STUDIES ON OVINE POLIOENCEPHALOMALACIA

by

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A thesis presented for the degree of Doctor of Philosophy

in the University of Edinburgh, 1975.

CONTENTS.

Acknowledgements.

I wish to thank all the people who made this work possible, with especial thanks to Dr. R.M. Barlow and Dr. G.H.K. Lawson for advice and supervision. I am grateful to Miss P. Wooding and Mr. E.W. Gray for technical instruction, Mrs. S.F. Cairns and Mr. G. Newlands for technical assistance, and Mr. J. Wight, Mr. B.J. Easter and Mrs. P. Gray for the preparation of photographs. I also wish to thank Dr. B.S.W. Smith, Dr. N.S. Suttle, Mr. W. Smith, Mr. H. Wright, Dr. D.L. Mould and Mr. C.C. Renwick for their advice and assistance. I am indebted to Miss S. Fulton and Miss M. Mitchell for preparation of the type-script.

Abstract.

Polioencephalomalacia (PE), also known as Cerebrocortical Necrosis, is a neurological disease of several ruminant species including sheep and cattle, which is diagnosed on the basis of characteristic brain lesions. Prior to the present investigation it had been established that brain lesions similar to those of natural PE, may be produced in calves and sheep by the oral administration of the thiamine antagonist amprolium. The similarity of these encephalopathies at the light microscopic level was considered to be evidence that the natural disease may be a consequence of thiamine antimetabolite production in the rumen of affected animals. Support for this proposal was then further provided by the demonstration, in the rumen of affected sheep and cattle, of an enzyme capable of producing such antimetabolites, thiaminase type I.

In this thesis the mechanisms responsible for the brain lesions of natural PE are examined, and compared with those of amprolium poisoning encephalopathy, at the ultrastructural level. The distribution of thiaminase type I, the source of the enzyme, and reasons for its accumulation in the gut contents of sheep have also been investigated.

Of 21 sheep suffering from PE, diagnosed by identification of characteristic brain lesions, 19 were found to have thiaminase type I activity in their rumen contents. Two cases of PE, which did not have detectable thiaminase activity, indicated that factors other than impaired thiamine metabolism may occasionally be responsible for the lesions.

Ultrastructural studies of the brains of PE cases revealed that the primary morphological change in the cerebral cortex is oedema of astrocytes, and that degeneration of other cortical elements is probably secondary. No changes were observed consistently in tissues other than the brain.

Intoxication of pre-ruminant lambs with amprolium resulted in a complex syndrome after 3 to 4 weeks treatment, one feature of which was brain lesions resembling those of natural PE. Electron-microscopy of the cerebro-cortical changes demonstrated that the primary morphological change was again swelling of astrocytes. This similarity to PE was considered to provide further evidence for the implication of thiamine antimetabolites in the aetiology of the natural disease.

In addition to the encephalopathy, amprolium-poisoned lambs had diarrhoea, in common with some cases of natural PE, but investigations failed to establish the mechanisms responsible. In the terminal stages of the intoxication these lambs also developed extensive systemic haemorrhages, which predominated in actively mobile tissues. The haemorrhages were attributed to a thrombocytopenia demonstrated during the 2 to 3 days prior to the onset of neurological disturbance. Consequent examination of the bone-marrow revealed severe degeneration of megakaryocytes, and this was considered to account for the reduced numbers of circulating platelets. Cellular depopulation of the bone-marrow, also observed in the amprolium treated animals, was associated with reduced numbers of mitotic figures in

bone-marrow cells, suggesting that amprolium may have arrested cell division in this tissue. Bone-marrow changes have not been reported in natural PE.

The brain lesions of amprolium poisoning were preceded by decreased cerebro-cortical transketolase activity, indicating that they may be a consequence of impaired thiamine metabolism in this area of the brain. In spite of the differences observed between amprolium poisoning and natural PE, it was concluded from this study that the overall similarity of the 2 conditions supported the implication of thiaminase type I in the cause of the natural disease.

Thiaminase assays were carried out using a ^{14}C-labelled-thiamine method devised by other workers, but modified by the author to improve its sensitivity. Faecal thiaminase estimations of clinically normal animals, in flocks involved in outbreaks of PE, demonstrated that this enzyme may be wide-spread. Over half of the lambs examined in one such flock showed faecal thiaminase activity on at least one occasion during a 5 week period. In three groups of sheep the administration of certain anthelmintics, which are known to react with thiamine in the 'thiaminase type I reaction', appeared to induce further cases of PE.

Of a small number of clinically normal sheep with ruminal fistulae, some showed intermittent ruminal thiaminase which appeared as peaks of activity of 3 to 10 days duration, and in one animal peaks were repeated during a period of several months. Thiaminase was not detected in the faeces of these sheep even when ruminal enzyme activity was high, which indicates that faecal thiaminase studies

could under-estimate the 'thiaminase status' of flocks. It was concluded from this work, that PE is a clinical manifestation of a much more extensive condition, characterised by the presence of thiaminase type I in the alimentary tract.

Samples taken from feedstuffs in use during outbreaks of PE showed no evidence of thiaminase, which suggests that in the outbreaks investigated the enzyme was probably not ingested by the sheep but synthesised in the alimentary tract.

A subsequent search for the source of thiaminase in the rumen contents and faeces of sheep affected with PE resulted in the isolation of bacteria which produced thiaminase type I activity in vitro. After taxonomic studies they were considered to be strains of Bacillus thiaminolyticus. The thiaminases produced by these organisms were found to have similar co-factor specificities to the naturally-occurring enzymes and they mediated in the base-exchange reaction implicated in the aetiology of PE. It was thus proposed that thiaminase type I present in sheep may be synthesised by strains of B. thiaminolyticus. This represents the first reported account of the isolation of B. thiaminolyticus in Britain.

Attempts to confirm that these organisms are responsible for the presence of thiaminase, by the administration to sheep of ovine strains of B. thiaminolyticus, failed to influence ruminal activity of this enzyme. Subsequent studies demonstrated that these organisms are inhibited by a range of other bacterial species, and by a number of volatile fatty acids (VFAs).

Concurrent studies by other workers have resulted in

the isolation of strains of Clostridium sporogenes from sheep affected with PE. It was found in the present investigations that these organisms, which produce thiaminase type I similar to that of B. thiaminolyticus, are also inhibited by the same bacterial species and VFAs as B. thiaminolyticus. It was therefore concluded that a similar range of influences on rumen function could control thiaminase type I production, and consequently the occurrence of PE, irrespective of which of these 2 bacterial species was involved.

SECTION I.

LITERATURE REVIEW.

Polioencephalomalacia (PE) was first described in the USA in 1956 (Jensen, Griner and Adams), and next in New Zealand in 1958 (Mullins, Hartley and Salisbury). An almost identical condition was reported in Britain in 1959 (Terlecki and Markson) and given the name Cerebro-cortical Necrosis (CCN). Zlotnik, Nisbet and Campbell (1963) proposed that PE is more appropriate and recently a number of workers have used the two names as synonyms (Markson and Terlecki, 1968; Little, 1969; Edwin and Lewis, 1971). At present the disease is referred to as CCN in Britain and certain European countries (Wegner and Wegner, 1969; Sinha, 1971), whilst PE (or PEM) is generally used elsewhere (Little, 1969). In an attempt to avoid further confusion the term PE has been adopted for the present work.

PE was first described as an acute neurologic condition of cattle and sheep (Jensen, Griner and Adams, 1956), but the clinical picture varies considerably in severity and rate of onset (Terlecki and Markson, 1961). In sheep, symptoms usually commence with aimless wandering, with or without circling, followed by a rapidly progressive ataxia, frequently accompanied by partial or complete blindness. At this stage the animal may stagger and sway or stand with feet wide apart and with its head raised or drawn back in convulsive spasms (Terlecki and Markson, 1961). There is often nystagmus, trismus and hypersensitivity followed by recumbency when the animal may lie motionless for long

periods or show intermittent violent galloping movements. Affected animals become gradually weaker, the opisthotonus persistent and more severe followed by coma and death (Terlecki and Markson, 1961).

The dramatic clinical signs are associated with equally dramatic brain lesions. The most consistent feature is malacia of the cerebral cortex, but other areas, including the thalamus, hippocampus, mesencephalon, cerebellum and medulla may also be involved (Little, 1969). Gross examination may reveal swelling of the cerebral gyri, particularly the dorsal and dorsolateral regions, which are often slightly soft, and discolouration and softening may also be detectable in the cerebellum (Terlecki and Markson, 1961).

Histological examination of the brains of sheep which have died within 24 hours of the onset of symptoms, reveals that the cerebral lesions are fairly constant. These appear as focal or laminar areas of spongy texture involving part or all of the thickness of the cortical grey-matter. The spongy texture is attributable to enlarged peri-vascular spaces, perineuronal vacuolation and a general looseness of the matrix (Terlecki and Markson, 1961).

Most neurons in the centre of affected areas are shrunken and angular, sometimes triangular in outline with strongly acidophilic cytoplasm. Small perivenular haemorrhages are not uncommon, whilst meningeal blood vessels, particularly over the affected parts of the cortex, are frequently congested. The meninges may be moderately infiltrated with lymphocytes, monocytes and occasionally neutrophils (Terlecki and Markson, 1961).

In animals which have survived longer than a day there is progressive disruption and disintegration of the affected areas but capillary beds remain largely intact, and there may be an apparent increase in the number of capillaries present (Terlecki and Markson, 1961). There is often marked infiltration by macrophages which commences in the submeningeal zone. No abnormality has been found in the spinal cord (Terlecki and Markson, 1961).

The lesions are bilateral but not always symmetrical, and vary in severity from case to case and within the same brain. The correlation between the histopathological changes and the duration and severity of symptoms is not always clear (Terlecki and Markson, 1961) and the morphogenesis of these lesions has not yet been determined (Markson and Terlecki, 1968).

Lesions are found consistently only in the brain, although epicardial haemorrhages and an excess of pericardial fluid are frequently observed (Little, 1969). Myocardial degeneration has been described in some outbreaks (Jensen, Griner and Adams, 1956; Zlotnik, Nisbet and Campbell, 1963; Little, 1969). Diarrhoea may also be a feature of some outbreaks of PE in sheep (Little, 1969), but no specific pathological changes have been associated with it.

PE occurs sporadically and the true incidence and distribution of the disease in Britain has not been established. In the only epidemiological study to be published in Britain it was reported that most ages of sheep are susceptible with cases ranging from 6 weeks to 7 years of age, and morbidity from 1 to 6% with 98% of affected

animals dying (Spence, Stevens, Saunders and Harris, 1961). This disease has been reported in almost every country of the world, and it represents a major problem in some states of the USA (Little, 1969). It has also been described in several ruminant species besides cattle and sheep, including goats, antelope and deer (Little, 1969). Although PE was not described until 1956, Little (1969) considers that it may have occurred in New Zealand and Canada prior to this time.

The cause of PE remained a complete mystery for nearly 10 years after the first report, but there was considerable speculation on this subject (Markson and Terlecki, 1968). In 1965, Davies et al found that the parenteral administration of thiamine to a calf affected with PE produced a rapid clinical improvement. There have since been many reports of the value of thiamine in the treatment of PE (Little, 1969) but this therapy is less effective in sheep than in cattle (Markson and Terlecki, 1968). The latter workers postulated that this may be attributable to the more extensive involvement of the thalamus and cerebellum in sheep.

Affected animals were shown to have low tissue thiamine concentrations and biochemical examination of the blood revealed evidence of impaired thiamine metabolism (Pill, 1967). Lambs reared on a thiamine deficient diet develop severe diarrhoea associated with neurological disturbance (Draper and Johnson, 1951), but they fail to show the characteristic and severe brain lesions of PE (Lewis et al, 1967). Calves reared on similar diets also develop severe

diarrhoea, but show no clinical or pathological evidence of neurological disturbance (Little, 1969). These findings suggest that the disease does not represent a state of simple thiamine deficiency (Edwin and Lewis, 1971).

Further progress was made by Pill et al. (1966) when they administered the thiamine antagonists pyrithiamine and amprolium to a pre-ruminant calf and produced a condition "indistinguishable" both clinically and pathologically, from natural PE. The administration of amprolium to sheep has also been found to result in lesions resembling those of PE (Sinha, 1971; Loew and Dunlop, 1972). Therefore, whilst it has not been possible to induce PE-like lesions by dietary thiamine deficiency, the administration of thiamine analogues is capable of initiating such lesions.

After an examination of the factors involved it was postulated by Edwin, Lewis and Allcroft (1968) that thiaminase type I might be associated with this condition. Subsequently thiaminase was demonstrated in the ruminal contents of calves and sheep affected with PE (Edwin, Spence and Woods, 1968), and was found to be of type I (Edwin and Jackman, 1970). Pill et al. (1966) postulated that a thiamine antagonist could be responsible for PE and it was concluded by Edwin, Lewis and Allcroft (1968) that such a compound could be produced in the rumen, from thiamine in the presence of a suitable co-factor, by the action of thiaminase type I.

Two types of thiaminase, I and II, have been recognised (Fujita, Nose and Kuratina, cited by Edwin and Lewis, 1971), which catalyse different reactions involving thiamine. The structure of this vitamin is shown in Figure 1, and it

is seen to consist of a pyrimidine ring joined by a methylene bridge to a thiazole ring.

The mode of action of thiaminase II is a hydrolytic fission of the pyrimidine and thiazole moieties of thiamine, at the methylene bridge, to yield free thiazole and pyrimidine molecules.

Thiaminase I, however, mediates in a base exchange reaction and requires an amine as a co-substrate, without which no reaction occurs. In this reaction the co-substrate is substituted for the thiazole portion of the thiamine molecule, producing a 'pyrimidinyl-co-substrate product' as shown in Figure 1. For the reaction shown in Figure 1, alpha-picoline was selected as the co-substrate, as it results in the production of a compound very similar in structure to amprolium (Figure 2).

Edwin, Lewis and Allcroft (1968) succeeded in isolating picoline-like bases from rye grass, and proposed that the production in the rumen of compounds similar to amprolium, by the action of thiaminase type I, could cause PE. These compounds would presumably act in a similar manner to amprolium, by competing with thiamine in metabolic reactions because of the similarity of their molecular structures to that of thiamine (Edwin, Lewis and Allcroft, 1968). The proposal that this would be due to competitive inhibition is supported by the observation that the administration of thiamine may prevent the effects of amprolium in calves (Markson et al., 1972). However the nature of the thiamine antagonist responsible for natural PE has yet to be established.

Naturally occurring thiaminases have been detected in a wide range of situations including a variety of plants

(Murata, 1965). In a search for the source of the thiaminase associated with outbreaks of PE, Edwin, Lewis and Allcroft (1968) detected this enzyme in materials such as rye grass, decaying wood, mouldy barley and concentrates obtained from farms on which the disease had occurred. It has not been established whether ingested thiaminase from such sources provides the thiaminase activity present in gut contents of affected animals. Bracken contains high levels of thiaminase type I activity, but it has not been implicated in outbreaks of PE (Little, 1969). Loew, Smith and Dunlop (1972) failed to demonstrate thiaminase production by fungi isolated from mouldy straw associated with outbreaks of PE, and the source of this enzyme in such outbreaks has yet to be found.

OUTLINE OF THE WORK.

