability or biochemical parameters.

Nippostrongylus brasiliensis has been used widely in these screens. Adult parasites were examined for changes in motility when incubated with various anthelmintics but failed to show any activity for the benzimidazoles (Coles and McNeillie, 1977). The development of fourth stage larvae (L_4) to the adult stage has also been used as an anthelmintic screen and was successful in detecting a wide range of different drugs including the benzimidazoles and avermectins (Jenkins, Armitage and Carrington, 1980). This work used an undefined medium which was capable of maintaining the *N. brasiliensis* adults for a number of weeks. In this study the effects of both the benzimidazoles and avermectin were gradual. A study was also done using a fully defined medium originally devised for the maintenance of the free-living nematode *Caenorhabditis briggsae* (Jenkins and Carrington, 1982).

Fourth stage *Nematospiroides dubius* larvae recovered from mice and allowed to develop to young adults *in vitro* have also been used as an anthelmintic screen (Jenkins and Ibarra, 1984) as well as the tissue stages of *Trichinella spiralis* for drugs active against this parasite (Jenkins and Carrington, 1981).

Other workers used parasites against which the anthelmintics were likely to be used, arguing that this would be closer to the situation *in vivo*.

Leland *et al* (1971; 1975) devised a method using *in vitro* development of *Cooperia punctata* but it was cumbersome and required a complex medium.

The motility of third stage larvae of Haemonchus contortus was used to detect avermectin and levamisole effects but again did not detect the activity of newer benzimidazoles (Boisvenue et al, 1983). However there were problems with the solubility of these substances and drug solutions were not obtained.

The effect of drugs on the development from egg to third stage larvae in a faecal suspension culture medium as a test system has also been proposed. Work was done on Ostertagia ostertagi, Haemonchus contortus and Trichostrongylus colubriformis as well as N. brasiliensis and Nematospiroides dubius, both rodent parasites, and was successful in detecting activity of all the major broad-spectrum anthelmintics. Usually compounds showed a variety of ovicidal or larvicidal effects, or slowed the development of the parasites. Levamisole was not ovicidal but was larvicidal. Ivermectin was unique in that it showed neither effect but produced marked inhibition of the development and motility of the larvae. O. ostertagi was found to be most susceptible to the benzimidazoles (Ibarra and Jenkins, 1984).

Fourth stage larvae of T. colubriformis were used in a screen devised by Rapson, Jenkins and Topley (1985). These were obtained from gerbils and maintained in N. brasiliensis medium (NB medium) (Jenkins, Armitage and Carrington, 1980) where they moulted to become young adults. This screen also detected activity of all the major broad-spectrum anthelmintics by monitoring development and motility. All of the drugs affected both parameters equally except ivermectin which inhibited

moulting at much lower concentrations than were necessary to render the larvae moribund.

The development of second stage larvae, hatched from eggs of Ascaris suum to third stage, and in vivo derived late third stage to early fourth was also used similarly as an anthelmintic screen. The effects of ivermectin was inhibited by the presence of serum in the support medium (Rew, Urban and Douvres, 1986) but this was not reported by others. (Jenkins, Armitage and Carrington (1980), Jenkins and Carrington (1982)).

However, the fact that all these screens take a great deal of time and can be very subjective was recognised by two different developments.

The secretion of the enzyme acetylcholinesterase (AChE) by fourth larval stage and immature adults of N. brasiliensis was found to be inhibited by all major anthelmintics having a broad spectrum of activity. Organophosphorus compounds are also potent inhibitors of this enzyme but some are also anthelmintically active e.g. haloxon. The ease of measurement of AChE and the ability to store medium samples for measurement later is useful in this technique (Rapson, Chilwan and Jenkins, 1986). A number of other species have been shown to secrete AChE, particularly at the fourth larval (L_4) and adult stages so it is possible that this technique could be used with other parasites; however the difficulty of obtaining L_4 from other larger and more expensive hosts may make N. brasiliensis a more feasible parasite to use.

A micromotility meter has been designed to allow a less subjective assessment of larval and adult nematode motility and its response in the presence of different drugs. A number of

different species were able to be used in this system providing a simple and rapid analysis of the parasites' motility. The meter is based on the reflection of light from the meniscus of the medium containing the parasites to a photodiode. Motility of the parasites produces a fluctuation in the light reflected and a motility index can be calculated (Bennett and Pax, 1986; 1987; Folz et al, 1987).

Previous workers in this field devised similar instruments for assessing the motility of schistomes (trematodes) and the effect of drugs on their motility. One instrument utilised fibreoptics (Hillman and Senft, 1973) , another used ultrasound (Brown et al, 1973).

Materials and methods

Larvae

The larvae used in these experiments were obtained as described previously. Generally, O. circumcincta larvae were used, but Cooperia oncophora were also tested.

Assay techniques

(i) Larval migration

Initially, the method as described by Douch et al (1983) was followed.

The larvae to be used were exsheathed by addition of sodium hypochlorite solution (Milton 2 sterilising fluid, Richardson-Vicks Ltd.). 5% v/v of sodium hypochlorite was added to the larvae and shaken to mix. A sample was taken onto a microscope slide for the exsheathing process to be observed. When exsheathment had taken place, approximately five minutes, water

was added to dilute the hypochlorite solution and the larvae centrifuged at 1500 x g for ten minutes. The supernatant was removed and fresh water added. This was repeated three times to remove a large percentage of the hypochlorite from the solution containing the larvae. The concentration of larvae was adjusted to 1500/ml to be used in this method.

1 ml of larvae in water was incubated with 2 ml of the test substance of mucus or faecal homogenates for three hours. 1 ml of Noble agar (Difco Lab. Ltd., 1.4 g/100 ml water) was added to the incubated mixture and 3 ml of the mixture placed in a sieve made from a plastic ring and 100 um nylon mesh (Simonyl). The sieve had been previously frozen to a glass plate using a small amount of water to prevent leakage. Once the agar had set, the sieve was placed on a wire ring in 40 mm petri dishes, previously scored on the underside to make a grid, containing 3 ml of normal saline (0.85%). Migration was allowed at room temperature for 24 hours. The sieve was removed and the migrated larvae in the saline counted using a microscope. The percentage migration was assessed. This method was laborious and the results obtained were very variable, which was also seen in the results of Douch et al (1983). It was thought that this method was not ideal and certainly not suitable as a possible screen for anthelmintic drugs which was a secondary aim of this work. The method was therefore modified.

The larvae were exsheathed as described previously. An agar layer of 0.25 ml Noble agar (0.35 g in 100 ml) was made in 1 ml plastic syringes barrels which had had the narrowing area of the nozzle removed. The syringes were stoppered to prevent the agar

being lost before gelling. The stopper was then removed and a small piece of 100 um mesh secured around the bottom of the syringe and the agar shaken down to contact the mesh. 100 ul containing a known number of larvae were placed on top of the agar after incubation with various test substances and the apparatus placed in a 5 ml syringe barrel containing 2 ml of normal saline, so that the saline was in contact with the mesh and agar. At the end of the migration period the saline was collected and larval numbers counted and the percentage migration estimated. Each test was repeated a number of times. Although this method was more capable of assessment of larger numbers of test substances, the results were still very variable. Results with mucus and faecal homogenates and various anthelmintic drugs were obtained using this method. To test some substances that needed longer contact with the larvae to show their effect e.g., benzimidazole anthelmintics, a support medium was necessary to promote larval survival during the incubation. Caenorhabditis briggsae maintenance medium (Gibco Biocult) a fully defined medium was used supplemented with benzylpenicillin, streptomycin sulphate and 5-fluorocytosine to prevent bacterial and fungal growth. All results obtained using this technique with various anthelmintic drugs used this medium unless stated otherwise.

However, the results obtained were very variable and often little effect was seen after long incubation times with various anthelmintic drugs.

Despite changing incubation and migration times, agar concentration, larval position, either in or on the agar gel, drug solvent and counting larvae accurately as placed on the agar, little improvement in the variability of results was obtained.

Finally, it was decided to assess larval motility only after incubation with test substances without proceeding through a migration step. The variation in results was less and fewer preparations were necessary. It was also able to detect changes caused by a wide variety of anthelmintic drugs that was impossible with the previous method.

(ii) Larval motility

Support medium

The support medium was changed to a N. brasiliensis medium (NB medium) adapted from Jenkins, Armitage and Carrington (1980). The larvae were able to survive longer in this medium and appeared more active in the NB medium compared to the C. briggsae defined medium. The NB medium was composed of:-

2 g tryptic digest of casein (Difco)

1 g yeast extract (Beta-lab)

0.5 g D-glucose

0.08 g Di-potassium hydrogen orthophosphate

0.08 g Potassium dihydrogen orthophosphate

This was made up to 100 ml using deionised water and the pH adjusted to 7.2 with 10N potassium hydroxide. The solution was transferred to universal bottles and autoclaved at 20 p.s.i. for 15 minutes.

Prior to use, the solution was supplemented with 60 mg sodium benzylpenicillin, 100 mg streptomycin sulphate and 3 mg natamycin per 100 ml of NB medium to prevent bacterial and fungal growth.

Assessment of larval motility

Multiwell plates (Sterilin) were used which had previously been scored on the underside to form a grid.

Larvae were used ensheathed and numbers were adjusted to approximatly 100 per 10 ul.

10 ul of larvae in water were placed in each well. 1.97 ml of NB medium was added and 20 ul of the test substance. The plates were covered and incubated at 37°C for variable periods. The plates were examined under a microscope and in each well, the percentage of larvae that were motile was calculated. Controls were run in each experiment, containing 10 ul of the solvent used with the test substance. Each test concentration was run in duplicate.

It was possible with this method to assess the effect of different types of anthelmintics, e.g. avermectins and benzimidazoles, as well as other substances such as meclofenamic acid and omeprazole, the results of which are shown elsewhere.

Preparation of test substances

(i) Mucus homogenates

The mucus samples investigated using the larval migration technique were collected from the first three metres of small intestine from sheep at necropsy. Gross food contamination was gently removed by washing. The mucus was carefully collected by scraping with the edge of a microscope slide.

An equal volume of phosphate buffered saline (PBS) was added and homogenised using a borosilicate hand-held homogeniser. The homogenate was centrifuged at 2500 x g for 20 minutes in a cooled centrifuge (Chilspin, MSE). The supernatant was removed and stored at -20°C until tested.

(ii) Faecal homogenates

These were also assessed using the larval migration technique. 2 g of ovine faeces were obtained and homogenised with 10 ml of phosphate buffered saline as described above. The supernatant was stored at -20°C until tested.

Classification of mucus and faecal homogenates

It was not possible to accurately ascertain the immunity status of the sheep from which the samples were collected. Those samples classed as low immunity were obtained either from young lambs or from animals that had been housed for periods greater than three months. The samples classed as immune were from adult animals that had been grazing contaminated pasture for at least three months and had few parasites at necropsy. Only very small numbers of parasites were present at necropsy in all these animals.

Substances tested by the larval motility technique

A number of different substances were assessed for their effect on O. circumcincta larvae (L_3) using this technique. The results are shown here and elsewhere in this thesis.

The different solvents used in these substances were all included in the controls for each experiment and because of the small volume added, little effect was observed on the larvae.

(i) Prostaglandins. Methanol (HPLC grade) was used as the solvent for prostaglandin E_1 and prostaglandin E_2.

(ii) Leukotrienes. Phosphate buffered saline (PBS) pH 6.9 was used as a solvent for the leukotrienes B_4, C_4 and D_4.

Results of the larvae migration technique

Mucus and faecal homogenates

The results are shown in Tables 43, 44, 45 and 46.

There was a wide variation in the percentages recorded both within experiments and between different experiments. Using C. oncophora L_3, there was no significant difference between the migration after incubation in mucus from immune and low immunity animals (Table 43). However, both results were significantly lower than the control results that were incubated in water. The mucus was present as twice the volume of larvae in water during incubation. This was not seen in Table 44 when the migration of O. circumcincta L_3 from immune mucus was compared to controls. The results were not significantly different. Here, the mucus was present as half the volume of the larvae in water during incubation which may have below the concentration required for effect. However, when equal volumes of larvae and mucus were incubated together either for three or 24 hours, using O. circumcincta L_3, the migration from immune mucus was significantly lower than that from 'low immunity' mucus at three hours (Table 45), whereas at 24 hours the difference between migration from faeces from immune and low immunity animals was significantly different. However, after 24 hours incubation the difference between the migration from the two different types of

mucus was not statistically significant (Table 46).

Anthelmintic drugs

The results using a variety of different types of anthelmintic drugs for variable incubation periods are shown in Tables 47, 48, and 49. It was very difficult to show any consistent effect of the benzimidazole anthelmintics in this assay system. Ivermectin and levamisole did show some reduction in larval migration but the results obtained were very variable and the controls often did not show a high percentage of larval migration.

Little effect of albendazole sulphoxide or oxfendazole was seen after 24 hours incubation (Table 47). Thiabendazole appeared to show some activity at this point with 57.3% migration for 80 ug/ml. Ivermectin also showed some activity with 57.3 and 65.7% migration for 20 and 80 ug/ml respectively. There was an indication that incubation of ivermectin with larvae in a glass vessel produced a lower migration percentage than when incubation was carried out in plastic.

A similar result was obtained for 48 hours incubation (Table 48) when again the ivermectin incubated with larvae in glass produced a lower migration percentage. However, some inhibition of migration was seen as a result of incubation of ivermectin with larvae in plastic vessels, 68.3 and 64.3% migration for 20 ug/ml and 80 ug/ml respectively. Again, no obvious effect was seen for albendazole sulphoxide, oxfendazole or thiabendazole at this stage.

After 72 hours incubation (Table 49) some inhibition of larval migration was seen with the benzimidazoles, with migration reduced to 50% but this was very variable. Ivermectin showed a more pronounced effect, with only 18.3% migration after incubation with 80 ug/ml. In contrast to the two previous tables, ivermectin incubated with larvae in glass showed little inhibition of larval migration. Changing the solvent to DMSO for the test substances did not alter the results obtained with albendazole sulphoxide (Tables 50, 51 and 52). Some effect was seen after 72 hours (Table 52). Ivermectin produced less inhibition of migration when compared to the results using methanol as a solvent. Levamisole at 80 ug/ml after 24 hours, allowed 54% of larvae to migrate (Table 50). The migration increased, 59.7% at 48 hours (Table 51) and 60.7% at 72 hours (Table 52).

Results of the larval motility technique

Anthelmintic drugs

Table 53 shows a comparison of the effects of various concentrations of ivermectin on O. circumcincta L_3 as assessed by larval motility and larvae migration techniques. Although the motility was assessed only once at each concentration, a greater degree of correlation between ivermectin concentration and motility was seen than between concentration and larval migration.

Table 54 shows a further assessment of the effects of ivermectin using the larval motility technique. There was little variation between the results at each concentration compared to the results obtained using larval migration. The difference

between the results obtained with the larval motility technique and ivermectin on the two occasions may be due to the fact that the second experiment did not utilise C. briggsae medium, which was present in the first.

Prostaglandins

Table 55 shows the motility of O. circumcincta L_3 after incubation with prostaglandins E_1 and E_2 in the presence of NB medium. After two hours the motility of larvae incubated with 50 ug/ml of prostaglandins E_1 and E_2 was significantly lower than the control larvae. At three hours, larvae incubated with 10 ug/ml of prostaglandin E_1 showed significantly lower motility than the controls.

Leukotrienes

Table 56 shows the motility of O. circumcincta L_3 after incubation with leukotrienes B_4, C_4 and D_4 in the presence of NB medium. There was little difference between the motility of the larvae incubated with leukotrienes and the control larvae.

Tables 57 and 58 show the motility of sheathed and exsheathed larvae during incubation with leukotriene C_4 in water. No medium was present. After two hours incubation there was a reduced motility of both sheathed and exsheathed larvae incubated with leukotriene C_4 compared to controls. Towards the end of the experiment, the motility of the control larvae was markedly reduced due to lack of support medium.

The motility of larvae incubated for two hours with the leukotriene was not statistically significantly lower than the controls, but this may have been due to the small number of

results.

Discussion

There would appear to be inhibition of larval migration when larvae are incubated with mucus from immune animals compared to that from animals with low immunity. This work also detected some effect due to substance(s) present in faeces from immune animals. Cooperia oncophora larval migration was not significantly altered however. This parasite was obtained from calves and although the effect of the mucus is thought not to be specific, it might show a reduced effect for a parasite normally resident in a different species.

The results obtained using O. circumcincta would seem to indicate that mucus and faeces from immune animals do have some effect on larval migration. The inhibition caused by incubation with immune mucus was seen at three hours, but a 24 hour incubation was necessary to see the effect of the substance(s) in faeces.

The anthelmintic studies produced disappointing results. It was very difficult to detect effects of the benzimidazoles after 24 and 48 hours incubation. Seventy-two hours incubation produced a greater effect of the benzimidazoles but results were very variable. These anthelmintics are thought to produce their effects slowly, by their effects on microtubules (Lacey, 1985) but this assay system did not detect their effects convincingly.

Ivermectin also needed long incubation with larvae to show some effect, but even after 72 hours this was not marked. It has been suggested that ivermectin binds to plastic (Rew, Urban and Douvres, 1986) but this was not always apparent in these

experiments.

There was no improvement in the results obtained when the drug solvent was altered, or by changing incubation and migration times.

Levamisole showed some inhibition after 24 hours, which was consistent with its rapid effect on parasites.

The results obtained from the assessment of motility were less variable when compared to migration results for ivermectin. The effects of thiabendazole, albendazole sulphoxide and levamisole were also detected by this simpler method (see Table 38).

The results obtained using assessment of larval motility after incubation with prostaglandins and leukotrienes were very interesting. The inhibitory effect of immune mucus on larvae has been attributed to SRS-A and therefore leukotrienes C_4, D_4 and E_4 (Douch et al, 1983) but there was no detectable inhibition when various leukotrienes were incubated with larvae in the presence of NB medium. It is possible that constituents of this medium bound the leukotrienes, or protected the larvae, as a greater effect was seen in the experiment conducted without medium. To be important as a substance that prevents larvae penetrating through the mucus, it must produce any effect within a few hours. An inhibition of motility was seen with leukotriene C_4 after two hours' incubation and also with prostaglandins E_1 and E_2, which is contrary to results obtained by Douch et al (1983). It was not necessary for the larvae to be exsheathed to see this effect.

Therefore it would seem that prostaglandins E_1 and E_2 and leukotriene C_4 have a deleterious effect on larval motility and one or more could be the substances present in immune mucus that protect the mucosa from larvae.

TABLE 43

Number of Cooperia oncophora larvae (L_3) that migrated after three hours incubation with ovine duodenal mucus. Incubation was carried out 2 : 1 v/v mucus : larvae. The results are expressed as a percentage of the total number of parasites

	Control	Low immunity Mucus	Immune Mucus
	93	52	62
	75	42	21
	93	47	10
	70	42	47
	99		
	100		
Mean ± SEM	88.3 ± 5.19	45.8 ± 2.39	35 ± 11.88

TABLE 44

Number of Ostertagia circumcincta larvae (L_3) that migrated after three hours incubation with ovine duodenal mucus. Incubation was carried out 1 : 2 v/v mucus : larvae. The results are expressed as a percentage of the total number of parasites

	Control	Immune Mucus
	39	7
	14	21
	49	24
	49	27
	75	69
	52	17
	40	12
	69	29
	87	75
Mean ± SEM	52.7 ± 7.28	31.2 ± 8.06

TABLE 45

Number of *O. circumcincta* L_3 that migrated after three hours incubation with ovine duodenal mucus and faeces. Incubation was carried out 1 : 1 v/v mucus : larvae. Results are expressed as a percentage of the total number of parasites

Control	Low immunity mucus	Immune mucus	Low immunity faeces	Immune faeces
100	36	16	46	49
92	62	23	46	36
95	59	30	56	72
73	75	22	83	19
77	72	28	93	19
83	50	50	100	63
34		28		
55		34		
51		16		
55		21		
		30		
		9		
		21		
		47		
Mean ± SEM 71.5 ± 6.93	59 ± 5.9	26.8** ± 3.04	70.7 ± 9.90	43 ± 9.09

** $p < 0.01$

TABLE 46

Number of *O. circumcincta* L_3 that migrated after 24 hours incubation with ovine duodenal mucus and faeces. Incubation was carried out 1 : 1 v/v mucus : larvae. Results expressed as a percentage of the total number of parasites

	Control	Low immunity mucus	Immune mucus	Low immunity faeces	Immune faeces
	100	100	20	65	13
	72	13	0	75	30
	62	49	13	66	16
	70	60	28	100	50
	100	31	31	100	25
	73	47	22	75	60
			44		
			69		
			9		
Mean ± SEM	79.5 ± 6.67	50 ± 12.03	26.2 ± 6.86	80.3 ± 6.43	32.3 ± 7.70

TABLE 47

Number of *O. circumcincta* L_3 that migrated after 24 hours incubation with various anthelmintic drugs in methanol. Results expressed as a percentage of the total number of parasites

Test substance	Concentration (ug/ml)	Migration (%)			Mean ± SEM
Albendazole sulphoxide	20	82	93	87	87.3 ± 3.18
Albendazole sulphoxide	80	71	89	84	81.3 ± 5.37
Albendazole sulphoxide +	80	61	33	100	64.7 ± 19.45
Oxfendazole	20	95	88	51	78 ± 13.67
Thiabendazole	80	71	51	47	56.3 ± 7.43
Ivermectin	20	49	67	56	57.3 ± 5.25
Ivermectin	80	84	51	62	65.7 ± 9.71
Ivermectin +	80	37	42	58	45.7 ± 6.34
Control (Methanol)		71	40	71	60.7 ± 10.35

+ incubation carried out in glass tubes instead of plastic.