The investigations described in this thesis are presented in 4 sections. In the first (Section II) sheep affected with PE were examined for the presence of alimentary thiaminase type I, and the cerebro-cortical changes of this disease were examined by light and electron-microscopy in order to establish the mechanisms responsible for their characteristic appearance. The brain lesions produced by amprolium intoxication of lambs were studied and compared with those of natural PE at the ultra-structural level (Section III, part one). The biochemical mechanisms responsible for 'amprolium poisoning encephal-opathy', were examined, along with further studies of the haemorrhagic and diarrhoeic syndromes found to be associated with this experimental condition (Section III, part two). Attempts to assess the extent of the distribution of thiaminase type I in clinically normal animals are presented (Section IV), followed by attempts to establish the source of this enzyme in sheep (Section V, parts one to four).

SECTION II.

PATHOLOGICAL AND BIOCHEMICAL STUDIES OF NATURAL OVINE POLIOENCEPHALOMALACIA.

Introduction.

Animals affected with Polioencephalomalacia (PE) have characteristic brain lesions (Terlecki and Markson, 1961) and thiaminase type I activity may be demonstrated in their rumen contents (Edwin and Jackman, 1970). This section consists of an investigation of field cases of ovine PE, in which detailed consideration is given to the distribution, histochemistry and ultrastructure of the brain lesions with a view to extending our understanding of the pathogenesis of the disease. Thiaminase estimations were also conducted to establish whether the presence of this enzyme in the rumen contents represents a consistent feature of the condition.

Several methods for the assay of thiaminase activity are available, but that described by Edwin and Jackman (1970) provides a very sensitive means of demonstrating the enzyme in crude preparations of rumen contents, faeces or broth cultures and large numbers of such samples may be handled readily (Edwin and Jackman, 1974). This method was modified for improved sensitivity and economy in the use of substrate.

Materials and Methods.

Source of Materials.

Fixed brain material, from animals with a range of

neurological disorders, was available through the Moredun Institute Neuropathological Diagnostic Service. Live sheep suffering from central nervous system disturbance were obtained via the Scottish Veterinary Investigation Service and the Moredun Institute flocks.

Selection of PE Cases.

Fixed Brain Material. The cerebro-cortical changes, described by other workers as characteristic of PE (see literature review), were accepted as diagnostic of the disease, and were used to differentiate it from other conditions.

Live Sheep. The majority of animals were initially selected for study when found to be exhibiting clinical symptoms similar to those described by other workers in PE. However, the diagnosis was ultimately dependent upon the detection of the cerebro-cortical lesions considered characteristic of the disease.

Routine Pathological Procedures.

Formalin Fixed Brain Material. Areas of the brain examined were those routinely prepared for diagnostic purposes. After 3 to 6 weeks immersion fixation in 10% formol-saline, coronal sections were cut at the following levels:- head of the caudate nucleus, infundibulum, hippocampus, superior colliculus, mid-cerebellar peduncle, mid-pons, and medulla at the area postrema. A sagittal block adjacent to the mid-line, was cut from the posterior half of the cerebellum. The blocks were then immersed in saturated mercuric chloride for 48 hours, and processed

through graded alcohols, toluene, soft and hard wax. Six μm-thick sections were cut and stained with haematoxylin and eosin for light microscopy.

This material was used to provide sections for a study of the lesion distribution and severity in 44 cases of PE (Table 1), each being scored by a subjective assessment of lesion 'severity' in the cerebral-cortex, hippocampus, thalamus, superior colliculi and cerebellum.

Sheep Affected with PE. Material was obtained from 21 cases of PE and 11 clinically normal sheep (Table 2) and used as follows:- Prior to post-mortem animals were anaesthetised with sodium pentobarbitone and exsanguinated.

Brain: The brains were prepared for routine light microscopy unless alternative fixation was required for special procedures (see below).

Other Tissues: A post-mortem examination was carried out, but histological examination was confined to 8 cases for which the following organs were used; thyroid, heart, somatic muscle (longissimus dorsi), liver, spleen, kidneys, adrenals, abomasum, duodenum, lower ileum, mesenteric and mediastinal lymph-nodes and the eyes. These tissues were fixed for 4 to 5 weeks in 10% formol-saline, with the exception of the eyes which were fixed for 6 weeks in Davidson's fluid (Appendix No.1), and then processed as for the brain.

Other Samples: Rumen contents and faeces were collected at post-mortem and stored at -80°C prior to thiaminase assay.

Thiaminase Assay Procedure.

The assay method of Edwin and Jackman (1970) depends

on the release of ^{14}C-labelled thiazole from labelled thiamine, in the thiaminase reaction. The released thiazole is extracted into ethyl acetate, in which thiamine is almost insoluble, and the ^{14}Carbon activity in the extract is estimated by liquid scintillation counting.

The thiaminase type I reaction requires a co-factor, for which Edwin and Jackman recommended nicotinic acid, at a concentration of 36 μg/ml (0·29 mM) of substrate solution. In the present study this was increased to 500 μg/ml (4·06 mM) which resulted in improved sensitivity, the rationale for which is presented later.

Buffer Preparation. The buffer was made by adding 0·1 M citric acid to 0·1 M disodium hydrogen orthophosphate, with pH adjustment to 6·4 using a Pye Unicam pH meter. This will be referred to as citrate/phosphate buffer.

Sample Preparation. Rumen liquor mixed with an equal weight, and faeces samples with 3 times their weight, of citrate/phosphate buffer, were left to extract at 4°C for 18 to 24 hours, and then centrifuged at 4000 g for 15 minutes. Very viscous samples were centrifuged at 18,000 g for 1 hour at 4°C. The supernates were then assayed.

Substrate Solution. A stock solution containing 10 mg thiamine (thiazole-2-Carbon 14)* and an activity of 5 μCi/ml was diluted for use 1:100 with buffer and nicotinic acid added to give a concentration of 500 μg/ml. To study the effect of the nicotinic acid concentration on the reaction, substrate solutions were also prepared with a range of concentrations of this co-factor. These solutions were stored at -20°C and thawed prior to use.

* The Radiochemical Centre, Amersham.

Assay Procedure. 0·25 ml of the solution to be assayed was mixed by agitation with an equal volume of substrate solution in a test tube, and incubated for 1 hour at 37°C in a water bath. One ml of ethyl acetate was then added and the tube shaken vigorously for 1 minute, centrifuged at 4,000 g for 5 minutes and 0·5 ml of the upper ethyl acetate phase transferred to 10 ml of NE 233 scintillation fluid. The amount of activity present as Carbon-14 in the extract was estimated in an automatic liquid scintillation counter (Nuclear Enterprise, 8310 Edinburgh) set to count for a minimum of 300 seconds per sample. The absolute activity as disintegrations per second (DPS) was calculated using an External Standardisation correction for variation in counting efficiency by means of an appropriate computer programme.

Blanks.

Heat Killed Sample Blanks: 1 ml aliquots of the rumen content or faeces extracts were heated to 100°C for 30 minutes to destroy enzyme activity, and then assayed.

Buffer/Substrate Blanks: Buffer was substituted for the sample in the assay procedure.

Thiaminase Standard.

This was provided by the use of supernate from a nutrient broth culture (24 hours at 37°C) of Bacillus thiaminolyticus (NCTC 10760) prepared as described in Section 5, page 76. This supernate was stored in 2 ml aliquots at -80°C and thawed prior to use in the assay procedure.

Calculation of Units of Thiaminase Activity.

The thiaminase unit adopted was similar to that recommended by Edwin and Jackman (1974), and was calculated from the DPS of the sample assay, minus the DPS of a heat killed blank for that sample (100°C for 30 minutes) divided by a 'conversion factor' of 3·42. The calculations from which the conversion factor derived are presented in Appendix No.2.

One unit of thiaminase activity was considered as that which will yield 1 x 10^{-1} nmoles thiazole ml^{-1} $minute^{-1}$ at 37°C using the conditions of assay described above and a nicotinic acid concentration of 500 µg/ml of substrate solution, unless otherwise stated.

Selection of Nicotinic Acid Concentration in the Final Substrate Solution.

A 1:4/3 dilution of the thiaminase broth supernate was assayed with substrate solutions containing 1000, 500, 250, 125, 62·5, 31·3, 15·6 and 7·8 µg nicotinic acid/ml and an incubation or 1 hour at 37°C. The nicotinic acid concentration was found to limit the amount of thiazole released up to approximately 500 µg/ml and at this concentration the activity in the ethyl acetate extract was about 4 times that achieved with 36 µg/ml (Figure 3). Substrate solutions containing 500 µg nicotinic acid/ml were tested with various enzyme concentrations and periods of incubation. The reaction was found to be roughly linear for time of incubation up to 2 hours (Figure 4), and thus 1 hour incubation was considered suitable for the assay. The reaction was also found to be linear for enzyme

concentration up to a limiting level of thiaminase activity of about 32 units (Figure 5). This limit was possibly due to substrate exhaustion in the presence of high thiaminase activity. Thus to obtain an accurate assessment of thiaminase activity in such preparations a series of dilutions would be required. In the present study samples with activity in excess of 32 units were reported as such (>32 units).

Confirmation that Thiaminases were of Type I.

Thiaminase type II will cleave thiamine on its own, whilst the type I enzyme requires the presence of one of a range of co-factors for the reaction to proceed. Thus thiaminase type I may be distinguished by the exclusion of such co-factors (eg nicotinic acid, pyridine, alpha-picoline) from the reaction mixture; this will result in loss of activity which may be restored by the addition of a co-factor. Assays of enzyme preparations were therefore conducted in the absence of co-factors by a) the exclusion of nicotinic acid from the substrate solution and b) dialysis of such substances out of the preparations as follows:- 2 to 5 ml aliquots of rumen liquor or faecal extracts were dialysed in Visking dialysis tubing, against 3 changes of 500 ml citrate/phosphate buffer over a period of 48 hours at 4°C.

Methods Used in Further Studies of the Cerebro-cortical Lesion.

Special Histological Stains.

These methods were applied to sections of the parietal cerebral cortex of sheep PE/4, 5, 7 and C/3, 4, 5, details of which are presented in Table 2. The methods

applied included the following:-

From Histological Technique (Drury and Wallington, 1967):

Cajal's Gold Chloride-Sublimate method for astrocytes.

Holmes' Method for axons.

Holzer's Method for fibrous glia.

Penfield's Method for neurologia.

Periodic acid-Schiff Reaction with and without Diastase.

Weil-Davenport Method for microglia.

Luxol Fast Blue method for myelin.

From Selected Histochemical and Histopathological methods (Thompson, 1966):

Ninhydrin-Schiff reaction for free alpha amino acids.

Enzyme Histochemical Techniques.

These were applied to sections of parietal cerebral cortex from sheep PE/12, 14, 17, 18 and C/6 and 7 (Table 2). Two to 3 mm-thick coronal sections were cut and immersed for 24 hours in cold neutral Baker's formol and processed by the following methods, of which the first 2 were derived from 'An Introduction to Histochemical Technique', (Bancroft, 1967);

Acid Phosphatase. Gomori Lead Method.

Alkaline Phosphatase. Gomori Calcium Method.

Thiamine Pyrophosphatase. Lead Nitrate Method of Novikoff and Goldfischer (1961).

Electron Microscopy. This work was confined to studies of the early malacia in 2 affected sheep (PE/1 and 3), whilst 2 normal sheep (C/1 and 2) were used to provide control material (Table 2). Both affected animals had thiaminase

type I activity in the rumen liquor but the controls were not assayed. The brains were prepared for electron microscopy as follows:-

Intravenous heparin was administered at the rate of 0·5 mg/kg body weight, in aqueous solution (5 mg/ml). The animal was then anaesthetised with sodium pentobarbitone, laid on its back, the common carotid arteries exposed by blunt dissection through a long cutaneous incision and a 50 mm segment of one artery gently clamped at each end. A metal cannula was inserted in this portion of the artery and tied in with braided nylon. The clamps were removed from the vessel and the perfusion fluid, consisting of 3% glutaraldehyde in 0·1 M sodium cacodylate buffer pH 7·4, was run into the cannula via a drop chamber from a height of 100 cm above the cannula. The jugular vein was then incised and a similar procedure carried out on the carotid and jugular vessels of the opposite side.

This procedure resulted in a continued supply of blood to the brain via one carotid and the circle of Willis until perfusion fluid was introduced, thus minimising the risk of cerebral ischaemia prior to fixation. The head was left on the body throughout and four litres of perfusate used for each animal.

The brain was removed 1 hour later and cut into 5 mm-thick coronal slices. Alternate blocks of the cerebral cortex were transferred to fresh perfusion fluid, and from the remaining tissues, selected material was given 2 hours fixation in du Bosq fixative at 60°C, and embedded in paraffin wax. Six μm-thick sections were stained with

haematoxylin and eosin (H and E) and examined by light microscopy within 18 hours. On the basis of these observations areas from the tissue stored in perfusion fluid for 18 to 24 hours, were selected for araldite embedding. Small blocks, approximately $1mm^3$ were cut and immersed for 2 hours in fresh perfusate, washed for 24 hours in cacodylate buffer at pH 7·4, post-fixed in 1% osmium tetroxide in cacodylate buffer, and embedded in araldite. One µm-thick sections were stained at 60°C in Giemsa stain for examination by light microscopy. Ultrathin sections were cut with glass knives on LKB and Reichert ultratomes, stained with lead citrate and examined and photographed in a Siemens Elmskop I electron microscope.

Results.

Pathology of Fixed Brain Material from the Diagnostic Service.

This material was often accompanied by an incomplete history but PE was generally suspected on the basis of clinical symptoms. A diagnosis of PE was derived from the appearance of brain lesions resembling those described by other workers in cases of the disease. The severity of these changes and their distribution varied considerably, and the lesions in the cerebral cortex and other areas of the brain are described below:-

Cerebral Cortex. The lesions were bilateral but not necessarily symmetrical, predominantly in the dorsal and dorso-lateral cerebrum and only the most severe were visible macroscopically, as areas of brown discoloration of tissue

sharply demarcated from the adjacent normal cortex. Light microscopic examination of affected cortex revealed a range of changes from minimal spongy transformation to almost complete degeneration of most parenchymal elements. The earliest change appeared to be spongy transformation of a laminar or pseudo-laminar nature (Figures 6 and 7), consisting of perineuronal and perivascular spaces with a foamy neuropil (Figures 8 and 9). Within spongy areas some neurones showed degenerative changes including cytoplasmic shrinkage and increased eosinophilia, with nuclear pyknosis. The changes progressed to lesions with almost complete loss of the cortical elements with sharp delineation of these areas from the adjacent tissue (Figure 10). In a few cases there was liquefaction, macrophage infiltration and cavitation of the cerebral cortex in affected areas (Figure 11).

Other Areas Affected. Other parts of the brain showed focal malacic changes including foamy change in the ground substance, neuronal and glial degeneration and fragmentation proceeding to almost complete tissue destruction. Other than the cerebral cortex, areas found to be involved were:- the hippocampus and dentate gyrus: bilaterally symmetrical, focal areas of the thalamus with variable extension of the lesions into the lower brain-stem: superior colliculi: cerebellum in which either a) the nodule and uvula were involved, this being associated with cerebellar coning due to brain swelling or b) malacic changes throughout the 'core' of the cerebellum. These changes have been described in more detail by other workers.