TABLE 48

Number of *O. circumcincta* L_3 that migrated after 48 hours incubation with various anthelmintic drugs in methanol. Results expressed as a percentage of the total number of parasites

Test substance	Concentration (ug/ml)	Migration (%)			Mean ± SEM
Albendazole sulphoxide	20	100	71	80	83.7 ± 8.58
Albendazole sulphoxide	80	100	93	100	97.7 ± 2.34
Albendazole sulphoxide+	80	95	98	93	95.3 ± 1.46
Oxfendazole	20	100	100	81	93.7 ± 6.34
Thiabendazole	80	100	100	91	97.0 ± 3.00
Ivermectin	20	65	100	40	68.3 ± 17.42
Ivermectin	80	76	93	24	64.3 ± 20.78
Ivermectin +	80	44	35	98	59.0 ± 19.70
Control (Methanol)		87	100	98	95 ± 4.05

+ incubated in glass tubes.

TABLE 49

Number of *O. circumcincta* L_3 that migrated after 72 hours incubation with various anthelmintic drugs in methanol. Results expressed as a percentage of the total number of parasites

Test substance	Concentration (ug/ml)	Migration (%)			Mean ± SEM
Albendazole sulphoxide	20	56	13	76	48.3 ± 18.61
Albendazole sulphoxide	80	60	78	76	71.3 ± 5.70
Albendazole sulphoxide +	80	86	84	79	83 ± 2.08
Oxfendazole	20	100	14	-	57 ± 48.13
Thiabendazole	80	69	71	18	52.7 ± 17.36
Ivermectin	20	72	58	35	55 ± 10.80
Ivermectin	80	24	24	7	18.3 ± 5.67
Ivermectin +	80	86	86	44	72 ± 14.02
Control (Methanol)		73	76	80	76.3 ± 2.03

+ incubated in glass tubes.

TABLE 50

Number of *O. circumcincta* L_3 that migrated after 24 hours incubation with various anthelmintic drugs in dimethylsulphoxide (DMSO). Results expressed as a percentage of the total number of parasites

Test substance	Concentration (ug/ml)	Migration (%)			Mean ± SEM
Albendazole sulphoxide	80	78	100	84	87.3 ± 6.57
Ivermectin	80	68	73	85	75.3 ± 5.05
Levamisole	80	62	55	45	54 ± 4.94
Control (DMSO)		75	86	94	85 ± 5.51

TABLE 51

Number of *O. circumcincta* L_3 that migrated after 48 hour incubation with various anthelmintic drugs in dimethylsulphoxide (DMSO). Results expressed as a percentage of the total number of parasites

Test substance	Concentration (ug/ml)	Migration %			Mean ± SEM
Albendazole sulphoxide	80	70	79	81	76.7 ± 3.39
Ivermectin	80	100	69	81	83.3 ± 9.04
Levamisole	80	64	63	52	59.7 ± 3.85
Control (DMSO)		81	84	31	65.3 ± 17.21

TABLE 52

Number of *O. circumcincta* L_3 that migrated after 72 hours incubation with various anthelmintic drugs in dimethylsulphoxide (DMSO). Results expressed as a percentage of the total number of parasites

Test substance	Concentration (ug/ml)	Migration (%)			Mean ± SEM
Albendazole sulphoxide	80	74	53	72	66.3 ± 6.70
Ivermectin	80	93	82	100	91.7 ± 5.25
Levamisole	80	54	56	84	60.7 ± 5.70
Control (DMSO)		67	74	74	71.7 ± 2.34

TABLE 53

Comparison of the effect of various concentrations of ivermectin on *O. circumcincta* L_3 as assessed by larval motility and larval migration after three hours incubation. Results expressed as a percentage of the total number of parasites

Concentration of test substance (ug/ml)	Motility (%)	Migration (%)			Mean ± SEM of Migration
Control (DMSO)	90	74	39	49	54 ± 10.42
Ivermectin 0.1	89	15	24	51	30 ± 10.83
0.25	87	69	11	34	38 ± 16.88
0.5	55	44	9	36	29.7 ± 10.60
0.75	26	16	38	20	24.7 ± 6.77
1	23	13	1	2	5.3 ± 3.85
2.5	5	3	53	3	19.7 ± 16.69
5	7	6	0	15	7.0 ± 4.36
10	1	3	4	7	4.7 ± 1.20
80	1	6	0	3	3.0 ± 1.73

The larvae were incubated in *C. briggsae* medium.

TABLE 54

Number of *O. circumcincta* L_3 that were motile after three hours incubation with various concentrations of the anthelmintic drug ivermectin. Results expressed as a percentage of the total number of parasites

Concentration of test substance (ug/ml)		Motility (%)					Mean ± SEM
Control (DMSO)		90	98	91	97	100	95.2 ± 1.98
Ivermectin	0.1	91	90	97	100	97	95 ± 1.92
	0.25	93	93	96	100	98	96 ± 1.38
	0.5	95	89	96	99	98	95.4 ± 1.75
	0.75	91	97	98	97	93	95.2 ± 1.35
	1	86	92	87	94	94	90.6 ± 1.72
	2.5	20	38	42	56	46	40.4 ± 5.90
	5	5	8	22	32	14	16.2 ± 4.89
	10	3	4	16	10	6	7.8 ± 2.37
	80	0	2	4	3	4	2.6 ± 0.75

The larvae were incubated with water and were **not** maintained in medium.

TABLE 55

Mean motility (expressed as percentage of control motility) of *O.circumcincta* L_3 after incubation with various concentrations of prostaglandins E_1 and E_2 (PGE_1 and PGE_2)

Test substance	Time (hours) 0.5	2	3	4	6	24	30
PGE_1 50 ug/ml	107.2	80.3*	82.5	105.3	136.9	111.8	122.7
PGE_1 10 ug/ml	90.6	89.0	63.7*	94.9	100.4	110.7	92.7
PGE_2 50 ug/ml	98.9	63.2**	85.9	120.5	92.2	105.9	103.6
PGE_2 10 ug/ml	102.4	94.6	83.4	82.2	119.6	103.5	121.6

TABLE 56

Mean motility (expressed as percentage of control motility) of *O. circumcincta* L_3 after incubation with leukotrienes B_4, C_4 and D_4 (LTB_4, C_4 and D_4)

Test substance	Concentration (ug/ml)	Time (hours) 0.25	1	2	4	6	24
LTB_4	0.1	101.2	118.8	115.4	105.1	107.4	113.6
LTC_4	0.1	100.4	104.9	109.8	97.7	110.0	92.4
LTD_4	0.1	100.7	108.4	111.8	83.1	93.8	102.0

Incubation took place in the presence of NB medium.

* $p < 0.05$

** $p < 0.01$

TABLE 57

The mean motility of sheathed and exsheathed O. circumcincta L_3 after incubation with water and leukotriene C_4 (0.1 ug/ml). Results expressed as a percentage of the total number of parasites

	Time (hours) 0.5	2	4	6	8
Sheathed larvae					
Control	98.5	72.0	61.3	52.6	38.7
0.1 ug/ml LTC_4	95.4	46.8	52.5	28.8	29.0
Exsheathed larvae					
Control	92.7	80.9	60.5	45.1	62.0
0.1 ug/ml LTC_4	93.0	52.6	45.4	26.6	40.7

TABLE 58

The mean motility of sheathed and exsheathed O. circumcincta L_3 expressed as a percentage of control motility after incubation with leukotriene C_4 (0.1 ug/ml)

	Time (hours) 0.5	2	4	6	8
Sheathed larvae	96.8	65.0	85.6	54.8	74.9
Exsheathed larvae	100.3	65.0	75.0	59.0	65.7

In vitro STUDIES INTO BENZIMIDAZOLE RESISTANT STRAINS OF *Ostertagia circumcincta*

INTRODUCTION

Resistance of nematodes to anthelmintics

Currently there is a great deal of interest in this subject with work being carried out in many different areas of the world.

A number of reviews have been published in this area (Kelly and Hall, 1979; Prichard *et al*, 1980; Waller and Prichard, 1986).

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. It is genetically expressed and inherited (Kelly and Hall, 1979).

Resistance has been detected in *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Ostertagia circumcincta* in sheep and goats to benzimidazole, levamisole and morantel anthelmintics. A strain of *Nematodirus spathiger* has also been found to be resistant to benzimidazoles (Martin, Anderson and Jarrett, 1985). Benzimidazole resistance has also been detected in the small strongyles (cyathostomes) of horses.

Practical problems with resistance seem to be centred on areas where *H. contortus* is endemic and where the climate is generally adverse to survival of free-living stages of nematodes, e.g. certain areas of Australia.

History of resistance in nematodes

Resistance was discovered in nematodes to the different broad-spectrum anthelmintics in the same order as they appeared

on the market. Therefore, resistance to phenothiazine was detected first, then to thiabendazole, the first of the benzimidazole group which was introduced in 1961. Its activity was superior to that of phenothiazine. Resistance to thiabendazole did not take long to be reported. In 1964 resistance was described in H. contortus in sheep in the USA (reviewed by Donald, 1983).

As different members of the benzimidazole group were introduced, it became apparent that parasites resistant to the effects of one benzimidazole, showed side-resistance to other members of the group even if they had never been previously exposed. Initially, however, the newer, longer acting benzimidazoles e.g. fenbendazole and oxfendazole had greater activity against nematodes resistant to thiabendazole (Hall et al, 1978; Donald, 1983) but resistance across the group soon developed.

Side-resistance is defined as resistance to a compound as a result of selection by another compound with a similar mode of action.

Cross-resistance is resistance to a compound as a result of selection by another compound with a different mode of action (Prichard et al, 1980).

Strains of H. contortus, T. colubriformus and O. circumcincta resistant to benzimidazoles were described in Australia showing side-resistance. Similar resistance has been detected in New Zealand (Kemp and Smith, 1982; Kettle et al, 1983), South Africa (Berger, 1975) and the Netherlands (Boersema, Lewing-Van der Wiel and Borgsteede, 1982; Eysker et al, 1983)

together with isolated reports from other areas of the world.

In Great Britain, a strain of O. circumcincta was the first to be described as being resistant to benzimidazoles (Britt, 1982; Britt and Oakley, 1986) followed by other reports (Cawthorne and Whitehead, 1983).

Later, in a survey of farms in the south-east of England, Cawthorne and Cheong (1984) detected benzimidazole resistance in H. contortus.

Resistance to levamisole/morantel anthelmintics was the next to be reported generally. It took some time to appear in H. contortus, in which all the initial reports were concerned with benzimidazole resistance. In contrast, the early reports of resistance in O. circumcincta and T. colubriformis were as common to levamisole/morantel as to the benzimidazoles.

Generally, if resistance is detected to levamisole, side resistance to morantel is also present. However, Waller et al (1986) isolated a strain of T. colubriformis from goats resistant to morantel, but highly susceptible to levamisole. In most of the above reports, changing to an anthelmintic with a different mode of action, resulted in efficient removal of the parasites.

Multiple-resistance however has been reported in a few instances. It is defined as resistance to two or more different anthelmintic groups, either as a result of selection by each group independently, or as a result of cross-resistance (Prichard et al, 1980). It has been reported in some sheep flocks and goat herds in Australia, with resistance to benzimidazoles, levamisole and morantel. Doubt has been cast on the reports of multiple

resistance in goats. Studies of the pharmacokinetics of anthelmintics in goats treated at the recommended, sheep dose, in Australia have shown much lower plasma concentrations and faster excretion for levamisole when compared to sheep. Therefore reports of failure of levamisole may be due to inadequate dosing (McKenna, 1984; Waller et al, 1986). Kettle et al (1983) also suggests that inadequate dosing would allow partially resistant parasites to survive and subsequently increase the resistance of the population.

There has recently been a report of ivermectin resistance in a strain of H. contortus in sheep in South Africa (Carmichael et al, 1987).

There are no convinving reports of resistance in any nematodes of cattle, although there have been a few reports of a decrease in efficacy of levamisole and thiabendazole against certain strains of O. ostertagi (Anderson, 1977; Anderson and Lord, 1979; Lyons et al, 1981). The lack of definite reports of resistance may be due to less intensive anthelmintic treatment in this species (Donald, 1983). It has been speculated that inhibited development of O. ostertagi may be partially drug induced (Kelly and Hall, 1979).

Important factors in the development of resistance

The most important factor in the development of resistance to an anthelmintic is the contribution that survivors of anthelmintic treatment make to succeeding generations. This can depend on a number of variables.

Before any anthelmintic is introduced, genes for resistance to its action are already present in the parasite population but

at a very low frequency. They therefore cannot confer any additional survival advantage otherwise they would be more common.

However, there have been reports of greater fecundity and pathogenicity of resistant strains (Kelly and Hall, 1979; Borgsteede and Couwenberg, 1987). In insects resistant to pesticide, it has been reported that there can be a general modification of the background genome around the resistant allele(s) to produce a greater degree of fitness in a pesticide-free environment (Martin, 1987) which may explain these findings.

Resistant individuals are selected by treatment with anthelmintic and the level of resistance of the population increases by increasing the number of resistant individuals (Prichard et al, 1980). If selection is withdrawn for many generations, the population would be expected to become more like the original i.e. susceptible. This process is termed reversion (Kelly and Hall, 1979).

The frequency of anthelmintic administration and its relationship to the prepatent period has been shown to be very important in development of resistance. A direct correlation has been found between treatment frequency and level of resistance (Martin et al, 1982; Barton, 1983; Kettle et al, 1983; Martin et al, 1984).

Laboratory production of resistant strains has also been achieved by a high frequency of anthelmintic treatment. This has been reported by a number of workers (for example, Kates, Colglazier and Enzie, 1973) but there are a number of differences

in laboratory studies that make extrapolation to the field situation difficult.

These are:-

(i) small gene pool. If a laboratory strain becomes resistant, then it is likely that the strain in the field also has the potential for resistance. If resistance is not developed, it could be because of the small population size.

(ii) anthelmintic dose rates are carefully controlled and administered. In the field, dosage is tailored usually to the heaviest animal and estimation of weights may be incorrect.

(iii) laboratory studies use discrete parasite generations, whereas in the field there is substantial overlap. The environmental conditions in the field also have to be survived by free-living stages before a contribution can be made to the next generation (Waller and Prichard, 1986).

As the frequency of anthelmintic treatment increases, the ability of susceptible parasites to multiply and contribute to succeeding generations becomes less and the number of resistant individuals in the population increases.

In Western Europe, frequent administration of anthelmintic (greater than three times annually) is less common and occurs only in some horses, sheep and goats. In this situation the grazing management in relation to anthelmintic treatment probably has a greater influence on the development of resistance. The association of anthelmintic treatment and relocation to so-called clean or safe pastures could allow parasites surviving treatment to seed the new pasture and make a substantial contribution to succeeding generations, depending on the number of parasites

already present on the pasture (Michel et al, 1983). Resistance arising from this sort of grazing management has been reported in O. circumcincta (Martin, 1987) and obviously depends on the importance of dosed animals as a source of relevant pasture contamination.

Selection only occurs in the parasitic stages within the host, the free-living stages escape selection. Individuals of a population that escape selection are defined as being in refugia. If treatment of animals harbouring parasites is carried out when there are large numbers of free-living parasites on the pasture, then the selection pressure is less than if only a few parasites escape treatment. This has also been shown experimentally (Martin, Le Jambre and Claxton, 1981).

As stated previously, if selection by anthelmintic treatment is stopped, it would be expected that the population would revert to susceptibility. Studies on this subject have produced different results.

Reversion in a strain of H. contortus originally resistant to thiabendazole has been detected. However, when thiabendazole was reintroduced, resistance quickly established (Kelly and Hall, 1979). Some reversion has also been detected in a strain of T. colubriformis (Simpkin and Coles, 1978). Re-establishment of resistance in T. colubriformis after some reversion has also been found (Waller et al, 1985). It has been suggested that some benzimidazole resistant parasite strains revert towards susceptibility more quickly when treated with levamisole (Donald et al, 1980; Prichard et al, 1980).

However strains of H. contortus and T. colubriformis were studied and no reversion was detected (Herlich, Rew and Colglazier, 1981; Hall, Ritchie and Kelly, 1982). They concluded that any reversion that may occur was likely to be very slow. Similar results were obtained with H. contortus, where a partially resistant strain actually increased its level of resistance while not subjected to anthelmintic treatment (Le Jambre, Martin and Jarrett, 1982).

Mechanisms of resistance

The only studies of the mechanism of levamisole resistance have been in the free-living nematode, Caenorhabditis elegans, where resistant individuals were found to have fewer acetylcholine receptors than susceptibles (Waller and Prichard, 1986).

Benzimidazole resistant parasites have been shown to be less affected, with respect to inhibition of the fumarate reductase system that is involved in anaerobic metabolism, than susceptibles when in the presence of benzimidazoles (Prichard et al, 1980). It has also been reported that there is a decreased uptake of benzimidazoles by resistant parasites but this has not been repeated and is thought unlikely. In association with the suggestion that the primary site of action of benzimidazoles is on tubulin within parasite cells and the inhibition of its polymerisation into microtubules, it has been found that parasites resistant to benzimidazoles allow less drug to bind to their tubulin than susceptibles (Lacey, 1985; Lacey et al, 1987). This could be the primary cause of benzimidazole resistance in parasites.

Genetics of resistance

In general, little is known about the genetics of parasite resistance to anthelmintics. Difficulties arise from not being able to sex free-living stages and breeding experiments having to take place within the host.

It is thought that resistance of H. contortus to benzimidazoles is a polygenic trait i.e. it is inherited through alleles at several loci and is not sex-linked (Le Jambre, Royal and Martin, 1979; Herlich, Rew and Colglazier, 1981; Martin, Le Jambre and Claxton, 1981). It would appear that there can be a greater maternal influence on resistance than paternal which could be explained by extra-chromosomal inheritance present in the egg cytoplasm, or by partial mitosis, endomitosis, multiplying the number of maternal chromosomes (Le Jambre, 1985).

There is a report of one strain of T. colubriformis resistant to levamisole that appears to be caused by a single gene (Waller et al, 1986).

It has been argued that, due to the non-persistant nature of most broad-spectrum anthelmintics, a number of small changes in the parasites' physiology or biochemistry could allow it to survive the short time of anthelmintic presence. Longer acting anthelmintics or continuous administration would require a major gene change to allow survival (Le Jambre, 1985).

Methods of detection of resistance

Egg hatch assay

This in vitro technique was first described by Le Jambre (1976) and Coles and Simpkin (1977) as a way of assessing

resistance to the benzimidazole group of anthelmintics, utilising the ovicidal properties of these drugs. The assay has been widely used (for example, Hall, Campbell and Richardson, 1978; Whitlock et al, 1980; Cawthorne and Whitehead, 1983) often with modifications, making comparison of results from different workers difficult.

Basically, undeveloped nematode eggs are incubated in serial concentrations of benzimidazoles, usually thiabendazole, and the percentage of eggs that hatch at each concentration is determined. Log-dose probit lines for egg mortality are calculated (Le Jambre, 1976), or arc-sine transformation used (Cawthorne and Whitehead, 1983), and the ED_{50}, the concentration of benzimidazole required to prevent 50% of the eggs hatching obtained. The responses are then compared to known susceptible strains.

The differences in technique include:-

(i) incubation times, from 24 hours to 72 hours.

(ii) the inclusion of larvated eggs either in the percentage hatched (or developed) or in the percentage that fail to hatch.

(iii) the use of commercial anthelmintics or pure drug and solvents for stock solutions.

Possibly because of these different techniques a wide range in the ED_{50}s obtained for benzimidazole susceptible strains is observed. Boersema (1983) cites values for the ED_{50} for benzimidazole susceptible Haemonchus contortus from 1.1 ug thiabendazole/ml (Le Jambre, 1976), 0.47 ug thiabendazole/ml (Donald et al, 1980) to 0.023 ug thiabendazole/ml (Hall, Campbell

and Richardson, 1978). Obviously, reference to standard susceptible strains would be preferable (Waller and Prichard, 1986).

Initially it was thought necessary to allow only a couple of hours to elapse from the faeces being passed to commencement of the assay (Le Jambre, 1976). However, Smith-Buijs and Borgsteede (1986) showed that maintenance of parasite eggs for 72 hours did not alter the results obtained from the egg hatch assay. This made the assay more practical for field work. However, it has been found that once the eggs have started to embryonate, the benzimidazole is less able to prevent hatching (Le Jambre, 1976; Weston, O'Brien and Prichard, 1984).

Day to day variation has also been observed when a single strain has been assayed (Boersema, 1983). Borgsteede and Couwenberg (1987) studied resistant and susceptible strains of H. contortus throughout the patent period of the infection. They observed similar variation in both strains, the ED_{50} starting at a low level, rising and then falling towards the end of the infection.

This technique has been used mainly to monitor resistance in experimental infections where the parasites were administered as a single dose and only one species present. However, it has been used to assess resistance in field infections where the egg hatch assay is combined with differentiation of the first-stage larvae (L_1) or allowed to develop to third-stage to aid identification (Whitlock et al, 1980).