Distribution and Severity of Brain Lesions. The cases of PE were diagnosed on the basis of the appearance of the cerebro-cortical lesions. The severity of these lesions and those found elsewhere in the brain are presented in Table 1, and may be summarised as follows:- in 44 cases of PE lesions of varying severity were observed in,

Hippocampus of 31 cases,
Superior colliculi " 22 ",
Thalamus " 18 ",
Cerebellum " 25 ".

Clinical and Pathological Studies of Sheep Affected with PE.

Clinical Findings. Sheep subsequently diagnosed as cases of PE exhibited a range of clinical symptoms, including anorexia, lethargy, ataxia, in-coordination and severely affected animals were recumbent with intermittent convulsions, nystagmus, opisthotonus and extensor spasms. Many showed signs of abdominal pain on handling which included grinding of the teeth, hypersensitivity of the abdominal wall and stertorious breathing. A few had loose faeces, younger animals being more prone to diarrhoea. The duration of the neurological signs for each case is presented in Table 2. One sheep (PE/21) had a pronounced odour of hydrogen sulphide for 2 days prior to being killed in the terminal stages of neurological disturbance.

Pathology.

Brain: Light microscopic examination of H and E stained sections of the brains of these animals revealed minimal to severe lesions of PE resembling those already described.

Other Tissues: No consistent changes were observed.

Thiaminase Assays.

The assay procedure gave repeatable results for thiaminase estimations with rumen contents, faeces and broth cultures of B. thiaminolyticus. The mean activity of heat killed blanks from rumen contents of the 21 PE cases (Table 2) was 4·0714 DPS (SD ± 0·545; SE ± 0·1189), whilst the mean of 21 buffer blanks was 3·1904 DPS (SD ± 0·3176; SE ± 0·0693). The heat killed blank mean exceeded the buffer blank mean by 0·8810 DPS which was found to be significantly higher by Students 't' test ($p = <0·001$). Thus heat killed blanks give a more accurate assessment of 'non-enzymic' contribution to the scintillation count, than buffer blanks. Thiaminase activity in excess of 1 unit (more than 6 standard deviations in excess of the heat killed blank mean) was considered significant.

Details of ruminal and faecal thiaminase activities of sheep examined are presented in Table 2. Of the PE cases 19/21 had thiaminase activity in the rumen contents, 9/12 had the enzyme in the faeces. Dialysis of the extracts of all these samples resulted in loss of thiaminase activity when assayed in the absence of nicotinic acid, which was restored on addition of this co-factor; this demonstrated that the extracts contained only type I thiaminases (EC 2.5.1.2.).

Effect of Nicotinic Acid Concentration on the Sensitivity of the Assay. Six rumen liquor extracts in which thiaminase was detected when tested using a substrate solution containing 500 µg nicotinic acid/ml were also assayed

with 36 μg/ml. This revealed that the higher concentration of this co-factor gave increased sensitivity as enzyme was not demonstrated in 2 of the 6 samples using the lower concentration (Table 3).

Further Studies of the Cerebro-cortical Malacia.

The brain lesion of PE may be attributable to other factors besides impaired thiamine metabolism. Further detailed studies of PE cases were thus restricted to those animals in which thiaminase type I was detected in the rumen, in an effort to exclude cases arising from other causes. As the cerebro-cortical lesion is used in the diagnosis of this condition and no other areas of the brain appeared to be consistently involved these more detailed studies were also confined to the cerebral cortex.

Special Stains. Cajal preparations revealed that astrocytes were intact in areas of early spongy change (Figure 12) but showed progressive damage and loss in areas of more severe malacia (Figure 13). Adjacent to some blood vessels in degenerate tissue amorphous, eosinophilic, P.A.S. and ninhydrin-Schiff positive material, indicative of glycoprotein, was found. These accumulations may be the result of leakage of blood proteins through the vessel walls.

Enzyme Histochemical Stains. Thiamine pyrophosphatase staining revealed fragmentation of the Golgi apparatus proceeding to complete loss of activity of this enzyme in degenerate neurones (Figures 14 and 15). The method to demonstrate acid phosphatase indicated reduced numbers or loss of lysosomes in degenerating neurones. The most

striking change was found in sections stained to demonstrate alkaline-phosphatase activity. In areas of malacia the lesions were delineated by a zone of dark brown to black reaction product at the periphery, with absence of enzyme activity in the ground substance enclosed by this zone, whilst blood vessels retained their normal high activity of the enzyme (Figure 16).

Light Microscopy of Araldite Sections. The earliest evidence of cerebro-cortical malacia observed by light microscopy in paraffin sections, was considered to be spongy change consisting of perineuronal and perivascular spaces with foamy transformation of the ground substance (Figures 7 and 9). In Giemsa-stained araldite sections the spaces around neurones and blood vessels were found to be divided by membranes, thus appearing as a series of clear vacuoles, whilst the ground substance contained numerous irregular spaces (Figure 17). Many neurones in these areas showed no evidence of degeneration, whilst swollen glial cells (Figure 17) were numerous.

Electron Microscopy. In electron micrographs the majority of swollen glial cells were recognised as astrocytes by the presence of bundles of glial fibrils whilst remaining organelles were dispersed in the watery hyaloplasm (Figure 18). Clusters of glycogen granules were often concentrated in the swollen astrocyte processes which ramified in the surrounding neuropil (Figure 19). Oligodendroglia were generally well preserved in moderately oedematous cortex.

The clear spaces observed in the neuropil in 1 µm-thick araldite sections were readily identified in electron

micrographs, and found to be membrane-bound (Figure 20). They were considered to be swollen astrocyte processes because of the presence of glial fibrils in many of them, their irregular outline, and the absence of microtubules, neurofilaments or synaptic vesicles. A small proportion were considered to be distended axon terminals because they contained synaptic vesicles. Oedematous neuropil showed a marked increase in the numbers of glycogen granules compared with control material. Most of the glycogen granules were located within swollen astrocyte processes.

The perineuronal vacuoles were also found to be membrane-bound, but were less easily classified as many contained only bizarre membraneous remnants and glycogen granules (Figure 22). They appeared to be cell processes but did not show any evidence of synaptic junctions or vesicles. Those which were less severely distended occasionally contained mitochondria, rough and smooth endoplasmic reticulum and bundles of glial fibrils in addition to glycogen granules. These observations suggest that the perineuronal vacuoles were also astroglial. Between these distended processes were normal axon-terminals with axo-somatic synapses (Figure 22).

The perivascular spaces observed by light microscopy were found to be membrane bound, many contained mitochondria and rough e.r. and occasionally clumps of glial fibrils and glycogen. They were considered to be the grossly swollen end-feet of astrocytes.

Many neurones surrounded by large swollen processes showed no evidence of degeneration in electron-micrographs,

but appeared to be compressed by these processes (Figure 22). In some areas there was partial or almost total occlusion of capillary lumina, apparently due to compression by distended astrocyte end-feet.

Control Material.

These animals were showing no evidence of neurological disturbance, and the brains appeared normal by light and electron-microscopy, revealing a compact morphology, with no evidence of spongy change (Figures 6, 8, 21 and 23). No thiaminase activity was detected in the rumen contents of faeces of these animals (Table 2).

Discussion.

It was found in the present investigation that the majority of sheep diagnosed as cases of PE, on the basis of neuropathology, had thiaminase type I activity in their rumen contents. These findings provide further support for the proposal of Edwin and Jackman (1970) that this enzyme may have aetiological significance in PE.

In certain cases of PE the absence of thiaminase may have been attributable to lack of sensitivity in the assay method, loss or destruction of enzyme prior to assay or the induction of PE in some other way in these individuals. Of the 2 sheep with appropriate brain lesions, but no thiaminase activity in the gut contents, one had shown a temporary response to thiamine injections and thus could have been an example of enzyme loss prior to assay. The other case showed no such response to therapy, even in the early stages of the condition, but it differed from all the

other sheep studied in that it was associated with a strong smell of hydrogen sulphide during the 2 to 3 days preceding its death. Therefore the possibility of PE being caused by factors other than thiaminase cannot be excluded.

The thiaminase assay method was found to give consistently repeatable results, and was easy to carry out even with crude enzyme preparations as suggested by Edwin and Jackman (1974). The concentration of nicotinic acid recommended by these workers was sufficient to allow the reaction to go to completion, but appeared to be insufficient to detect very low levels of thiaminase activity. The increased concentration of this co-factor adopted in the present work did not apparently have any adverse effect on the procedure, and increased the sensitivity markedly. In this work the 'thiaminase units' used were similar to those applied by Edwin and Jackman (1974) but the results are still not directly comparable due to differences in co-factor concentration.

In previous neuropathological studies of PE, which incorporated the use of a range of special histological staining procedures, the mechanisms responsible for the characteristic appearance of the cerebro-cortical lesions was not determined (Markson and Terlecki, 1968). The histological methods used for the light microscopic examination of these lesions in the present work, confirmed many of the changes reported previously by other workers, but they did not provide the means to determine the mechanisms involved in the development of the early malacia.

The earliest evidence of cortical malacia observed by

light microscopy was a foamy appearance of the neuropil with distended perineuronal and perivascular spaces and swollen glial cells. Examination of these changes in araldite sections by light and electron-microscopy revealed that all these changes may be attributable to swelling of astrocytes. This was considered to be a state of oedema confined to the intracellular compartment, and as such may be classified as a 'cytopathic oedema' (Klatzo, 1967).

Markson and Terlecki (1968) proposed that the basic lesion in PE is necrosis of neurones with associated perineuronal and pericapillary oedema. The present study indicates that neuronal necrosis may be secondary to an oedema of astrocytes, as many neurones in oedematous tissue showed no evidence of degenerative change even at the ultrastructural level.

Swollen watery astrocytes occur in a wide range of conditions, and also result from poor fixation (Lee and Bakay, 1966). The excellent correlation between the morphology of oedematous tissue in paraffin and araldite sections and the absence of watery astrocytes in control material indicates they are not artifacts, but evidence of early oedema in PE. The greater susceptibility of the cell processes to swelling, as compared with the cell body, may be due to surface-volume relationships (Birks, cited by Cornog et al., 1967).

Cells may swell due to defects of volume control mechanisms or in response to alteration of the external environment (Ginn et al., 1968). Support for the suggestion that a defect of cell volume regulation is responsible for

the oedema of PE is provided by work conducted on rats treated with ouabain (Cornog et al, 1967). The changes described in astrocytes in the cerebral cortex of these rats closely resembled those observed in early oedematous process of PE. These workers attributed glial swelling to impaired fluid control as a result of inhibition of Na^+, K^+-activated, membrane-bound ATP-ase, which is believed to be related to, if not identical with, the electrolyte transport mechanism (Ginn et al, 1968).

Cellular volume control depends upon energy for the active transport of electrolytes and it has been proposed that any factor which reduces the availability of ATP may thus produce oedema (Sato, et al, 1969). There is considerable evidence that an aberration of thiamine metabolism plays a major role in the pathogenesis of PE (Edwin and Lewis, 1971). Thus the early oedema may be the result of reduced ATP-production following a defect of carbohydrate metabolism in the astrocyte. This hypothesis is supported by the suggestion that glial cell swelling represents the earliest morphological change in thiamine deficiency neuropathy of rats (Collins, 1967; Robertson et al. 1968).

It has been proposed however that astroglial swelling may occur as a response to extensive neuronal depolarisation as a result of the considerable release of K^+ and its retention by astrocytes (de Robertis, et al, 1969). Thiamine per se is believed to have a role, independent of its co-enzyme functions, in nerve impulse conduction and sodium transport (Cooper et al. 1963; Itokawa and Cooper, cited by Edwin and Lewis, 1971). Extensive neuronal depolarisation

may thus be brought about by either impaired neuronal energy production, or impaired neuronal membrane function, arising as a consequence of defective thiamine metabolism. The oedema may therefore be secondary to a primary neuronal defect at the biochemical level.

Tellez and Terry (1968) disputed the proposal that glial swelling represents the first morphological change in thiamine deficiency neuropathy of rats. These workers described primary alterations in axons and presynaptic terminals and suggested that earlier workers had killed their animals either too late or too early. This argument has yet to be resolved, but examination of cases of PE in an even earlier clinical or a preclinical state may provide further information.

Pill et al (1966) argued that in PE compression of arterioles may lead to tissue anoxia with consequent cortical necrosis. Vascular compression was a feature in areas of early spongy change. Arterioles were patent, but many capillary lumina were almost completely obliterated by swollen astrocyte end-feet, and thus impaired blood flow may contribute to the progress of the brain lesions.

The increased numbers of osmiophilic granules in areas of early malacia were considered to be glycogen on the basis of staining characteristics and morphology (Revel, et al, 1960; Revel, 1964). Similar accumulations of glycogen, described within glial cells in thiamine-deficient rat cerebellum, were attributed to thiamine deficiency and consequent impairment of carbohydrate metabolism (Collins and Converse, 1970). A similar proposal may be made for the increased glycogen in PE.

However, glycogen accumulation within glial cells has also been attributed to a lowered rate of glucose utilisation by neurones with piling-up of unused glucose in the surrounding astrocytes (Klatzo et al, 1970). Thus impaired neuronal thiamine metabolism, or neuronal damage due to local ischaemia following capillary compression, may account for the presence of increased glycogen in PE.

In sheep with severe cortical malacia there was evidence of vascular leakage in the form of extravasated serum protein. Increased intracerebral pressure may be due to the disturbance of fluid exchange mechanisms associated with vasogenic oedema (Klatzo, 1967), but a correlation between the degree of swelling and the extent of vascular breakdown in PE has yet to be established.

Pill et al, (1966) produced lesions, indistinguishable from those of PE at the light microscopic level, by the administration to a calf of the thiamine antagonist amprolium. Since then several workers have examined these lesions by light microscopy in calves (Markson, Terlecki and Lewis, 1966; Little, 1969; Markson et al, 1972), and sheep (Sinha, 1971; Loew and Dunlop, 1972). The similarity of the lesions produced by amprolium and those of PE has been used in support of the hypothesis that PE is the result of thiamine antimetabolite production by thiaminase type I in the rumen (Edwin and Jackman, 1970). The next stage of the present investigation was to compare brain lesions of amprolium poisoning with those of PE at the ultrastructural level.

SECTION III.

EXPERIMENTAL AMPROLIUM POISONING OF PRE-RUMINANT LAMBS.

General Introduction.

The purpose of this section of the work was to establish the mechanisms responsible for 'amprolium poisoning encephalopathy' of sheep, and to compare this entity with natural PE. It was intended to kill animals serially during the clinical syndrome and thus a consistent response to amprolium was necessary. Other workers using adult sheep reported considerable variation between individual animals, in the duration of amprolium administration required to produce the encephalopathy (Sinha, 1971; Loew and Dunlop, 1972). An experimental system was therefore designed to achieve a 'uniform' state of amprolium intoxication, and this was then used for a range of pathological, biochemical and haematological investigations.

PART ONE.

AMPROLIUM POISONING EXPERIMENT ONE.

Introduction.