Methods have also been developed by analysis of log dose-probit motility lines which allows the identification of sub-populations of resistant and susceptible individuals within a single strain and their proportions within that strain (Martin et al, 1984).

The results of egg hatch assays have been compared with another method of resistance detection, the faecal egg count reduction test (FECR) in sheep and goat flocks in Australia and they are found to correlate well (Presidente, 1985).

Therefore, this in vitro test represents a rapid, sensitive and economic method of assessment of benzimidazole resistance.

A similar assay, based on the paralysis of first-stage larvae within the egg which prevents hatching has been used for detection of levamisole resistance. In this situation the eggs are allowed to develop to a point just prior to hatching and then exposed to serial concentrations of levamisole for a short time before termination of the assay. Resistant nematodes are able to hatch at higher concentrations of levamisole than susceptible strains (Dobson et al, 1986). Previously, the paralysis of infective (L_3) larvae by levamisole and also morantel was used to detect parasite resistance to these drugs (Martin and Le Jambre, 1979).

Faecal egg count reduction (FECR)

This test has the advantage over the in vitro egg hatch assays in that all the different types of anthelmintics may be used. Little specialised equipment is needed and might be considered the first test to be used where resistance is suspected.

Generally, faecal egg counts are obtained prior to treatment and this is repeated five to ten days post treatment and again ten to 14 days post treatment. Only one species may be resistant and therefore the differentiation of hatched larvae has to be undertaken.

The disadvantages of this test are:-

(i) the faecal egg count is often not related to parasite numbers, this is particularly the case with Ostertagia spp.

(ii) the test does not detect immature worms which may survive treatment and develop.

(iii) the faecal egg count may be lowered but this may not correspond to a drop in parasite numbers.

(iv) levamisole treatment of an Ostertagia spp infection has been found not to reduce the faecal egg count even after repeated treatment, but when isolated and tested in vivo the strain was found to be fully susceptible (Waller, Dobson and Donald, 1983). It has been found that in populations of T. colubriformis resistant to levamisole, a higher number of females remain after levamisole treatment. This may be because of metabolic differences or mode of attachment in small intestine, i.e. the females may occupy a more 'protected site' (Dash, 1985).

(v) If faecal egg counts are low pre-treatment, resistance may not be detected by this method.

If the faecal egg count is not reduced considerably post-treatment then resistance may be suspected (Prichard et al, 1980; Waller and Prichard, 1986).

Controlled tests

In these, the adult worm populations are compared at post-mortem between treated and untreated individuals. Again all anthelmintics may be tested and it is possible to use serial doses to ascertain the response to the anthelmintic. At least five days should elapse post treatment before necropsy as there is some evidence that partly resistant parasites may take longer to be removed (Prichard et al, 1980). Evidence for this has also been shown in Cooperia curticei in sheep treated with ivermectin. C. curticei, Cooperia oncophora and Nematodirus spp have been reported as requiring higher dose rates of ivermectin to be removed compared to abomasal parasites e.g. O. circumcincta. The anthelmintic efficacy of ivermectin assessed at seven and 14 days post treatment resulted in 61.1% and 90.4% of C. curticei being removed. There was evidence that the parasites had moved distally in the small intestine after treatment (Bogan et al, 1987).

It is possible for this test on T. colubriformis to be performed in guinea-pigs rather than sheep to reduce expense. Generally, however, it is a very expensive and a laborious method of testing for resistance.

Tubulin binding assay

This provides a measurement of the extent a labelled benzimidazole binds to a crude tubulin extract of a parasite strain. Strains resistant to benzimidazoles have been found to bind less benzimidazole than susceptible parasites.

Basically the assay involves the incubation of parasite tubulin with tritiated benzimidazole until equilibrium has been reached. The free drug is removed by charcoal absorption and precipitation. The tubulin bound label is then counted (Lacey, 1985; Lacey et al, 1987). This assay may be carried out on parasite eggs, infective larvae (L_3) or adults. It has been found to give similar resistance factors as those obtained with the egg hatch assay and is a relatively quick assay to perform but needs specialised equipment. It also requires a minimum of 25,000 L_3 which could limit its usefulness.

MATERIALS AND METHODS

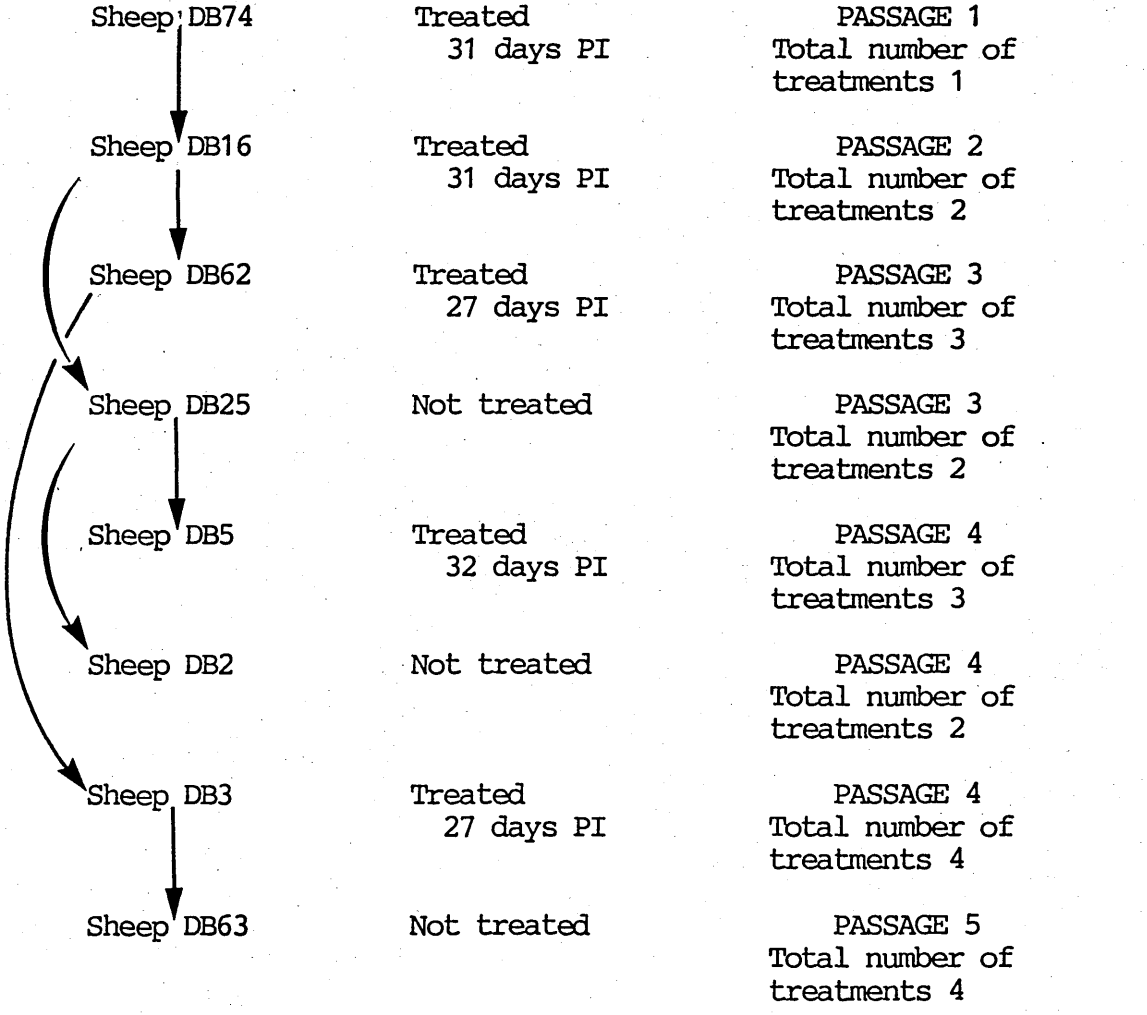
This study was part of a much wider project to investigate resistance in Ostertagia circumcincta.

Animals

All the animals used for passage of both benzimidazole resistant strains and the benzimidazole susceptible strain of O. circumcincta were male Dorset-cross lambs which were housed individually. They were previously parasite-naive.

Experimental design

Experimental design of studies into HFRO resistant strain of *O. circumcincta*



PI - post infection

Strains of *O. circumcincta* used

The benzimidazole susceptible strain used in these assays had been maintained at Glasgow University Veterinary School for a number of years. This was termed the Bearsden strain.

Two benzimidazole resistant strains were used. The main study centred on the HFRO strain, isolated from a farm owned by the Hill Farming Research Organisation. The other resistant

strain had been isolated from the Moredun Institute (Moredun strain). Both sites are in East Scotland.

Collection of parasite eggs and preparation of larval inoculum for further passage

Faeces known to contain parasite eggs were collected and incubated as described previously. Lambs were infected with 10,000 third stage larvae (L_3) as a single dose. The eggs collected from the resistant HFRO strain were isolated one week after treatment of the animal with fenbendazole.

Collection of parasite eggs for the egg hatch assay

Faeces were collected as previously described.

Parasite eggs were separated from fresh faeces by a method modified from that of Le Jambre (1976).

3 - 4 g of faeces were processed at one time. Approximately 10 ml of saturated salt solution was added to the faeces and homogenised for three minutes. The faecal suspension was then passed through a 250 um sieve and the filtrate collected in a plastic tray. The residue was washed with saturated salt. This was repeated until the tray was filled to an approximate depth of 6 - 8 cm. A plastic sheet was floated on top of the faecal suspension and as the specific gravity of the parasite eggs was less than most of the faecal matter they floated and adhered to the sheet. The sheet was lifted after approximately five minutes and the eggs washed into a beaker with water. This process was repeated a number of times. The eggs in water and salt solution were then passed through a 38 um sieve, the eggs collected on the sieve and were washed into a sedimentation flask with water. The eggs were allowed to settle for two hours. The supernatant was

then poured off. Often at this stage there was still some faecal contamination of the eggs so the residue was centrifuged at 1500 x g for five minutes to reduce the volume. The supernatant was discarded, saturated salt 2 : 1 v/v with the residue was added and mixed. Centrifugation was repeated and the salt supernatant containing the eggs again collected onto 38 um sieve and washed into a beaker. The egg concentration could then be adjusted by dilution or further centrifugation.

Fenbendazole treatment

The lambs that were treated while infected with the HFRO strain of O. circumcincta received the recommended dose rate of 5 mg/kg fenbendazole per os on one occasion. Some animals were infected with HFRO strain of O. circumcincta and were not treated in order to study the fluctuations throughout an unpressurised infection.

Egg hatch assay

This technique was used to estimate the level of resistance throughout the series of passages of the resistant HFRO strain of O. circumcincta. Also the level of resistance in the Moredun strain of O. circumcincta was assayed and compared to results obtained with the benzimidazole susceptible Bearsden strain of O. circumcincta. Eggs were collected as described and used immediately. The assay took place using plastic multiwell plates (Sterilin). Approximately 100 eggs were added in water with serial solutions of thiabendazole into each well. The solutions of thiabendazole were made from pure drug dissolved in methanol and diluted with water to produce the final concentration. Controls containing methanol in water were run with each assay.

Each concentration of thiabendazole was assessed in duplicate.

The eggs were incubated at 28°C for 48 hours. At the end of this period a small amount of iodine was added to kill the eggs and larvae. The numbers of unhatched eggs, larvated eggs and larvae were counted and the percentage of eggs that failed to develop calculated (i.e number of eggs as a percentage of the total) and corrected for natural mortality using the results from the control wells. The ED_{50}, i.e. the concentration of drug required to prevent development of 50% of the eggs, was calculated from a plot of probits against log dose concentration. The ratio of ED_{50} of the resistant strains to that obtained for the susceptible Bearsden strain was used as a 'resistance ratio' (Le Jambre, 1976).

RESULTS

HFRO strain of O. circumcincta

The ED_{50}s obtained using the egg hatch assay throughout the five passages are shown in Table 59.

First passage

The egg hatch assay on day 23 post infection produced an ED_{50} of 1.527 ug thiabendazole/ml. When compared to the result obtained with the susceptible Bearsden strain of O. circumcincta of 0.164 ug thiabendazole/ml, gives a resistance ratio of 9.31.

Second passage

The results for the second passage are also shown in Figure 40. The ED_{50} 25 days post infection was 3.360 ug thiabendazole/ml which was more than twice the figure obtained in the first passage. This animal was treated with fenbendazole on

day 31 post infection. The ED_{50} remained high one day post treatment but then fell to 1.924 ug thiabendazole/ml by three days post treatment and by eight days post treatment had reduced further to 0.980 ug thiabendazole/ml.

Third passage

The results for the third passage, treated with fenbendazole (Animal DB62) are shown in Figure 41. Another animal (DB25) was infected and sampled in parallel but was not treated. The results are shown in Figure 42.

Twenty-seven days post infection, the ED_{50} was higher than at the end of the previous passage at 2.808 ug thiabendazole/ml. After treatment it rose, one day post treatment it was 2.833 ug thiabendazole/ml and it reached a maximum two days post treatment at 3.062 ug thiabendazole/ml. The ED_{50} then fell to 1.396 ug/ml three days post treatment, only to show a rise again 44 days post infection, 17 days post treatment, to 3.457 ug thiabendazole/ml.

The results from the untreated infection were similar 27 days post infection, ED_{50} 2.441 ug thiabendazole/ml. This fell slightly during the time of treatment of the sheep run in parallel, rising slightly to 1.910 ug thiabendazole/ml on day 30. The high ED_{50}s seen in sheep DB62 post treatment were not observed in this animal. Fourty-four days post treatment the ED_{50} had fallen further to 1.301 ug thiabendazole/ml much lower than that seen in the treated infection at this stage. The ED_{50} continued to fall to 0.185, 51 and 55 days post infection, the level of resistance was only slightly higher than the ED_{50} obtained for the susceptible Bearsden strain. By 73 days post infection the ED_{50} had risen to 0.665 ug thiabendazole/ml.

Fourth passage

Two animals DB5 and DB2 were infected from DB25 the untreated infection. Twenty days post infection the ED_{50} for DB5 was only slightly above the last ED_{50} recorded in DB25 at 0.761 ug thiabendazole/ml (Figure 43). This rose to reach a maximum at 27 days post infection of 1.963 ug thiabendazole/ml and then fell before treatment with fenbendazole. There was no rise immediately post treatment until day 7 post treatment, ED_{50} 1.132 ug thiabendazole/ml. The ED_{50} then generally rose to reach 1.699 23 days post treatment.

The infection in DB2 was not treated. The ED_{50} at 20 days post infection was similar to that of DB5 at 0.764 ug thiabendazole/ml. The ED_{50} showed a similar rise as that recorded in DB5 to reach a maximum of 2.087 ug thiabendazole/ml 27 days post infection that fell slightly to 1.854 ug thiabendazole/ml 29 days post infection.

Animal number DB3 was infected from the treated infection of animal DB62 and was further pressurised by fenbendazole treatment in this animal. The few results obtained for this infection show an ED_{50} of 2.468 ug thiabendazole/ml six days post treatment, falling dramatically to 0.811 ug thiabendazole/ml seven days post treatment but rising to 1.343 by day 11 post treatment.

Fifth passage

The one egg hatch assay carried out after treatment on four occasions with fenbendazole in previous passages produced an ED_{50} of 1.483 ug thiabendazole/ml, a resistance ratio of 9.04 when compared to the susceptible Bearsden strain.

Stored larvae

Larvae that had been stored in the refrigerator (+4°C) for four months were used to infect sheep number DB4. The ED_{50}s obtained are shown in Table 60. Twenty-two and 30 days post infection, the ED_{50}s were 1.416 and 1.546 ug thiabendazole/ml respectively. The mean of these two results would give a resistance ratio of 9.03 compared to the susceptible strain.

Moredun strain of O. circumcincta

Egg hatch assays were carried out during a treated infection in animal B2 to compare the resistance with that found in the HFRO strain (Table 61).

Twenty-six days post infection the ED_{50} was 1.289 ug thiabendazole/ml which rose to 1.480 ug thiabendazole/ml 28 days post infection and then slowly fell. After treatment, the ED_{50} rose very slightly one day post treatment to 1.039, fell to 0.675 one day later then rose to reach 2.041 ug thiabendazole/ml 14 days post treatment (Figure 44).

Bearsden susceptible strain of O. circumcincta

The ED_{50} obtained for this strain of O. circumcincta was 0.164 ug thiabendazole/ml.

Table 62 shows the ED_{50}s for the various strains of O. circumcincta used in these experiments. The first ED_{50} obtained in a patent infection was used for comparison. The values obtained for the HFRO resistant strains were very similar for the first and fifth passages and also for the larvae stored for four months, a mean of 1.475 ug thiabendazole/ml. The ED_{50} for the Moredun resistant strain at the beginning of the patent infection was slightly lower than those obtained for the HFRO

strain at 1.289 ug thiabendazole/ml. The susceptible strain had an ED_{50} of 0.164 ug thiabendazole/ml.

Discussion

The results of the egg hatch assay have confirmed that both the HFRO and Moredun strains of O. circumcincta are resistant to benzimidazoles. Studies carried out in conjunction with this work by E. Scott using albendazole in this assay produced similar results for the level of resistance to these. However the fluctuations in the ED_{50} were greater using albendazole than thiabendazole. This could possibly be because the resistance was selected for using fenbendazole and although side resistance to all benzimidazoles would occur, albendazole is much closer to fenbendazole in structure than thiabendazole. Fenbendazole is insufficiently soluble to be used in the egg hatch assay although it is ovicidal (Kirsch, 1978).

The ED_{50} obtained for the susceptible strain was high at 0.164 ug thiabendazole/ml in comparison to other O. circumcincta strains assayed by other workers. Cawthorne and Whitehead (1983) gave ED_{50}s of 0.036 and 0.038 ug thiabendazole/ml. Coles and Simpkin (1977) also assayed a strain of O. circumcincta at 0.03 ug thiabendazole/ml. The differences could be due to differences in technique. Boersema (1983) compared the ED_{50} for susceptible strains of H. contortus and found a range of values from 1.1 to 0.023 ug thiabendazole/ml. The result obtained with albendazole was similar to that for thiabendazole at 0.2 ug albendazole/ml. It may also be possible that the susceptible (Bearsden) strain had developed a low level of resistance during

the 15 years it has been maintained at Glasgow University Veterinary School.

A criticism of this work would be that the susceptible strain was not assayed on each occasion with the resistant strains. It was very difficult, however to do this, as the egg collection often took a couple of hours, particularly when low faecal egg counts were recorded, and during the summer months care had to be taken to prevent embryonation of the eggs.

Despite this, a resistance factor of 9.31 was recorded for the first passage of the HFRO resistant strain of O. circumcinta. It is of interest that the fifth passage produced a very similar resistance ratio to that obtained in the first passage. Therefore, the pressurisation of the strain by four treatments of fenbendazole had not caused the resistance to increase.

This has been found by other workers using O. circumcincta (Martin et al, 1984) and T. colubriformis (Kelly and Hall, 1979). It has been assumed that once a limit is reached, that the population is homozygous for resistance, but often indications from log dose-probit lines are that there are two stable subpopulations, one resistant, one susceptible (Le Jambre, 1985).

The Moredun resistant strain showed a slightly lower level of resistance than the HFRO strain and it is interesting to speculate if the level of resistance of this strain could have been increased by anthelmintic treatment.

The result obtained from larvae stored in the refrigerator for four months indicated that no reversion in resistance had occurred during this period.

Within each passage there was a great deal of variation in the ED_{50} from day to day. This has also been recorded in H. contortus by Borgsteede and Couwenberg (1987) but the pattern was slightly different. In our studies, the level of resistance was generally high at the start of patency but declined thereafter. Borgesteede and Couwenberg (1987) found in H. contortus, each strain, one benzimidazole resistant, one susceptible, that the ED_{50} started at a low level; and rose until days 40 to 60 post infection and then fell.

The fluctuation could be caused by the resistant individuals maturing and producing eggs in a greater proportion than susceptibles at the point where the resistance level of the eggs rises, assuming the resistant population is not fully homozygous, or the resistance of the individuals could alter during the infection, declining towards the end of the infection. It is also possible that the host exerts some influence on the resistance level of the parasite eggs. It has also been suggested that senility of the worms towards the end of the infection could cause the resistance of the eggs to drop (Borgsteede and Couwenberg, 1987). Fluctuations of up to 2.4 ug thiabendazole/ml were observed in a single passage of the HFRO strain.

Therefore, if the Bearsden susceptible strain had been studied throughout an infection, it would also be expected to exhibit fluctuations in the level of resistance. This gives greater importance to running the resistant infection alongside a susceptible and subjecting both to egg hatch assays at the same time.

The ED_{50} immediately post fenbendazole treatment did not yield consistent results in these studies, except that the number of eggs that failed to hatch in the control wells was high, reaching up to 75% two days post treatment compared to the normal value of less than 5%.

Generally, the level of resistance decreased post treatment to rise again later, although there were small rises in resistance seen two days post treatment in the third passage and one day post treatment in the fourth. It would be expected that the fenbendazole would bind to the tubulin within the eggs and less thiabendazole would be required to prevent hatching. The cause of the slight rises in resistance is unknown but may be due to dominance of the most resistant females at this time.