Amprolium (1-(4 amino-2-n propyl-5 pyrimidinylmethyl)-2-picolinium chloride hydrochloride) is a competitive thiamine antagonist and its effects on the brain are inhibited by thiamine administration (Markson et al, 1972). Ruminating sheep obtain the major part of their thiamine requirement as a by-product of rumen fermentation (Austin, 1947). Therefore, in order to keep available thiamine at a low and approximately constant level, the ruminal source was eliminated by the use of pre-ruminant lambs reared on a semi-synthetic liquid diet of moderately low thiamine content. To reduce intestinal synthesis of thiamine by bacteria, the lambs received phthalylsulphathiazole, and slatted floors were used to reduce coprophagy. Amprolium was administered on a body-weight related basis.

The brain lesions produced by amprolium intoxication, of lambs reared under this system, were then studied by light and electron microscopy.

Materials and Methods.

Experimental Animals and System of Rearing.

Twenty-one Suffolk x Dorset lambs, having received colostrum for 24 hours, were reared on a proprietary milk substitute diet (Nutrilamb - S.A.I. Ltd). The diet was prepared to give a final concentration of 0·25 mg thiamine per litre, and fed ad libitum from an automatic feeder.

The lambs were housed on slatted floors in loose boxes at an ambient temperature of 8 - 15°C. Because of the sudden change of diet at weaning each lamb received 11 mg/kg oral oxytetracycline/day for 3 days to reduce the risk of intestinal bacterial infection, and then phthalylsulphathiazole was administered orally at the rate of 0·3 g/kg throughout the remainder of the experiment. The animals were kept as 2 groups in adjacent loose boxes, with 12 in one group and 9 in the other. The larger group were given 280 mg/kg body weight of amprolium/day, which was commenced on the fifth day after weaning, and was administered orally. Of the 9 control animals, 4 received 1 mg thiamine/day orally in aqueous solution, throughout the experiment and the other 5 were untreated. The lambs were weighed at weekly intervals, and the amprolium and phthalylsulphathiazole intake adjusted on the basis of these weights each week.

Experimental Regimen.

With 2 exceptions the lambs were killed at approximately 2, 8, 18 or 36 hours after the onset of neurological symptoms, along with the appropriate controls (Table 4) as described below:-

Post Mortem Procedure.

Intravenous heparin was administered, the lambs anaesthetised and the brains perfused using the procedure described for the natural cases of PE (Section 2, page 17). The fixative was a mixture of 4% formaldehyde and 1% glutaraldehyde in 0·1 M Sorensens phosphate buffer pH 7·4, which had been found in concurrent work to improve the

preservation of most cell types and their organelles, this being demonstrated in Figures 23 and 24. Three litres of fixative were used for each animal, the blocks for electron microscopy, approximately 1 x 3 x 3 mm, were washed in buffer containing 10% sucrose prior to osmication. One µm-thick, and ultrathin, araldite sections were cut by the author on a Porterblum MT1 ultratome.

At post mortem the following tissues were prepared for light microscopy of H and E stained paraffin sections: heart, liver, spleen, pancreas, adrenal, somatic muscle, kidneys, thyroid, abomasum, duodenum, jejunum, ileum, caecum and colon, and the eyes. These were processed as described for the tissues selected from cases of natural PE.

Results.

Clinical Findings.

Details of sex and time on experiment are shown in Table 4. All the lambs grew well for the first 2 weeks but in the third week the average food consumption of the amprolium-treated group decreased and their weight gain declined. The controls grew well throughout and no marked difference in weight gain was recorded between those given a thiamine supplement and the untreated controls. During the fourth week 10 of the amprolium-treated animals developed a characteristic clinical syndrome. Initially there was diarrhoea and anorexia which lasted for 24 - 36 hours. Neurological signs developed within 1 to 2 days of the onset of diarrhoea. The ears drooped and the lambs became lethargic, then ataxic and finally recumbent with nystagmus

and intermittent convulsions, terminating in extensor spasms and opisthotonus. In recumbent animals convulsions could be precipitated by gentle dorsal flexion of the neck. Recumbent animals developed a slow laboured respiration and tachycardia. The clinical course of neurological disturbance lasted approximately 36 hours.

Due to the consistency of the clinical syndrome, it was possible to sample serially in relation to the onset of the neurological disturbance (Table 4).

Gross Pathology.

All the amprolium treated animals showed petechiae and ecchymoses in most organs (Figure 25). In animals killed in extermis many haemorrhages were diffuse in tissue such as myocardium, bladder wall, subcutaneous loose connective tissue and intestinal serosa. Ten animals showed areas of cerebro-cortical malacia of variable extent but of similar appearance to that observed in natural PE. Haemorrhages of varying severity were present in the dorsal cerebral meninges and in the brain parenchyma in 5 cases. Gross evidence of brain swelling, including compression of cerebro-cortical gyri, tentorial herniation of the occipital poles, and cerebellar coning was present in 6 cases (Figure 26). Animals Nos. 11 and 12 were killed prior to the development of symptoms of CNS disturbance but following the onset of diarrhoea, in the hope of finding very early lesions. These 2 cases had small haemorrhages in most organs but no gross neuropathological changes were observed. These observations are detailed in Table 4.

Microscopic Findings.

Histological examination of tissues other than brain revealed consistent changes only in heart-muscle and duodenum. In the myocardium many dark-staining myocardial cells were observed. In the duodenum there were areas of flattened distorted villi with evidence of degeneration of the mucosal epithelium and severe distension of the underlying Brunner's glands.

Lesions in the brain were confined to the cerebral cortex, except in those lambs with severe cerebellar distortion in which there were also petechial haemorrhages and malacic foci in the herniated cerebellar uvula (Figure 27).

The description of the cerebro-cortical changes is divided into 3 sections:

The Early Malacia. The earliest evidence of cerebro-cortical malacia was spongy change of a focal or laminar nature, this type of change being most extensive in animals killed 2 to 8 hours after the onset of symptoms, and characterised by the presence of perineuronal and perivascular spaces with a foamy appearance of the neuropil (Figures 28 and 29).

Laminar lesions did not appear to be located in any particular cortical laminae and would often pass across a sulcus to continue in the adjacent gyrus. Malacic areas were generally present on both sides of a sulcus with one lesion often appearing slightly more advanced than the opposing one.

Electron microscopy revealed that the perivascular and perineuronal spaces and the foamy appearance of the

neuropil were attributable to watery swelling of astroglial cells (Figures 30 and 31). This astroglial change appeared to follow a uniform sequence, with perivascular end-feet being the first, and in some areas the only, structures to be affected. The processes in the neuropil and adjacent to neurones were the next to swell followed by distension of the perikaryon and nucleus. Severely distended astroglia then showed evidence of degeneration with clumping of organelles in the watery hyaloplasm and granular aggregation of chromatin principally at the nuclear membrane (Figure 31).

Many swollen astroglial cells and their processes contained clusters of glycogen granules. Counts of representative areas in experimental animals revealed a near three-fold increase in the number of granules as compared with the controls.

In areas of moderately severe astroglial swelling the blood vessels were well perfused and there was no evidence of capillary or arteriolar compression by distended astroglial end-feet. No increase in the extra-cellular space was observed. Most neurones and oligodendroglia in areas of early malacia had a normal appearance by light and electron microscopy. Those neurones undergoing degenerative change showed distension of the Golgi apparatus and mitochondria with or without distension of the endoplasmic reticulum. In subcortical white-matter adjacent to areas of early malacia many myelin sheaths were distended by an accumulation of electron-lucent fluid in the space around apparently normal axons. A few swollen myelin sheaths also showed

separation of myelin lamellae at the intraperiod line. This periaxonal and intramyelinic oedema contributed significantly to the malacia in these areas.

Further Development of the Malacia. In lambs killed at 18 and 36 hours after the onset of clinical signs the pathology had progressed to coagulative neuronal necrosis, distension and fragmentation of glia; changes closely resembling those of natural PE. The most notable difference between PE and amprolium poisoning was the more severe haemorrhage in several amprolium poisoned lambs (Figure 32). Haemorrhage was present in 5 animals as shown in Table 4, and resulted in extensive loss of blood from many small blood vessels into the brain parenchyma. In the other lambs no haemorrhage was observed but isolated vessels in lamb No.1 showed small amounts of extravasated plasma proteins in electron-micrographs.

In some areas of severe malacia there was extracellular oedema in the underlying white matter of the gyral cores with very little evidence of the swelling of myelin sheaths which were bathed in clear oedema fluid.

The Inflammatory Response. Inflammatory cell infiltration appeared to commence in the meninges overlying malacic cortex, and then progressively involved the deeper layers in the form of perivascular cuffs and accumulations of inflammatory cells at the periphery of severely degenerate tissue. In all animals killed at 2 to 8 hours after the onset of clinical symptoms there was a mixed population of polymorphonuclear leucocytes (PMNs), monocytes and macrophages in which the first predominated. At 18 hours there were

many more macrophages and the majority of PMNs were apparently degenerating. In 3 animals killed at 36 hours there was extensive infiltration by macrophages, and PMNs were not apparent.

Pathology of Control Lambs.

No significant pathological changes were observed in the 9 controls. The brains appeared normal by light and electron microscopy (Figures 29, 33 and 34). Astroglial cytoplasm was barely perceptible by light microscopy (Figure 29) but could be readily identified in electron-micrographs (Figure 34).

Discussion.

The success of this experiment in producing a uniform state of amprolium intoxication, was demonstrated by the development of cerebrocortical malacia in 10 of the 12 amprolium treated group after 23 to 28 days on experiment. This represents a more consistent result than that achieved in earlier reported experiments in which adult sheep were used; Sinha (1971), 9 - 38 days amprolium administration; Loew and Dunlop (1972) 18 - 44 days. The nature of the syndrome produced by this method permitted serial examinations through the clinical period.

The earliest evidence of cerebro-cortical malacia due to amprolium poisoning was found to be oedema of astrocytes leading to the development of perineuronal and perivascular spaces and foamy neuropil. This oedema was apparently identical to that described as the primary morphological change in natural ovine PE (Section II). Accumulation of

glycogen in astrocytes was observed in both conditions and further development of the malacia was also very similar, with degeneration of neurones and other parenchymal elements following the oedema in both cases. The striking resemblance of these 2 encephalomalacias at the ultrastructural level thus provides further evidence of their similarity to each other, thus supporting the hypothesis of Edwin and Jackman (1970) that natural PE may be a consequence of thiamine antimetabolite production in the rumen.

All the animals which developed CNS lesions, showed diarrhoea for 24 to 36 hours prior to the onset of neurological disturbance. Lambs 11 and 12 were slaughtered 24 hours after the onset of diarrhoea in the hope of finding early lesions. The absence of lesions in the brains of these 2 lambs, if they are representative of their fellows, indicates that the oedema develops 24 to 36 hours after the commencement of diarrhoea and thus has a fairly rapid onset.

It was proposed in Section II that astroglial swelling in PE may result from defects of cell volume regulation due to impaired energy metabolism within the astrocyte or as a response to change in the extracellular fluid bathing astrocytes. It was also suggested that the inhibition of thiamine in neuronal membrane metabolism might account for the astroglial oedema. These arguments may be applied equally to the primary oedema of amprolium poisoning encephalopathy.

Alteration of the composition of the extracellular fluid, with consequent astroglial swelling, may also be the

result of increased vascular permeability. The use of horse-radish peroxidase as a 'vascular tracer' has provided evidence to suggest that the early oedema in rat thiamine deficiency encephalopathy occurs in the absence of changes in vascular permeability (Manz and Robertson, 1972). This may also be the case in the early oedema of amprolium poisoning, but in the absence of further investigation, the pathogenesis of the early astroglial swelling remains unknown.

Haemorrhages were present in the brain parenchyma of 5 amprolium intoxicated lambs. Acute thiamine deficiency in man, mink, foxes, cats (Innes and Saunders, 1962) and rats (Manz and Robertson, 1972), is also associated with haemorrhages in the brain. These haemorrhages are, however, bilaterally symmetrical and predominate in the brain-stem, and thus in both respects differ from the lesions in the brains of amprolium poisoned lambs.

In natural cases of PE it was proposed that brain swelling may be due to vascular leakage and the consequent state of vasogenic oedema (Section II). In this experiment a relationship was observed between brain swelling and the haemorrhage in the brain parenchyma of amprolium poisoned lambs. There was one exception in that test animal No. 1 had brain swelling, but no haemorrhage. In this case, however, electron microscopic examination revealed that serum proteins had leaked from some vessels in the malacic cortex. Thus in both PE and amprolium poisoning, cerebral swelling may be the result of severe disturbance of fluid balance in the brain due to extravasion of blood proteins.

In this experiment a number of changes were observed which contrast with those described in natural PE (Section II) and amprolium toxicity of adult sheep (Sinha, 1971; Loew and Dunlop, 1972). The distribution of malacia in the amprolium intoxicated lambs was predominantly in the cerebral cortex, the only other change being in herniated cerebellar tissue. The cerebellar lesions were attributed to pressure as a result of cerebral swelling. The lesion distribution thus differed from that in amprolium poisoning of adult sheep (Sinha, 1971), in which they were reported in the cerebrum, brain-stem and cerebellum and in natural PE in which the hippocampus, brain-stem and cerebellum are also frequently affected (Section II).

The involvement of myelin sheaths in the early malacia was striking and has yet to be described in natural PE. The oedema fluid appeared to accumulate principally in the periaxonal space and less frequently within the myelin sheaths in the intra-period line. Thiamine is present in the axonal membrane (Tanaka and Cooper, 1968) and may have a role in this site independent of its co-enzyme function in nerve impulse conduction and sodium transport. Thus the inhibition of thiamine in the axonal membrane by amprolium may account for the periaxonal accumulation of fluid observed.

This experiment also demonstrated the characteristic nature of the inflammatory response, initially by PMNs, which were later replaced by a population of macrophages. The direct correlation observed between inflammatory-cell type and time of kill indicates that time of onset of clinical

signs provides a useful baseline for studying the progress of the brain lesions.

As a second experiment was then carried out to investigate the biochemical mechanisms responsible for the brain lesions, the opportunity was also taken to study in more detail the haemorrhagic and diarrhoeic syndromes associated with amprolium poisoning of pre-ruminant lambs.

PART TWO.

AMPROLIUM POISONING EXPERIMENT TWO.

Introduction.

Amprolium is a thiamine antagonist and is known to enter the brain (Markson et al, 1972). Inhibition of transketolase (TK) activity provides a very sensitive indicator of thiamine inadequacy (Brin, 1962). The aim of this experiment was to examine brain TK activity throughout the period of amprolium administration to determine whether biochemical evidence of impaired thiamine metabolism could be detected in the cerebral cortex prior to the onset of cerebrocortical malacia. The studies of the diarrhoeic and haemorrhagic syndromes were conducted within the confines of the experimental design imposed by this aim.

In the first experiment intestinal lesions were observed in the duoedenum, and thus pathological studies of the gut were concentrated on the first third of the small intestine. Blood vessel walls, clotting mechanisms and features of platelet function were also examined.

The system of rearing and amprolium intoxication used in the first experiment were repeated exactly. Groups of amprolium treated lambs, with their appropriate controls, were then killed for sampling tissues at weekly intervals throughout the period of intoxication.

Materials and Methods.

Experimental Animals and System of Rearing.

Two groups of 12 Suffolk x Dorset lambs reared as in experiment 1 (Page 32), were used. One group (group E)

received amprolium (280 mg/kg body weight/day) whilst the other 12 lambs (Group C) were untreated controls.

Experimental Regimen.

Three amprolium treated (Group E) and 3 control (Group C) lambs were killed on each of days 7, 14 and 21 of the experiment; the remaining 3 lambs from group E were killed as soon as they exhibited evidence of CNS disturbance, along with the appropriate controls. These killing groups are numbered 1 to 4 and lambs in each group identified as a, b and c as shown in Table 5.

Haematological Tests.

Venous blood samples were taken weekly for the Lee-White test of clotting time (Archer, 1965), from the lambs in groups E4 and C4. The clot-retraction was expressed as the percentage volume of serum produced from 4·5 ml of clotted blood after 24 hours at 37°C. Blood was collected into citrated tubes for the Quick one-stage prothrombin test (Archer, 1965) using both human thromboplastin (Symplastin - W.R. Warner Ltd. Eastleigh, Hants.) and sheep-brain thromboplastin which was prepared as described by Archer (1965). Platelet counts were carried out on samples, using EDTA as an anticoagulant, by the staff of the R(D)SVS Clinical Laboratory. On days 15 and 19 samples were also taken from lambs of groups C3 and E3 for the Quick test.

Post Mortem Procedure.

With the exception of lamb E4a, which died, each lamb was anaesthetised with sodium-pentobarbitone and a cysternal sample of cerebrospinal fluid (CSF) withdrawn via a 21 gauge x 1½" hypodermic needle, The CSF samples were frozen by

placing in a vacuum flask containing dry-ice, and then stored at -80°C prior to K^+ and Na^+ estimations. The animals were exsanguinated, the small intestine immediately removed, and a 5 cm segment cut from the proximal and distal ends of the anterior third. These segments were cut in half transversely, opened along the mesenteric attachment and one piece placed in 4% glutaraldehyde in 0·1 M phosphate buffer, pH 7·4 for electron microscopy, and the other portion into cold neutral Bakers' formol for enzyme histochemistry. The remainder of the first third of the intestine was stored on ice prior to preparation for enzyme assays. Faeces samples taken from the rectum were stored in plastic pots of -20°C prior to pH estimation.

The bone-marrow of the left femur was rapidly exposed by sawing through the bone at the level of the minor trochanter. A small block was excised with a scalpel, cut into 1 mm cubes and immersed in 4% glutaraldehyde in 0·1 M phosphate buffer and the adjacent 1 cm core of marrow immersed in 10% formol-saline. Bone-marrow smears from the femur and the left sixth rib were made onto glass slides, air-dried and fixed in acetone-free, methylated spirit. A 1 cm square segment of the ventral aspect of the urinary bladder was immersed in 4% glutaraldehyde and another in 10% formol-saline.

The brain was removed rapidly and cut into 3 mm-thick, coronal slices, alternate pieces being placed in 10% formol-saline, whilst the remaining tissue was placed in polythene bags and 'quenched' in a mixture of dry-ice and isopentane for storage at -80°C prior to preparation for enzyme assays.

Histological Processing Procedures.

The samples in 10% formol-saline were processed to paraffin wax, 6 μm sections cut and stained with haematoxylin and eosin, or Heidenhains Iron Haematoxylin. The material in 4% glutaraldehyde was fixed for 24 hours, washed in phosphate buffer and embedded in araldite, for examination by electron-microscopy. The samples in cold neutral Bakers' formol were fixed for 24 hours and subjected to the 'Gomori Calcium' and 'Azo-dye Coupling' methods for alkaline phosphatase (Bancroft, 1967) using 15μm-thick frozen sections. Bone-marrow smears were stained with Leishmans' stain and used for the differential counting procedure by Dr. Doxey (R(D)SVS). 'Total' counts were estimated by the author, using both araldite and paraffin sections, by counting the number of cells in 100 μm-sided squares, using an eye-piece graticule: areas were selected in the stroma, adjacent to the periphery of the marrow, in which fat-cells did not impinge on the field to be counted. The slides were randomised, and five fields were counted in both araldite and paraffin sections from each case. From these results a mean count was calculated for each lamb. This procedure has been called 'total count', for convenience but it only provides a figure for population density in the areas examined and takes no account of the number of cells per unit volume of marrow. The total number of mitotic figures were similarly determined using a 100 μm-sided square, except that paraffin sections were stained with Heidenhains Iron Haematoxylin to facilitate recognition of mitotic figures.

Enzyme Assays.

The activity of Alkaline Phosphatase in the intestinal mucosa was assayed by Dr. Coop (Moredun Institute) as described in the attached paper (Appendix No. 7).

Transketolase (TK) assays were carried out in collaboration with Dr. Coop as follows: homogenates were prepared from the dorso-medial parietal cortex of thawed brain slices after separation from the under-lying white-matter with a scalpel. Cerebellar samples were taken from the core of the cerebellum just dorsal to the corpus medullare. Weighed samples were homogenised in 0·1 M Tris-HCl buffer, pH 7·6, centrifuged at 10,000 g for 30 minutes at 4°C and made up to volume. TK activity was determined by measuring the rate of formation of sedoheptulose-7-phosphate from ribose-5-phosphate in glycylglycine buffer pH 7·8 for 30 minutes at 37°C as described by Novello and McLean (1968). The sedoheptulose-7-phosphate produced was assayed by the cysteine/sulphuric acid method of Dische (1953). TK activity is expressed as units per gram fat-free dry weight. (1 unit is defined as the amount of enzyme catalysing the formation of 1 μmole of product per hour at 37°C).

Other Procedures.

The concentration of Na^+ and K^+ in the CSF were estimated with an I.L. 343 Flame Photometer. Faecal pH was measured by immersing the electrode of a Pye Unicam pH meter in faeces mixed with an equal weight of distilled water.

Results.

Control Lambs (Group C).

The control lambs remained healthy throughout the experiment and post mortem examinations revealed no evidence of pathological change. The blood parameters appeared normal (Figure 35 and Table 6). The mean TK activity was 132 (SD ± 16·6) units per gram fat-free dry weight for cerebral cortex and 141 (SD ± 34·9) units per gram fat-free dry weight for cerebellum (Figure 36). The bone marrow appeared normal in histological sections (Figures 37 and 39) and the differential, 'total' and mitotic counts of the bone marrow are recorded in Table 7. There was no evidence of pathological change in the alimentary tract and the mean intestinal alkaline-phosphatase and lactase activities were 1192 (SD ± 392) and 127 (SD ± 21) milli-units respectively.

Amprolium Treated Lambs (Group E).

Clinical Findings and Gross Pathology. These animals were clinically normal for the first 14 days of amprolium administration and the post mortem examinations of the first 2 groups killed revealed no macroscopic evidence of pathological change. Between days 15 and 20 several amprolium treated lambs developed loose faeces and at post mortem examination on day 21, moderately extensive haemorrhages were found in lambs E3b and E3c.

On day 23 lamb E4a developed very severe, greenish mucoid diarrhoea and died on the morning of the 24th day with conjunctival ecchymoses and slight epistaxis. This lamb was found to have petechiae and ecchymoses in the intestinal tract, subcutaneous loose connective tissues,

skeletal muscles and bone-marrow and diffuse haemorrhages in the diaphragm, epicardium, endocardium and myocardium, urinary bladder submucosa and the retro-orbital tissues. These changes closely resembled those observed in the first amprolium experiment, in that the haemorrhages predominated in actively mobile tissues. Haemorrhages were also present in the dorsal cerebral meninges and there was gross evidence of brain swelling and cerebro-cortical softening.

On days 31 and 33 lambs E3b and E3c respectively were killed after the onset of ataxia following the appearance of severe diarrhoea during the preceding 2 to 3 days. Post mortem examination revealed very similar changes to those described in E4a. In the lambs with haemorrhages there was an excess of pale-yellow fluid in the pericardial sac.

Brain Pathology. Histopathological examination of the brain revealed the characteristic lesions of amprolium poisoning in lambs E4a, E4b and E4c. In all three lambs there was severe cerebro-cortical malacia, but only lamb E4a showed evidence of damage to the cerebellum which was associated with cerebellar coning. There was no evidence of neuropathological changes in the other experimental lambs.

The cerebro-cortical TK activity (units per gram fat-free dry weight) are presented in Figure 36 and summarised below:-

	Group E1	Group E2	Group E3
a.	163	188	101
b.	169	92	48
c.	158	101	65

The value of TK assays performed on cerebro-cortical material from the lambs in Group E4 is doubtful as this tissue was severely malacic. Two lambs in group E3 were considered to have significantly low TK activity in the cerebral cortex compared with the control values (without 4 Standard deviations of control mean).

Cerebellar TK activity of the amprolium treated lambs, apart from E1b (219 units) were all within 2 standard deviations of the control mean (Figure 36).

There was no significant difference in CSF Na^+ and K^+ levels or Na^+/K^+ ratio, between the treated and control groups of lambs.

Intestinal Pathology. In lamb E2b there were areas of flattened distorted villi with degeneration of mucosal epithelium and distension of the underlying Brunner's glands in the duodenum with intestinal alkaline-phosphatase and lactase activities of 372 and 63 milli-units per mg mucosal protein respectively. Histochemical examination also revealed a severe reduction of the alkaline-phosphatase activity in areas of flattened villi, in both the Gomori and Diazo preparations. These histological and biochemical changes were not seen in any other amprolium treated lambs.

The pH of the watery and mucoid faeces taken at post mortem from lambs E4b and E4c was 9·0 whilst that of their controls C4b and C4c was 7·2 and 7·1 respectively.

There were no other consistent differences of faecal pH between the lambs of groups E and C.

Vascular Histopathology. Examination of blood vessels in areas of haemorrhage in the intestine and bladder in both paraffin and araldite embedded material failed to reveal any vascular damage.

Haematological Tests. The first evidence of haematological disturbance was the failure of plasmas from lambs E4a, E4b and E4c to produce a clot in the Quick prothrombin test, using both the human (Table 6) and sheep thromboplastin. A normal response to this test was produced by these plasmas when 0·1 ml of plasma from the appropriate control was included in the incubation mixture. Plasmas from lambs E3a, E3b and E3c gave a normal response to the Quick test on days 15 and 19 (Table 6). The normal response to the Quick test was observed in plasmas from lambs E4b and E4c when sampled on day 29 (Table 6).

On day 23, lamb E4a showed impaired clot retraction and lambs E4b and E4c developed a similar defect associated with thrombocytopenia, during the 1 to 2 days prior to the onset of ataxia (Figure 35). The Lee-White test however revealed no differences between groups E and C, in which clotting times varied from 1 to 6·5 minutes.

Bone-marrow Pathology. In all five lambs with haemorrhages, histological examination revealed marked bone-marrow depopulation, in strong contrast to the controls (Figures 37 and 38). The majority of the remaining myeloid cells were mature eosinophils, PMNs, megakaryocytes and macrophages. Most of these cells appeared normal except for the

megakaryocytes, many of which showed a range of degenerative changes from moderate vacuolation of the peripheral cytoplasm to complete necrosis. These degenerative changes were best seen in araldite sections (Fig. 40), in which normal megakaryocytes showed finely granular cytoplasm (Figure 39). In paraffin sections the degenerate megakaryocytes were only recognisable by their increased cytoplasmic eosinophilia and nuclear pyknosis.

Differential cell counts on bone-marrow smears revealed that in the depopulated marrow of lambs in groups E3 and E4, there was a decreased proportion of erythrocyte precursors, and a concurrent increase in the proportion of eosinophil and neutrophil precursors in comparison with the control group of lambs (Table 7). The depopulation was confirmed by the 'total' bone-marrow cell counts. Mitotic counts revealed that there was also a considerable reduction in the number of dividing cells in the bone-marrow of lambs in the amprolium treated group, with only lambs E2a and E2c having mitotic counts within the range of the control groups (Table 7).

Discussion.

In this experiment it was found that 2 lambs which had developed severe diarrhoea and systemic haemorrhages after 3 weeks of amprolium administration, had no histological evidence of brain lesions but had a low cerebro-cortical TK activity. This observation supports the proposal that impaired thiamine metabolism in the cerebral cortex precedes the onset of the brain lesions. The cerebellar TK activity

of the amprolium treated lambs, including those with severe cerebro-cortical malacia, was apparently unaffected. Only one amprolium treated lamb had lesions in the cerebellum, and these were probably due to cerebellar distortion as a consequence of brain swelling. This restriction of primary brain lesions to the cerebral cortex may indicate regional differences of susceptibility to thiamine deficiency or antagonism as has been suggested for the rat (Dreyfus, 1965).

It was proposed that the early astroglial oedema of amprolium poisoning may be due to raised extra-cellular K^+ following the inhibition of thiamine either as a co-enzyme in neuronal energy-metabolism or as a metabolite in neural membranes (Section III, Experiment 1). However, in this second experiment there was no significant difference between amprolium treated and control lambs in the CSF K^+ or the K^+/Na^+ ratio. As the CSF is probably in direct communication with the brain extra-cellular space (Brightman, 1968) it seems unlikely that the extensive depolarisation of neuronal membranes is the cause of this astroglial swelling.

It was found in the first experimemt that in the amprolium treated lambs, all of which had severe diarrhoea, there was mucosal flattening and distension of Brunner's glands in the duodenum. In this second experiment these changes were observed in only one lamb, which had received amprolium for only 2 weeks, and was showing no signs of diarrhoea. This finding represents a marked difference between these 2 experiments, which in every other respect

were apparently very similar. There was no evidence of pathological change in the duodenum of any of the control lambs in this or the first experiment and the association of the mucosal flattening and distension of Brünner's glands with amprolium poisoning is unknown.

The diarrhoea may be attributable to the specific anti-thiamine activity of amprolium, as it is also a feature of experimental thiamine deficiency in lambs (Draper and Johnson, 1951). Amprolium does not produce diarrhoea in adult sheep (Sinha, 1971; Loew and Dunlop, 1972) which suggests that age may be a factor in the response of the alimentary tract to amprolium poisoning.

The in-vitro clotting times were normal throughout this experiment and thus the Lee-White test appears to be of little value for the diagnosis of this type of haemostatic defect. The significance of plasma clot failure in the Quick test in 3 amprolium treated lambs is unknown especially as they were clinically normal at the time when this defect was apparent.

The onset of haemorrhages was associated with a marked thrombocytopenia, attributable to severe degeneration of bone-marrow megakaryocytes. The functions of blood platelets include the maintenance of normal haemostasis by adhesion and aggregation in injured blood vessels (Born, 1970). The haemorrhages may have been the consequence of an insufficiency of platelets for the repair of minute holes in capillary walls incurred as a result of physiological trauma. This hypothesis is supported by the observation that the majority of haemorrhages occurred in

mobile tissues in which the capillaries might be more susceptible to damage. Extensive examination of blood vessels failed to show any morphological evidence of vascular damage, indicating that the leakage points may be very small.

In addition to the megakaryocyte degeneration, the bone-marrow of lambs with haemorrhages was depleted of many cellular elements and the differential counts showed that erythrocyte precursors were the most severely affected. Since the remaining cells in the marrow of the amprolium treated lambs showed little evidence of degeneration, it seems likely that the marrow depopulation was due to decreased cell production rather than increased cell destruction. This proposal is supported by the finding that the number of cells containing mitotic figures was considerably reduced even in lambs which had received amprolium for only 7 days. Thus amprolium may inhibit cell division or be highly toxic to the blast cells, but its mode of action upon the bone-marrow has yet to be established.