The results obtained with albendazole were more consistent post treatment. The levels of resistance as calculated in the assays with albendazole were generally higher post treatment than pre treatment and then declined.

TABLE 59

The ED_{50} (ug thiabendazole/ml) obtained from egg hatch assays with a benzimidazole resistant strain (HFRO) of *Ostertagia circumcincta*

	Time (days)		ED_{50}
	Post infection	Post treatment	(ug thiabendazole/ml)
First passage			
Animal DB74	23	-	1.527
Second passage			
Animal DB16	25	-	3.360
	32	1	3.264
	33	2	2.011
	34	3	1.924
	39	8	0.980
Third passage			
Animal DB62	27	-	2.808
	28	1	2.833
	29	2	3.062
	30	3	1.396
	34	7	1.676
	38	11	1.604
	44	17	3.457
Animal DB25	27	-	2.441
	28	-	1.305
	29	-	1.308
	30	-	1.910
	44	-	1.301
	50	-	0.310
	51	-	0.185
	55	-	0.185
	62	-	0.718
	73	-	0.665

TABLE 59 (Cont'd)

	Time (days)		ED_{50}
	Post infection	Post treatment	(ug thiabendazole/ml)
Fourth passage			
Animal DB5	20	-	0.761
	22	-	1.002
	25	-	0.964
	27	-	1.963
	29	-	1.594
	32	-	0.796
	33	1	0.913
	34	2	0.706
	39	7	1.132
	41	9	1.496
	46	14	1.590
	48	16	1.463
	55	23	1.699
Animal DB2	20	-	0.764
	25	-	0.813
	27	-	2.087
	29	-	1.854
Animal DB3	33	6	2.468
	34	7	0.811
	38	11	1.343
Fifth passage			
Animal DB63	30	-	1.483

FIGURE 40

The variation in resistance, as indicated by the ED_{50}, during the second passage of the HFRO benzimidazole resistant strain of O. circumcincta in sheep DB16. The animal was treated with fenbendazole (FBZ) on day 31 post infection

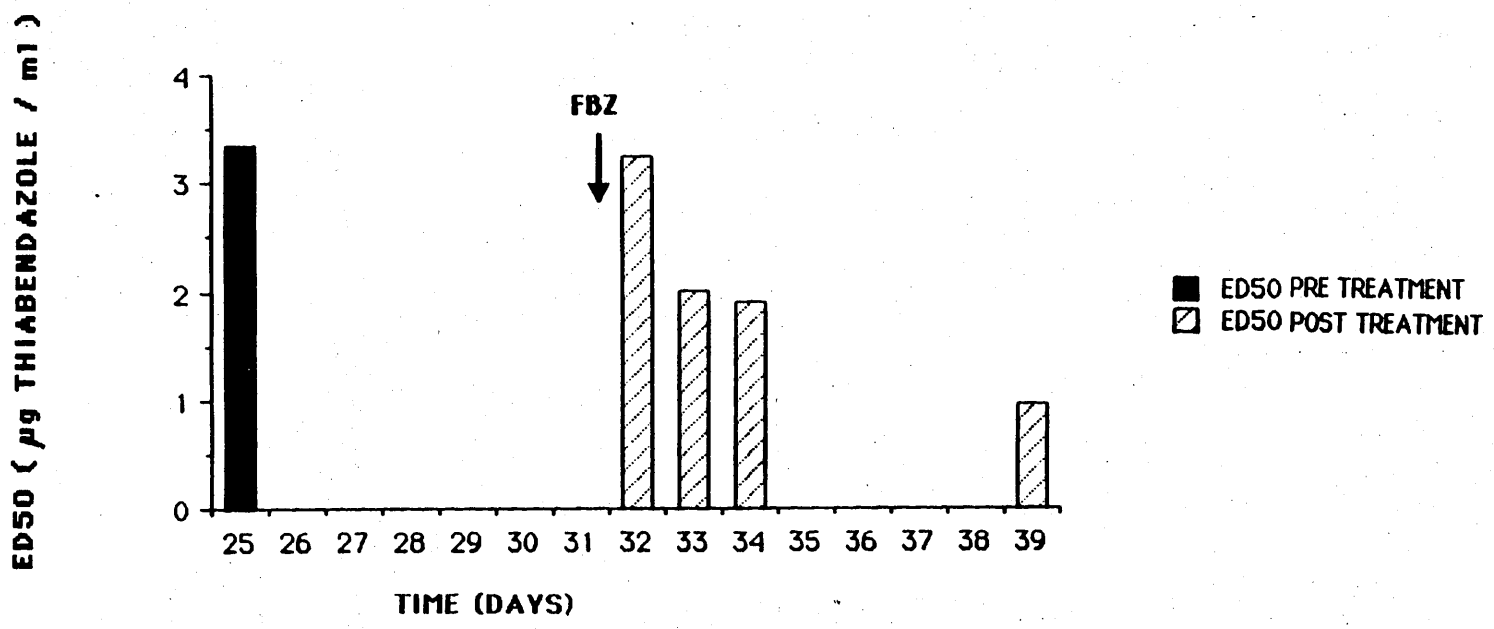

ED50 (µg THIABENDAZOLE / ml)
4
3
2
1
0
FBZ
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
TIME (DAYS)
ED50 PRE TREATMENT
ED50 POST TREATMENT

FIGURE 41

The variation in resistance, as indicated by the ED_{50}, during the third passage of the HFRO benzimidazole resistant strain of O. circumcincta in sheep DB62. The animal was treated with fenbendazole (FBZ) on day 28 post infection

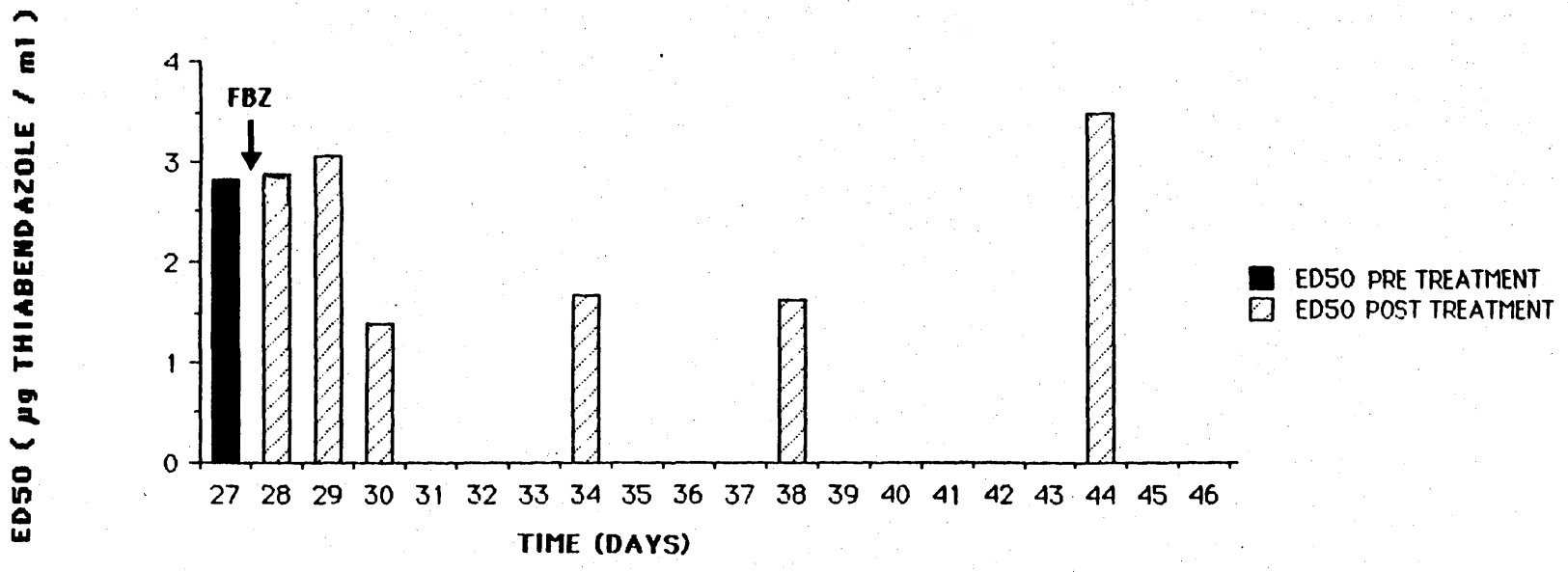

ED50 (µg THIABENDAZOLE / ml)
4
3
2
1
0
FBZ
27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46
TIME (DAYS)
ED50 PRE TREATMENT
ED50 POST TREATMENT

FIGURE 42

The variation in resistance, as indicated by the ED_{50}, during the third passage of the HFRO benzimidazole resistant strain of O. circumcincta in sheep DB25. The animal was not treated with fenbendazole.

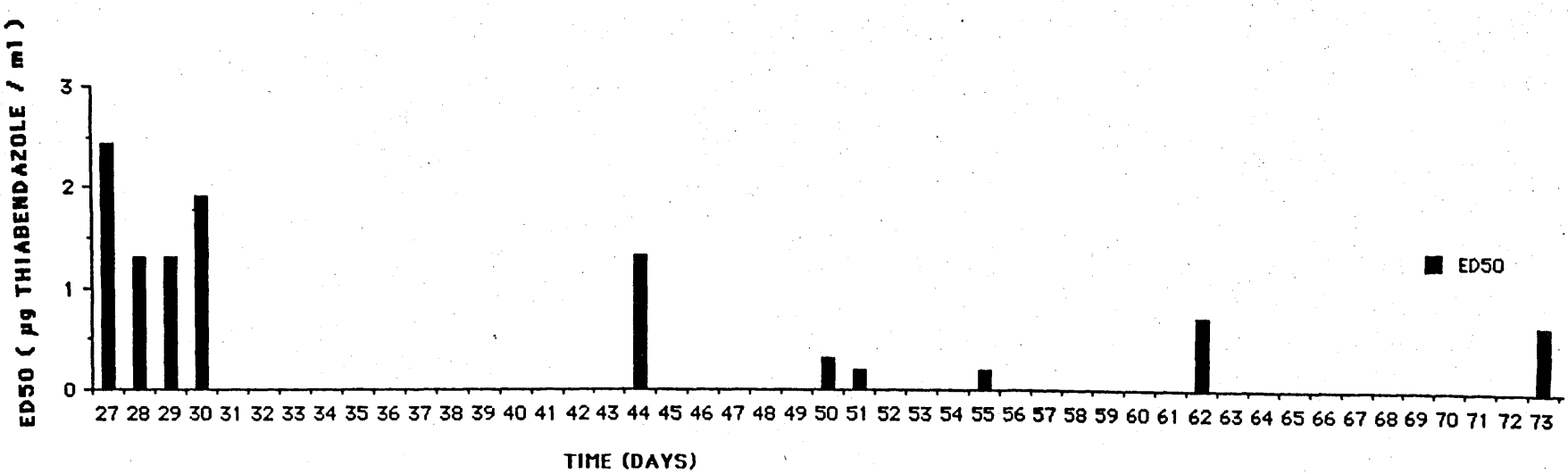

ED50 (µg THIABENDAZOLE / ml)
3
2
1
0
27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73
TIME (DAYS)
ED50