The neuropathology of amprolium toxicity thus closely resembles that of Polioencephalomalacia (Section II), but in the natural disease multiple haemorrhages and bone-marrow damage have not been reported. Thus conclusions about the nature of PE drawn from the results of experiments using amprolium should be treated with caution. Furthermore, workers using amprolium have referred to the neurological syndrome it produces as 'Polioencephalomalacia' (Loew and Dunlop, 1972) and 'Cerebro-cortical necrosis'

(Markson et al, 1972), whereas the term 'Amprolium poisoning encephalopathy' is more correct and its use would avoid confusion.

General Conclusions from Section III.

The similarity of the brain lesions of Polioencephalomalacia to those of Amprolium poisoning encephalopathy at the light microscopic level has been used as evidence for the hypothesis that PE is the result of thiamine antimetabolite production by thiaminase type I in the rumen (Edwin and Jackman, 1970). In the present investigation it was found that both conditions are associated with a primary astroglial oedema in the cerebral cortex, and that degeneration of other cortical elements is apparently secondary to this oedema. These findings thus strongly support the hypothesis of Edwin and Jackman (1970) that thiaminase type I may have aetiological significance in PE.

It was also found that the brain lesions of amprolium intoxication may be preceded by impaired thiamine metabolism in the cerebral cortex. If PE resembles Amprolium poisoning encephalopathy in this respect, it would account for the rapid response to thiamine injections shown by animals affected with PE. The failure of some to respond could be attributable to the oedema having reached a point beyond which it is not reversible.

Bone-marrow lesions, closely resembling those observed in the present work, are associated with Bracken poisoning of cattle (Naftalin and Cushnie, 1954). Bracken contains high concentrations of thiaminase type I and alpha-picoline, which in the presence of thiamine could result in the production of 'pyrimidinyl-alpha picoline' a compound very

similar to amprolium (Edwin, Lewis and Allcroft, 1968). It is thus conceivable that the bone-marrow toxicity of bracken is attributable to the production of compounds similar to amprolium. If this is the case, the presence of bone-marrow damage in the lambs used in the present work is not inconsistent with the 'thiaminase type I' hypothesis of Edwin and Jackman, for the cause of PE, but in fact would provide further supporting evidence in its favour. The absence of bone-marrow damage in natural PE could be attributable to the nature of the antagonist responsible, the age of affected animals or some other factor.

Diarrhoea is a feature of some cases of amprolium poisoning as observed in this work, and has also been reported to occur in 25% of cases of natural PE (Little, 1969). The mechanisms responsible have not been elucidated, but its presence in both conditions provides further evidence of their similarity.

On the basis of these findings it was concluded that there is considerable justification for the proposal that thiaminase type I may be responsible for ovine Polioencephalomalacia. Investigations were then carried out to assess the importance of this enzyme in sheep, and the factors which lead to its presence and activity in outbreaks of PE.

SECTION IV.

THIAMINASE IN CLINICALLY NORMAL SHEEP, AND FOODSTUFFS ASSOCIATED WITH OUTBREAKS OF POLIOENCEPHALOMALACIA.

Introduction.

Thiaminase type I activity has been detected in the faeces of clinically normal, in-contact animals associated with cases of PE (Edwin and Lewis, cited by Edwin and Lewis, 1971). Roberts and Boyd (1974) found that normal lambs at pasture have fluctuating amounts of thiaminase in their faeces, but it was not indicated whether the lambs were derived from flocks which had been affected with clinical PE. There are no other accounts of investigations assessing the proportion of normal sheep which have thiaminase in the faeces. The presence of thiaminase in the faeces may be associated with impaired thiamine metabolism, and could represent a state of "sub-clinical PE" (Edwin, Spence and Woods, 1968) or "sub-clinical deficiency of thiamin" (Roberts and Boyd, 1974). The detrimental effects of sub-clinical thiamine antagonism in sheep are unknown, but an attempt has been made here to establish the extent of the distribution of the enzyme in in-contact sheep.

Faecal thiaminase estimation may not accurately reflect the 'thiaminase status' of sheep as the enzyme may be present in the rumen and not in the faeces (Table 2). However faeces are more readily accessible than rumen contents when sampling large groups of sheep at pasture.

As ruminal fistulae provide easy access to the rumen a small number of sheep with such fistulae were used to follow both ruminal and faecal thiaminase activity.

Thiaminase activity has been found in foodstuffs on premises on which PE has occurred and this could provide a source of the enzyme (Edwin, Lewis and Allcroft, 1968). A range of foodstuffs were thus examined for thiaminase activity. Sheep with faecal thiaminase were selected for either daily or weekly sampling, and were subjected to a change of foodstuff and environment, or injections of thiamine in an attempt to find factors which might favour the accumulation or loss of this enzyme in the faeces.

Materials and Methods.

Source of Materials.

Flock Studies. Three flocks, in which one or more sheep had succumbed to PE, were used for faecal thiaminase studies. Faeces were collected daily or weekly from selected sheep. Herbage, corn, concentrate and beet-pulp nuts, and hay which had been fed to animals in these flocks were also assayed.

Sheep with Ruminal Fistulae. Thirteen Blackface sheep (Table 8) with ruminal fistulae were used, being kept in individual metabolism crates (Suttle, 1974). The ruminal fistulae were made by Mr. B. Mitchell (Moredun Institute); the method of insertion was similar to that described by Alexander (1970) for the caecum of the horse, except that the fenestrated flange securing the body of the cannula to the rumen was retained in position by

welding a coil of alkathene to the thread of the cannula.

Collection and Storage of Samples.

Faeces were collected manually from the rectum into zip-top plastic bags, using a new plastic glove for each animal to avoid cross-contamination, and stored at -20°C for up to 2 weeks prior to thiaminase assay. Foodstuffs were placed in plastic bags and assayed within 24 hours of collection. Rumen contents were aspirated from sheep with fistulae via a plastic tube.

Thiaminase Assays.

Faeces, rumen contents and finely chopped foodstuffs were extracted into buffer and then assayed using 500 µg nicotinic acid/ml of substrate solution, as described previously. From each 'batch' of samples 4 or 5 with thiaminase activity were selected at random to check that the enzyme was of type I.

pH Estimation.

Rumen pH was measured by immersing the electrode of a Pye Unicam pH meter in freshly collected rumen liquor.

Results.

Flock Studies.

The work carried out with each flock is described in turn.

Flock 1.

About 120 ewes and lambs (2 to 4 months old) grazing permanent pasture near West Linton. Affected lambs were confined to one field.

Clinical History. Lambs were thriving well up to about 6 - 8 weeks of age, then many started to scour and

lose condition. They were treated with tetramizole (Nilverm) as a routine control measure for parasitic gastro-enteritis, but faeces samples collected by the Veterinary Practitioner revealed no evidence of nematode infestation, and the lambs continued to scour. During the 2 to 3 weeks subsequent to dosing 3 lambs died following signs of neurological disturbance. A fourth lamb killed in the terminal stages of a similar syndrome was found to have extensive brain lesions of PE in the cerebral cortex and cerebellum and the rumen liquor had thiaminase activity (Table 2; PE/19).

Faecal Thiaminase Estimations. Subsequently the flock was investigated by the author for faecal thiaminase activity. Of 46 lambs sampled, 15 (32·6%) showed thiaminase activity as shown in Table 9. No further investigations were carried out on this farm.

Conclusions. A significant proportion of sheep showing no evidence of neurological disturbance had faecal thiaminase activity.

Flock 2.

170 Greyface ewes grazing permanent pasture on the Moredun Institute farm at Roslin.

Clinical History. One sheep was found dead (PE/2, Table 2), histological examination of its brain revealed lesions of PE in the cerebral cortex and thiaminase activity was detected in the rumen contents and faeces. The other ewes were apparently normal and no other cases of PE occurred in this flock during the period of study.

Faecal Thiaminase Estimations. Faeces were collected initially from 39 sheep, 3 days after the case of PE was found and the remaining sheep (130) were sampled 10 days later. In the first group 7 samples contained thiaminase activity (17·9%) whilst in the second group 29 were positive (22·3%).

Conclusions. Again a significant proportion of sheep which appeared clinically normal had faecal thiaminase activity.

Further Studies with Flock 2. Small groups of sheep with faecal thiaminase activity were selected for sampling daily, or on alternate days; these animals were either left on the original pasture (Group A) or removed to the Moredun Institute and housed in loose boxes on a diet of hay, beet pulp and protein concentrate nuts (Groups B1 and B2) as indicated in Table 10. In addition selected animals were treated with a single intramuscular injection of 100 mg thiamine, to see if this influenced the presence of the enzyme in the faeces.

Details of the groups and the results of faecal thiaminase estimations are presented in Table 10. The results indicated that thiamine injections, a change of environment or diet had no apparent effect on faecal thiaminase levels, which fluctuated, declined and then disappeared within 8 days in the majority of the sheep studied. In one animal the enzyme was found in the faeces for 15 consecutive days, without any evidence of clinical abnormality. Once activity had gone from the faeces, in all but one case, there was no further detectable thiaminase up until the study ceased. It was thus concluded

from this work that sheep may have thiaminase in the faeces for 1 to 2 weeks without any apparent ill-effect. Furthermore, when individual sheep changed from being 'thiaminase excretors' to 'non-excretors' of the enzyme, the transition was abrupt.

Flock 3.

About 1000 Greyface ewes were kept with their cross Suffolk lambs on arable land near St. Boswells. The study was conducted in collaboration with Dr. K.A. Linklater, East of Scotland College of Agriculture, and the author did not visit the premises.

Clinical History. The flock was divided into groups of about 100 ewes with their lambs, and the outbreak of PE was initially confined to one group of 102 ewes with 180 lambs, on 15 acres of a 2 year-old pasture (Field X). The lambs were receiving 150 g whole barley daily as a supplement. One ewe was first affected with neurological disturbance, a profuse scour and abdominal pain, and despite thiamine injections it died. Post-mortem examination revealed brain lesions of PE. As the farmer attributed the scour to gastro-intestinal worms the ewes in Field X were all treated with oral thiobendazole. During the subsequent 5 days a further 7 ewes were affected with neurological disturbance, 3 died of PE and 4 responded to injections of thiamine.

The lambs (3 to 4 months of age) were then weaned and dosed orally with tetramizole as a routine control measure for parasitic gastro-enteritis, whilst the ewes were removed to another field. During the subsequent 7 days evidence of

neurological disturbance was observed in 12 lambs. They were given thiamine injections and responded favourably, and it was thus considered that they had probably been suffering from PE. However, no further cases occurred in the ewes which had been removed from field X. At this time no cases of PE had occurred in groups of sheep on other fields on the farm.

Thiaminase Estimations. Faeces samples were taken from about 100 lambs, selected at random, from those in field X, and of these 23 were found to contain thiaminase activity (23%).

Conclusions. Again a significant proportion of in-contact sheep which showed no evidence of neurological disturbance had faecal thiaminase. The PE cases in ewes and lambs in the majority of cases followed anthelmintic administration which was initiated by the diagnosis of parasitic gastro-enteritis based upon the presence of diarrhoea. The diagnosis of PE was supported by a favourable response to thiamine therapy. An attempt was then made to establish why the outbreak of PE was confined to sheep on one field.

Further Studies with Flock 3. Four groups of 20 lambs were selected for, approximately weekly, faecal thiaminase estimations, as follows:-

From the 100 lambs originally sampled on field X, 20 thiaminase positive (Group A) and 20 negative (Group B) were retained on field X. A further 20 negative (Group C) from the 100 were transferred to another field (field Y). Also 20 lambs from a further field (field Z) were selected

at random and put onto field X. The 60 lambs (Groups A, B and D) on field X were given barley supplementation and retained in 5 acres to keep the same stocking ratio. Group C did not receive barley.

These groups were sampled at approximately weekly intervals for 5 weeks with the following aims:-

Group A. To follow weekly thiaminase activity in known positive lambs on field X + barley supplement.

Group B. To follow thiaminase activity in a known negative group on field X + barley supplement.

Group C. To follow a known negative group on an 'unaffected' field (Y) with no barley supplement.

Group D. To follow the effect of taking lambs from an unaffected field with no barley to the affected field (X) + barley supplement.

The results of the thiaminase assays are presented in Table 11. Two further fatal cases of PE occurred, one on field X (PE/13 Table 2) and one on field Y (Table 11). Both animals had been thiaminase excretors preceding their death. During the 5 week period of sampling the faecal thiaminase activities of individuals within the 4 groups fluctuated (Table 11). These results are summarised in Figure 41, which shows that the positive and negative groups kept on field X remained distinctly separate with respect of thiaminase status until day 24. Also there was no marked difference between negative groups B and C, indicating that the pasture and barley supplement had not influenced thiaminase excretion between these groups. The randomly selected sheep from field Z were not adversely affected by their transfer to barley supplement on the 'affected' field.

This study failed to demonstrate why cases of PE were initially confined to field X, but it did show the large proportion of sheep which may have faecal thiaminase activity whilst appearing clinically normal; of the 80 lambs sampled repeatedly, 45 (56·25%) showed thiaminase activity on at least one sampling date. It also confirmed that faecal thiaminase activity of lambs at pasture fluctuates from week to week.

Thiaminase Estimations on Foodstuffs.

All foodstuffs including herbage, from fields involved in the outbreaks in flocks 1 and 3, and barley, hay, beet pulp and concentrate nuts used, proved negative for thiaminase activity.

Studies using Sheep with Ruminal Fistulae.

Details of these sheep are presented in Table 8.

Sheep on Hourly Feeding. Two sheep (C185 and C215) were maintained in metabolism crates fitted with automatic feed dispensers (designed and constructed by Mr. C. Hodgson, Moredun Institute), which delivered aliquots of a complete pelleted diet at 1 hour intervals to a total daily ration of 1 kg. Hourly feeding was employed to reduce the variations in the rumen fermentation pattern throughout the day. Water was available ad lib. To reduce environmental variation the crates were housed in a windowless room with continuous artificial lighting, maintained at 20°C ($\pm$ 2°C).

Ruminal and faecal thiaminase activity and ruminal pH were estimated daily for 30 days. Throughout the period of study one of the sheep (C185) was found to have thiaminase in the rumen as intermittent peaks of activity

(Figure 42). The other sheep (215) never exhibited any evidence of this enzyme in the rumen, and neither sheep showed faecal thiaminase activity.

The thiaminase peaks in C185 were not associated with a marked alteration of ruminal pH, though there was a slight reduction prior to each of the peaks (Figure 42). No environmental change was observed which could account for the occurrence of thiaminase peaks in C185, and both animals remained clinically normal throughout the period of study.

Sheep on Twice Daily Feeding. Eleven other fistulated sheep (Table 8) were sampled on alternate days for 3 weeks for ruminal and faecal thiaminase estimations. In 6 of these animals the enzyme appeared in the rumen (Table 8); 4 showed peaks of activity of similar duration to those observed in sheep C185, whilst in the other 2 the activity was lower and of shorter duration. Again no activity was detected in faeces samples and the sheep remained clinically normal.

Discussion.

The observation that clinically normal, in-contact sheep and lambs at pasture may have thiaminase activity in the faeces (Edwin and Lewis, cited by Edwin and Lewis, 1971; Roberts and Boyd, 1974) has been confirmed and extended in the present work. It was found in the flock studies that up to one third of the clinically normal, in-contact animals may be excreting thiaminase on any one day, and that over half the flock could be thiaminase excretors at some-time during an outbreak. Since thiamine

is important in many biological metabolic pathways and thiaminase type I both destroys this vitamin and manufactures 'anti-thiamine' compounds, the widespread occurrence of thiaminase may have significant implications in sheep, besides clinical PE.

One interesting feature of the flock studies was that in 3 instances the majority of PE cases occurred following the administration of tetramizole or thiobendazole. Both the latter anthelmintics are known to react with thiamine in the base exchange reaction catalysed by thiaminase type I (Roberts and Boyd, 1974). It is conceivable that the 'pyrimidinyl-product' from such a reaction could be responsible for cases of PE by acting as a competitive thiamine antagonist. This proposal would appear to merit investigation in view of the wide-spread use of anthelmintics of this type.

In fistulated sheep no enzyme activity was observed in the faeces even in those animals with high ruminal enzyme activity. It is not known of course whether this accurately reflects the situation in sheep at pasture, but if it does, faecal estimations may lead to underestimation of the 'thiaminase status' of flocks, and thus this enzyme may be even more wide-spread in sheep populations than the present study suggests.

The growth of certain ruminal micro-organisms is stimulated by thiamine (Hungate, 1966), and they might be influenced by thiaminase in their environment. Therefore this enzyme could affect such microbial populations and consequently rumen function. The interaction of thiaminase

type I with other factors in the rumen requires investigation.

Failure to demonstrate thiaminase in foodstuffs suggests they were not the source of this enzyme, a view supported by the observation that the frequency of feeding did not apparently alter the character of thiaminase peaks in sheep with ruminal fistulae. Thus, as the enzyme is probably not ingested, a search for the source of thiaminase in the rumen contents and faeces of sheep was instigated, and this is the subject of the next section.

SECTION V.

STUDIES ON THIAMINASE TYPE I-PRODUCING BACTERIA ISOLATED FROM RUMEN CONTENTS AND FAECES OF SHEEP.

General Introduction.

Naturally occurring thiaminases have been detected in a wide range of situations, including a variety of plants, moluscs and fish and in the faeces of humans (Murata, 1965). Of the 2 types of thiaminase, a number of bacterial species and fungi are known to produce the type II enzyme but the only bacteria known to manufacture thiaminase type I activity are Clostridium thiaminolyticus (sporogenes) and Bacillus thiaminolyticus (Murata, 1965).

Edwin, Lewis and Allcroft (1968) found thiaminase activity in decaying wood and mouldy grain on premises on which PE occurred; other workers failed to demonstrate thiaminase production by fungi isolated from mouldy straw on other affected farms (Loew, Smith and Dunlop, 1972). When the present study commenced there were no other preported attempts to find the source of the enzyme in affected animals. In view of the failure to demonstrate thiaminase activity in foodstuffs (Section IV), attention was concentrated on rumen contents and faeces for bacteria capable of producing the enzyme.

The first part of this section describes the search which resulted in the isolation of bacteria which produce thiaminase type I. In the second part evidence is presented which indicates that these bacteria are strains of Bacillus

thiaminolyticus, and finally this section describes studies carried out to assess the relevance of these organisms to ovine PE.

PART ONE.

THE ISOLATION OF THIAMINASE TYPE I-PRODUCING BACTERIA AND STUDIES OF THIAMINASES THEY PRODUCE.

Introduction.

Initial attempts to isolate bacteria, which produce thiaminase, involved culture on solid media inoculated with rumen contents or faeces. Individual colonies were sub-cultured into broth which was then assayed for thiaminase activity. As this procedure failed to isolate any thiaminase-producing bacteria, a modification of the nutrient infusion method of Kimura (1965) was adopted. The thiaminases produced by organisms recovered by this method were then compared with the thiaminases present in the rumen contents or faeces from which they were isolated. The bacterial thiaminases were also tested for their ability to mediate in the base-exchange reaction implicated by Edwin and Jackman (1970) in the aetiology of PE.

Materials and Methods.

Isolation Procedures.

Sources of Materials. Rumen contents from sheep affected with PE were obtained at post-mortem as follows:- the ventral abdominal wall was incised along the midline and held clear of the viscera, and the rumen wall incised with a sterile scalpel. The rumen liquor was poured into sterile Universal bottles, and either used immediately or stored at -80°C. Faeces samples were collected as described in section IV, and stored at -20°C. Rumen liquor from sheep

with ruminal fistulae, was obtained by aspiration using a sterile plastic tube, and stored at -80°C. Details of the sheep from which samples were obtained are listed previously in Tables 2, 8 and 10.

Media. Broths used were APT (Difco No.0655-02), peptone water containing 1% Bacteriological Peptone (Oxoid No. L 34) and 0·5% salt, and nutrient infusion broth (Cruikshank, 1969). Solid media included Hartley's digest agar with 5% horse blood (Cruikshank, 1969), nutrient agar (Oxoid No.CM 271), APT agar and MacConkey agar (Oxoid No. CM 7). Blood agar plates with added streptomycin (100 μg/ml of medium) and oxytetracycline (25 μg/ml) were prepared by adding the appropriate concentration of antibiotic in saline, previously sterilised by millipore filtration, to the molten media at 56°C.

Methods of Incubation. 10 ml aliquots of broth cultures were incubated at 32°C or 37°C in Universal or McCartney bottles or in 4 oz medical flats, using screw caps or cotton wool plugs as required. Medical flats were incubated horizontally with media just covering the lower surface of the container ("aerated broth"). Solid media were incubated in air or in Mackintosh and Fildes jars with an atmosphere of air + 10% CO_2, or hydrogen + 10% CO_2 ("anaerobic").

Selection and Purification of Bacteria. Well separated colonies were picked with a straight wire and purified by subculture and reselection of single colonies on solid media. If colonies spread extensively, media containing 2% agar were used to prevent spreading. Stock cultures were stored

on nutrient agar slopes at 4°C and at -80°C in serum broth with 17% glycerol added.

Thiaminase Assays. Supernates of broth cultures of the strains isolated were assayed for thiaminase activity, after centrifugation at 18,000 g for 1 hour as previously described for rumen contents. Bacteria which did not produce thiaminase in broth were discarded.

For comparative purposes a strain of B. thiaminolyticus (NCTC 10760) was obtained from the National Collection of Type Cultures (Colindale, London).

Comparison of Naturally-occurring Thiaminases with those Produced by 'Ovine Strains of B. thiaminolyticus'.

Source of Thiaminase Preparations. Rumen liquor of 5 sheep with PE and faeces of 3 in-contact animals with the enzyme provided naturally occurring thiaminases (Table 14). These samples were extracted with buffer as described in Section II (Page 12). The 8 strains of B. thiaminolyticus (OBT) isolated from these samples of rumen contents and faeces, were used to prepare the bacterial thiaminases as follows:- the strains were grown individually for 48 hours at 37°C in 150 ml peptone water in Roux flasks placed horizontally to maximise aeration of the broth. Previous tests had revealed that enzyme yield was maximal if broth depth did not exceed about 1 cm. Bacteria were removed by centrifugation at 18,000 g for 1 hour at 4°C.

The enzyme preparations, from gut contents and broth cultures, were filtered through a 0·3 μm pore-sized Millipore filter, and tested for sterility by plating onto blood-agar and incubating for 72 hours at 37°C. The sterile supernates were stored in 2 ml aliquots at -80°C.

Test for 'Type I' Activity of Thiaminases. The enzyme preparations were dialysed and tested in the absence of co-factor (Nicotinic acid) for determination of thiaminase type I activity.

Examination of Co-factor Specificity. A range of potential co-factors were used to assess the activation they produce in dialysed thiaminase preparations. The compounds used were selected on the basis of previously reported activation they exhibit for a range of bacterial, and non-bacterial thiaminases (Murata, 1965). Those used were nicotinic acid, pyridine, aniline, cysteine, para-amino benzoic acid, meso-amino benzoic acid, hydroxyproline, lysine and phenylalanine.

For this comparison it was necessary to bring the different enzyme preparations to the same activity, against a standard co-factor by dilution. In Section II it was demonstrated that the relationship between thiazole release and enzyme concentration was linear up to a maximum level of the assay (approximately 110 DPS). A series of dilutions of the alimentary and bacterial enzyme preparations ~~were~~ was made and these solutions assayed with a substrate solution containing 500 µg nicotinic acid per ml (4·06 mM). From these 'dilution curves' (examples of which are shown in Figure 5) the dilution required to bring the activity to 45 DPS was calculated, and aliquots of each preparation was diluted with buffer accordingly. The relative success of these procedures to produce enzyme preparations of similar activity is presented in Table 14.

The enzyme preparations were then assayed using each

co-factor at a concentration of 1 μmole per ml of substrate solution. For the calculation of enzyme activity in units the mean of 4 substrate/buffer blanks was used for the 'blank subtraction' (Section II, page 14).

Tests to Establish that Pyrimidinyl-nicotinic Acid is Produced from Thiamine and Nicotinic Acid in the Reaction Catalysed by the Thiaminases of OBT Strains.

The products of the reaction between thiamine and nicotinic acid were studied after separation by paper chromatography.

Thiaminase Preparations. For this work it was found that very active enzyme preparations are required to produce sufficient product to give readily detectable spots in chromatograms. The enzyme in peptone water cultures of the ovine strains of B. thiaminolyticus and the NCTC strain (10760) was concentrated by ammonium sulphate precipitation of sterile supernates. The supernates were brought to about 70% saturation by the addition of 38 gm of solid, anhydrous $(NH_4)_2SO_4$ per 100 ml, with continuous stirring. This resulted in the formation, almost immediately, of a viscous mucoid material with very little thiaminase activity. This material was removed manually using a glass rod and the remaining solution left at 4°C for 24 hours. The fine suspension produced was separated by centrifugation and redissolved in a small volume of distilled water, and then dialysed against citrate/phosphate buffer, pH 6·4, to remove the ammonium sulphate. These preparations were found to have very high thiaminase activity, being in the range of 500 to 3,000 units/ml, and were suitable for the chromatographic procedures.

Reaction Mixture. The substrate solution, prepared as described by Murata et al.(1968), consisted of 0·1 M thiamine and 0·4 M nicotinic acid in 0·1 M citrate/phosphate buffer, pH 6·4, restored to pH 6·4 with 1N NaOH. 0·2 ml of 'concentrated' thiaminase solution was added to 0·8 ml of substrate solution, incubated at 37°C for 24 hours, and then used for paper chromatographic separation.

Paper Chromatography and Tests for Pyrimidinyl-nicotinic Acid. Chromatograms were run on Whatman No.4 paper, in glass tanks, at 30°C with a descending solvent of butanol:acetic acid:water mixture (12 : 3: 5). The papers were washed by pre-running with the solvent, and dried in a stream of warm air. Spots, of approximately 1 µl volume, were applied to the paper using a Finnpipette micropipette, and after running the solvent for 7 to 8 hours, the papers were dried and developed in Dragendorfs reagent (Block, Durrum and Zweig, 1958). Duplicate chromatograms were run for development in alkaline ferricyanide solution (Araki, 1965) and examination under a UV light for fluorescence. Pyrimidinyl-nicotinic acid may be synthesised by using sulphurous acid in place of thiaminase in the above reaction, and provides a suitable control for the thiaminase type I reaction (Edwin and Jackman, 1970); thus 0·5 M sulphurous acid was used for the reaction and the subsequent mixture subjected to the chromatographic procedures and staining described above.

Results.

Direct Plating.

Rumen contents and faeces, which contained moderate to high thiaminase activity, were plated conventionally onto a range of solid media, including blood agar, MacConkey agar and APT agar. These plates were incubated at 37°C, aerobically and anaerobically, and examples of the individual colony types which had grown after 24 to 48 hours, were inoculated directly into nutrient broths. These broths were incubated for 72 hours, assayed, and those showing thiaminase activity were plated onto solid media. The colonies produced after 24 to 48 hours incubation were purified by replating, and then tested for their ability to produce thiaminase in broth. All 92 bacterial strains, isolated from 11 samples of rumen contents and faeces in this manner, showed no thiaminase activity after purification. It was not established why purification resulted in loss of thiaminase production.

Nutrient Enrichment.

The procedure subsequently adopted followed that of Kimura (1965) for the isolation of thiaminase producing bacteria.

Approximately 1 gram amounts of faeces with thiaminase activity, were inoculated in duplicate into nutrient broths, which were then well shaken. The broths were subsequently heated to 80°C for 20 minutes in a water bath to kill vegetative cells, shaken again and divided into 2 aliquots of 10 mls, one of which was incubated aerobically and the

other anaerobically at 37°C for 48 hours, and then assayed for thiaminase activity. The results of these assays were:-

Thiaminase activity (units).

Source (Sheep)	Original faecal extract.	Aerobic broth	Anaerobic broth.
B 560	8·93	(x) 7·86	1·74
B 564	9·88	(y) 10·05	0
B 551	4·58	0	0
B 565	10·46	0	0

Because higher activity was produced in broths incubated aerobically, than those incubated anaerobically, subsequent investigations were confined to bacteria which grow under aerobic conditions. Broths marked (x) and (y) in the above table were then treated as follows:-

a) Inoculated with a wire loop (Small inoculum) directly into fresh nutrient broth which was incubated for 48 hours at 37°C and assayed for thiaminase activity.

b) One ml (Large inoculum) of the broth was inoculated directly into fresh nutrient broth and then treated as for (a) above.

c) Plated conventionally onto blood agar plates which were incubated at 37°C and examined daily for 3 days. Samples of the colony types observed on these plates were picked, and inoculated into nutrient broth, which was then examined for thiaminase activity after 48 hours incubation at 37°C. After 3 days incubation a sweep from each blood agar plate was inoculated into nutrient broth, in case organisms responsible for producing thiaminase in the

original broth had been missed during the selection of individual colony types. The results of thiaminase assays on all these broths after incubation was as follows:-

Inoculum	Thiaminase activity (units).			
	From broth (x). (Sheep B 560)		From broth (y). (Sheep B 564)	
Broth to broth.	Broth No.	Units.	Broth No.	Units.
Small inoculum -	x 1	5·15	y 1	6·15
Large inoculum -	x 2	5·65	y 2	6·49
Broth to agar to broth.				
Colony type A -	x 3	0	y 3	5·74
B -	x 4	0	y 4	6·55
C -	x 5	4·03	y 5	1·10
D -	x 6	0	absent	-
E -	absent	-	y 6	8·87
F -	absent	-	y 7	0
Sweep inoculum -	x 7	5·02	y 8	6·26

This table demonstrates that the size of the inoculum used had no apparent effect on the amount of thiaminase produced, following direct broth to broth subculture from thiaminase containing broths (Broths x1, x2, y1 and y2). However, broth to broth subculture did 'sustain' the thiaminase activity in cultures.

The cultures obtained via solid media (broth to agar to broth in the above table) were then studied further with the following results:-

1) Thiaminase positive broths (x5, y3, y4, y5 and y6) were plated on solid media and selected organisms retested in broth. These then failed to show activity as had

occurred previously with purification of individual colony types.

2) Broths inoculated with a 'sweep' (x7 and y8), both had thiaminase activity indicating that a thiaminase producing organism may have been present on the agar plates but had been missed by colony selection. Replating of these broths onto blood agar and daily re-examination resulted in the isolation of an organism which produced thiaminase activity, and which was subsequently found to do so in pure culture.