FIGURE 43

The variation in resistance, as indicated by the ED_{50}, during the fourth passage of the HFRO benzimidazole resistant strain of O. circumcincta in sheep DB5. The animal was treated with fenbendazole (FBZ) 32 days post infection

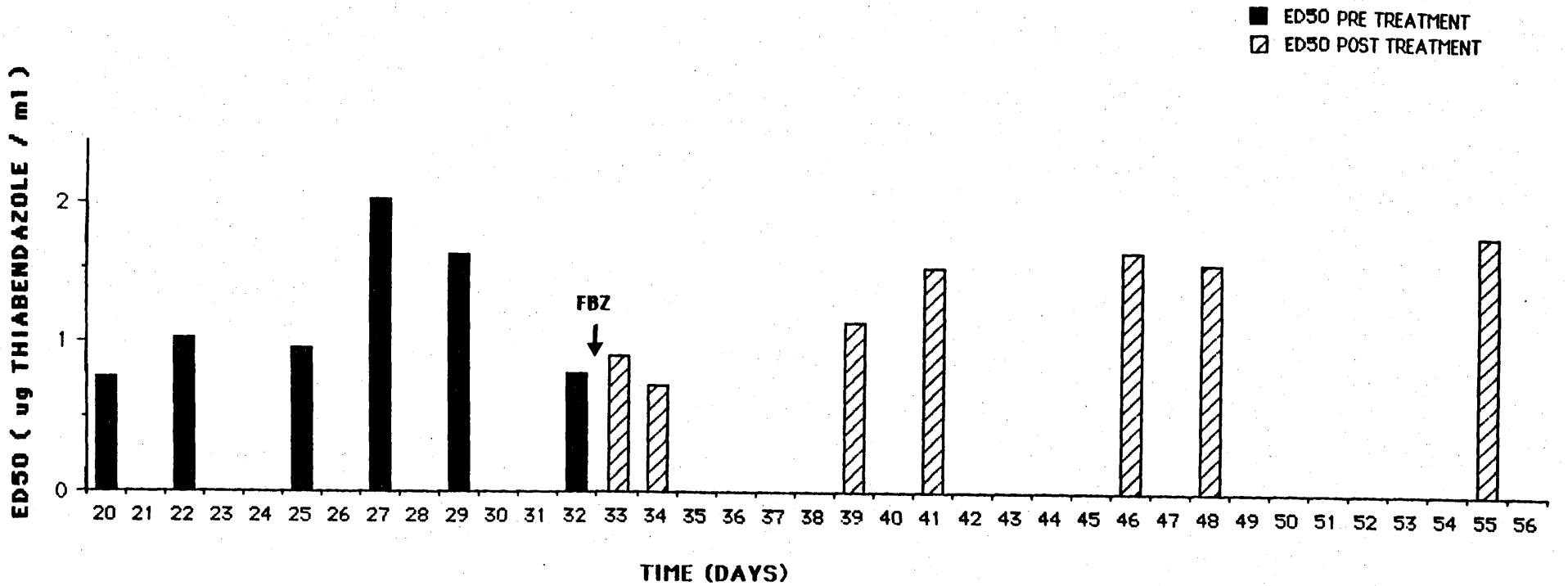

ED50 PRE TREATMENT
ED50 POST TREATMENT
ED50 (ug THIABENDAZOLE / ml)
0
1
2
FBZ
20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
TIME (DAYS)

TABLE 60

The ED_{50} (ug thiabendazole/ml) obtained from egg hatch assays with a benzimidazole resistant strain (HFRO) of Ostertagia circumcincta after storage at 4°C for four months

Animal	Time (days) post infection	ED_{50} (ug thiabendazole/ml)
DB4	22	1.416
	30	1.546

TABLE 61

The ED_{50} (ug thiabendazole/ml) obtained from egg hatch assays with a benzimidazole resistant strain (Moredun) of Ostertagia circumcincta

Animal	Time (days) Post infection	Post treatment	ED_{50} (ug thiabendazole/ml)
B2	26	-	1.289
	28	-	1.480
	35	-	1.292
	40	-	1.006
	41	1	1.039
	42	2	0.675
	43	3	1.000
	47	7	1.485
	49	9	2.035
	54	14	2.041

FIGURE 44

The variation in resistance, as indicated by the ED_{50}, during an infection of sheep B2 with the Moredun benzimidazole resistant strain of O. circumcincta. The animal was treated with fenbendazole (FBZ) 40 days post infection

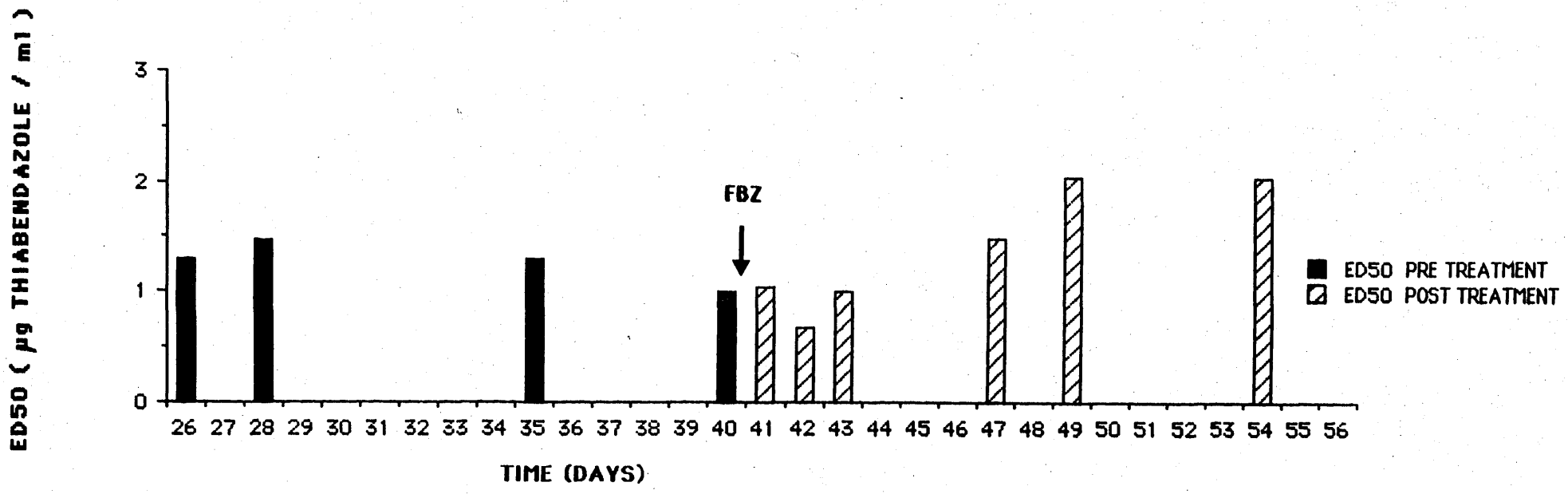

ED50 (µg THIABENDAZOLE / ml)
3
2
1
0
FBZ
26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
TIME (DAYS)
ED50 PRE TREATMENT
ED50 POST TREATMENT

TABLE 62

The level of resistance as determined by the ED_{50} obtained from egg hatch assays of a benzimidazole resistant HFRO strain, a benzimidazole resistant Moredun strain and a benzimidazole susceptible Bearsden strain of *O. circumcincta*

		ED_{50} (ug thiabendazole/ml)	Mean ± SEM
HFRO resistant strain	1st passage	1.527	
	5th passage	1.483	1.475 ± 0.03
	Stored larvae	1.416	
Moredun resistant strain		1.289	
Bearsden susceptible strain		0.164	

GENERAL DISCUSSION

The investigation of pharmacological mediators important in helminth infections of animals has become an increasing area of research, particularly as the assay of such mediators has become possible.

This work has centred on histamine, 5-hydroxytryptamine, prostaglandins and leukotrienes, looking at their effects on the parasites themselves *in vitro*, the effect on isolated gastrointestinal smooth muscle *in vitro* and inhibition of certain mediators on the responses of the host to parasites, *in vivo*.

Prostaglandins and leukotrienes appear to have inhibitory actions on *O. circumcincta* larvae (L_3) *in vitro*, which makes their presence in gastrointestinal mucus important in host protection.

The possibility of some substances causing changes in smooth muscle responsiveness and in that way, perhaps promoting parasite loss, or work in the parasites favour to aid their establishment was also investigated *in vitro*. Generally, ruminant smooth muscle was unresponsive compared to other tissues used in these systems. Prostaglandins did cause small degrees of contraction, the other mediators tested generally did not cause any response.

The study to inhibit prostaglandin production and monitor host changes and parasite establishment and administration of meclofenamic acid was difficult to interpret due to the possible direct effect of meclofenamic acid on the parasites. Parasite establishment was reduced in the treated animals. The plasma pepsinogen response, particularly the "hypersensitive" response seen in immune sheep did not seem to be altered, making it

unlikely that prostaglandins are involved in this.

The role of other components in the parasites' environment were studied by using drugs to alter the gastrointestinal mucus, the abomasal pH and generally affect the host's immune system with corticosteroids. The response to a raised abomasal pH was very interesting in that the plasma pepsinogen activity did not show the same pattern as would be expected in adult animals allowing only a small number of parasites to establish. This work warrants further study. The use of bromhexine hydrochloride as a mucolytic, in retrospect, was not ideal in that its effect on the mucus of the gut was not clearly established. Possibly a locally applied mucolytic into the ovine abomasum and subsequent challenge might allow a more exact measurement of the effects of disrupting the mucus layer.

The problems encountered working in ruminants, only serves to underline the difficulty of extrapolating results obtained with laboratory species. Much work will have to be repeated in these economically important species to determine the relevance of work already carried out using rats and mice etc.

The area of parasite factors altering its environment is an aspect of pharmacological mediation not dealt with in this thesis.

The final section of this thesis dealt with a study into a resistant strain of O. circumcincta isolated in Britain. A great many questions need to be answered about inheritance of resistance and factors important in this country in resistance development. The strain studied was highly resistant to

benzimidazole anthelmintics. The egg hatch assay was shown to be a convenient method of estimation of level of resistance, however care must be taken in its interpretation as, at a few points, the level of resistance was found to be very low, returning to higher levels later. This underlines the need for repeated assays during infection. This work was part of a much larger study that is still continuing.

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