Isolation of the First Strain of Thiaminase-producing Bacteria in this Study. The blood agar plate inoculated from broth x7 yielded a colony type which had not been observed previously in this work, and it was not apparent on this plate until the third daily examination. These colonies, of which there were several, were spreading, membraneous, adhered to the medium, and in Gram-stained smears were seen to consist of weakly Gram-positive slender rods. One of these colonies was picked, purified and inoculated into nutrient broth, and after 48 hours incubation at 37°C was found to produce thiaminase type I activity in excess of 32 units.

These organisms were studied further and found to have the following characteristics:- when incubated aerobically for 24 hours at 37°C they produced adhesive colonies, up to 1 cm in diameter, with a variable zone of complete haemolysis (Figure 43), peripheral to which was a narrow zone of incomplete haemolysis. Anaerobically they appear as a weak spreading growth, with branching extensions from the

periphery of colonies which were up to 5 mm in diameter after 24 hours incubation at 37°C. In Gram-stained smears the organisms were weakly Gram-positive, palisading, slender rods, approximately 2·5μ x 0·75μ (Figure 44), which produced, after 48 hours incubation on blood agar, at 37°C, large numbers of oval, thick-walled spores, centrally placed in terminal or sub-terminal sporangia. It produced both spreading and non-spreading colonies on blood agar (Figure 43), subcultures of either type resulted in a mixture of each type, and the organisms of either were indistinguishable in Gram-stained smears. It grew well on MacConkey agar, and its growth on this medium appeared to be enhanced by an atmosphere of 10% CO_2 in air. It also grew well on blood agar at 50°C. In peptone water at 37°C it produced a heavy even turbidity with variable pellicle formation.

Using Multodisk (Code S1) antibiotic discs with lawns of the organism grown on blood agar at 37°C it was found to be inhibited by chloramphenicol, erythromycin, cloxacillin, penicillin G, ampicillin and sulphafurazole, whilst being resistant to oxytetracycline and streptomycin.

During this work it was found that the thiaminase-producing strain isolated from the faeces of sheep B 560, and other similar strains subsequently isolated from other samples (Table 12), closely resembled B. thiaminolyticus (NCTC 10760); details of the comparison of these organisms are presented in part 2 of this section. The ovine thiaminase-producing strains in this work will therefore be referred to, for convenience, as 'ovine strains of B. thiaminolyticus'

(OBT), and numbered in order of isolation (First strain = OBT/1).

Selective procedures designed to facilitate the isolation of bacteria with cultural characteristics similar to those of OBT/1, were tried on a number of thiaminase containing samples of rumen contents from sheep which had been affected with PE. As a result a second strain, OBT/2, which was similar to OBT/1, was obtained after a number of manipulations which are summarised in Table 13. Again it appeared that direct broth to broth subculture favoured the growth of the thiaminase producing organism and subcultures from solid media using the sweep method retained thiaminase activity, whilst none of the colonies picked for subculture produced activity in broth after purification, except for those with OBT characteristics. The plate yielding OBT/2 required 96 hours incubation to produce a recognisible colony of this organism. As a result of this experience the following procedure was adopted for subsequent isolations:-

Untreated or heated rumen contents or faeces were inoculated into aerated nutrient broth and peptone water broth, which were then incubated at 37°C for 48 hours. Broths were then plated onto blood agar and MacConkey agar. The blood agar plates were incubated in air and the MacConkey plates in 10% CO_2 and air and were examined daily for 5 days for evidence of organisms resembling strains of OBT. This resulted in the isolation of a further 9 strains of OBT from 87 samples examined (Table 12), the majority of which were from rumen contents, but some rumen contents and many faeces samples which contained thiaminase failed to yield thiaminase-producing bacteria.

All the bacteria encountered in this investigation which were capable of producing thiaminase, were found to have similar cultural and morphological characteristics to those of OBT/1. A number of other strains superficially resembling OBT/1, which did not produce thiaminase, were not examined further.

Comparison of Alimentary and OBT Thiaminases.

The thiaminases produced by OBT isolates was of type I, resembling that demonstrated in rumen contents and faeces from which these strains were obtained. The enzymes from all these sources had similar co-factor specificities, pyridine being the most effective (Table 14), and in this they all resembled the thiaminase of B. thiaminolyticus (NCTC 10760).

Chromatographic Examination of the Products of the Thiaminase Type I Mediated Reaction between Thiamine and Nicotinic Acid.

Chromatographic separation of the products of the reaction between thiamine and nicotinic acid, gave a similar pattern for each of the ovine strains and the NCTC strain of B. thiaminolyticus thiaminases. This pattern consisted of four spots which stained orange with Dragendorfs reagent (Pattern a, Figure 47). Heat killed enzyme preparations and enzyme free substrate solutions produced one spot which had the same Rf as that of pure thiamine (Figure 47). Spots 1 and 2 were identified as pyrimidinyl-nicotinic acid on the basis of characteristics listed by Edwin and Jackman (1970), these being:-

a) They stain orange with Dragendorfs reagent.

b) They produce greenish-blue fluorescence under UV light when developed in alkaline ferricyanide solution.

c) The addition of 1 in 10, 1N HCl to the incubation mixture resulted in spot 1 predominating with spot 2 small or absent.

d) The substitution of sulphurous acid for the enzyme preparation in the incubation mixture resulted in the same pattern of spots (ie 1 to 4 on Figure 47), of which spots 1 and 2 had the same properties (a, b and c above).

Spot 3, which had the same Rf, as thiamine, probably represents the residue of this substrate and spot 4 is possibly equivalent to spot E of Edwin and Jackman (1970) (4-methyl-5 -hydroxyethyl thiazole), but a control for this compound was not used.

These findings indicate that the thiaminases produced by the OBT strains mediate in the base-exchange reaction implicated by Edwin and Jackman (1970) in the aetiology of PE.

Discussion.

B.thiaminolyticus has been isolated from the faeces of humans and a number of other species including rats, dogs, and cattle (Murata, 1965) but this is the first report of the isolation of similar organisms from sheep. These thiaminase-producing bacteria have been obtained previously almost entirely by workers in Japan, and the present work appears to be the first report of their isolation in Britain. The initial separation of the OBT strains proved difficult and similar problems have been inferred by the Japanese workers (Kimura, 1965). In the present study this was,

at least in part, attributable to the delay before visible growths appeared on solid media, which was in some instances overcome by prolonged incubation. The reason for the delay was not established. It was also found that aeration of the 'nutrient infusion step' facilitated their separation from mixed cultures, and represents another important feature of the isolation procedure.

Of the many hundreds of bacterial strains examined, only those resembling B. thiaminolyticus (NCTC 10760) were found to be capable of producing thiaminase. Though thiaminases of different origins vary widely in their ability to utilise individual compounds as co-factors in the base exchange reaction (Murata, 1965), the OBT strains produced thiaminases which had similar co-factor specificities and which resembled those of alimentary thiaminases. In each case, aniline, nicotinic acid and pyridine activated the enzyme, with pyridine being the most effective, whilst other compounds did not react. Ideally in the comparison of different enzymes, purified preparations should be used, as impurities may influence the reaction (Dixon and Webb, 1958). In the present work this was not possible, but the results obtained indicate a close similarity between the enzymes examined. Thus it was concluded that the thiaminase type I found in sheep may be of bacterial origin. Furthermore, it was found that the OBT thiaminases catalysed a reaction between thiamine and nicotinic acid, producing pyrimidinyl-nicotinic acid, which has been reported previously as a property of the thiaminases associated with cases of ovine PE (Edwin and Jackman, 1970). Therefore, some cases

of PE may be attributable to the *in-vivo* production of thiaminase type I by OBT strains.

The resemblance of the OBT strains to *B. thiaminolyticus* (NCTC 10760) was striking. The latter species has been implicated in a number of diseases associated with the presence of alimentary thiaminase type I (Kimura, 1965). It thus seemed important to establish the taxonomic position of the OBT strains, which is the subject of the following part of this section.

PART TWO.

TAXONOMIC STUDIES OF THIAMINASE TYPE I-PRODUCING BACTERIA ISOLATED FROM SHEEP.

Introduction.

Differentiation of species within the genus Bacillus was extremely difficult prior to publication of the key of Smith, Gordon and Clark (cited by Wolf and Barker, 1968) in 1946, and this led to the current classification (Wolf and Barker, 1968; Gordon, Haynes and Pang, 1973; Buchanan and Gibbons, 1974). At the time the present investigation started *B. thiaminolyticus* had not been accepted as a separate species in the classifications available (Breed, Murray and Smith, 1957; Wolf and Barker, 1968; Gordon, Haynes and Pang, 1973). Initially the classification of the genus Bacillus by Wolf and Barker (1968) was used but as neither the OBT strains nor *B. thiaminolyticus* (NCTC 10760) could be fully classified by it, additional tests were necessary.

Materials and Methods.

Bacterial Strains.

Eight of the OBT strains (1,2,3,4,6,7,8 and 9) purified as described on page 75, *B. thiaminolyticus* (NCTC 10760), *Bacillus alvei* (NCTC 6352), *Bacillus macerans* (NCTC 6355), *Bacillus polymyxa* (NCTC 10343) and *Bacillus circulans* (NCTC 2610) were examined.

Morphology.

Colonial appearance and texture were examined after 24 to 48 hours incubation on blood agar at 37°C. The appearance of vegetative cells and spores was examined by light microscopy in Gram-stained smears from blood agar plates. Vegetative cells and spores were prepared for electron microscopy from cultures grown on blood agar plates for 24 to 72 hours at 37°C. The plates were flooded with 1% glutaraldehyde in 0·1 M phosphate buffer pH 7·4. After 2 hours fixation the colonies were removed, cut into blocks 1 mm^3, and processed to araldite for the preparation of ultrathin section and examined by electron-microscopy as described for brain material (Section II).

Biochemical Tests. Where indicated the tests applied for identification of Group II members of the genus Bacillus (Table 15), were carried out as described in Cowen and Steel (C and S) (1965), and were as follows:-

Growth at 65°C at pH 7·0 or 60°C at pH 6·0; in nutrient broth (Oxoid, CM1), using a water bath.

Sugar fermentation tests; in peptone water broth with Bromthymol blue as an indicator (C and S, p.108).

Pathogenicity for insects; not tested.

Starch hydrolysis; starch agar and examined by method 1 of C and S (p.163).

Growth in 10% salt broth; at 37°C in nutrient broth (Oxoid CM1).

Voges-Proskauer reaction; Barritts' method (C and S), p.160).

Indole production; method 2 of C and S (p.158).

Catalase test; method 1 of C and S (p.150).

Antibiotic sensitivity tests were conducted with bacterial lawns grown on blood agar, using antibiotic sensitivity discs (Oxoid, Multodisk, Code S1).

Thiaminase in broth supernates estimated as in section II using cultures grown in nutrient Broth (Oxoid, CM1).

Polyacrilamide-gel electrophoresis. Polyacrilamide-gel electrophoresis (PAGE) of soluble proteins may assist in bacterial classification through the comparison of patterns produced (Lund, 1965; Morichi, Sharpe and Reiter, 1968; Morris and Park, 1973). PAGE was applied in an attempt to further clarify the taxonomic position of the thiaminase-producing bacteria. Strains were grown in peptone water for 24 hours at 37°C. The bacteria were harvested and washed 3 times in sterile 0·85% saline by centrifugation, and stored at -80°C prior to electrophoretic studies. PAGE was conducted by Dr. D.L. Mould (Moredun Institute) following the method of Foreshaw (1972) and the gels were photographed using the method of Fiske (1974). All strains to be compared were prepared and processed at the same time in order to minimise differences attributable to the technique.

Results.

Assessment of Purity.

This was based upon the consistency of colonial morphology, and appearance of the organisms in Gram-stained smears by light microscopy. Purified strains were subcultured using sweep inocula to avoid the selection of mutants (Meynell and Meynell, 1970).

OBT/1 differed from the other OBT strains in the production of 2 colonial types, spreading and non-spreading (Figure 43), which gave the appearance of a mixed culture. However, subcultures of either type onto blood agar resulted in a mixture of the 2 types of colonies. The organisms of spreading colonies were indistinguishable from those of the non-spreading ones in Gram-stained smears. It was thus assumed that OBT/1 was in pure culture inspite of the 2 colonial forms.

OBT Strains.

Colonial Characteristics. All these strains grew as adherent, smooth, flat, spreading (except for the non-spreading OBT/1), membraneous colonies on horse blood agar. Haemolysis, though generally weak, varied from complete to incomplete, with green discolouration and appeared to be affected by the batch of medium. All the strains tested however, produced similar haemolysis on any one batch of blood agar. Individual strains varied in the 'membrane strength' of colonies and their adherence to the medium, with OBT/4 consistently producing the softest and least adhesive colonies; OBT/4 was also the least prolific spore producer of these strains.

Morphology. Gram-stained smears of colonies on blood agar, after 24 hours incubation at 37°C, were found to consist of palisading, weakly Gram-positive, slender rods, approximately 0·75 μ x 2·5 μ (Figure 44); the measurements were confirmed by electron-microscopy. They produced large numbers of oval, thick-walled spores, centrally placed in swollen, terminal or sub-terminal, sporangia,

after 48 to 72 hours incubation (Figures 45 and 46). These features are consistent with those of Group II of the genus Bacillus in the classification proposed by Wolf and Barker (1968).

Group II Tests of Wolf and Barker (Table 16). For these tests, B. Alvei (NCTC 6352), B. macerans (NCTC 6355), B. polymyxa (NCTC 10343) and B. circulans (NCTC 2610) were used as controls.

The 4 control strains conformed to the characteristics defined for these species in Table 15, except that B. macerans (NCTC 6355) failed to produce gas in glucose broth (Table 16). Some strains of B. macerans may be erratic in this respect (Wolf and Barker, 1968).

The OBT strains were catalase positive, but the activity produced varied considerably between tests and OBT/3 gave only a very weak reaction or occassionally appeared negative. All the OBT strains produced considerable thiaminase type I activity in broth whilst the control strains showed no activity.

In the 'Group II tests' the OBT strains were readily distinguishable from B. polymyxa and B. macerans. They proved similar but distinct from the strains of B. alvei and B. circulans examined, in failing to produce acetyl-methyl carbinol and indole and not fermenting arabinose of xylose (Table 15 and 16).

At this stage Dr. J. Wolf was consulted, and he concluded after the examination of OBT/1 and 4, that they "belong morphologically to Smith's group 2, and since they grow anaerobically and ferment starch they are related

to B. circulans" (Wolf, 1974, personal communication). Consequently, 125 strains of B. circulans supplied by Dr. J. Wolf, were examined for their ability to produce thiaminase. The strains examined are listed in Appendix No. 3, and include examples of each of the sub-groups of the complex, distinguished on the basis of their spore antigens (Wolf and Chowdhury, 1971). No evidence of activity was detected in broth cultures of any of them, suggesting that production of this enzyme is not a feature of the B. circulans complex.

PAGE. The PAGE patterns for the 8 OBT strains revealed small individual differences within a basic pattern, which differed markedly from that of B. circulans (NCTC 2610) (Figure 50). The gel pattern of B. alvei (NCTC 6352) resembled those of OBT strains in the distal 2 thirds of the gel, but was different in the proximal third (Figure 50).

It was concluded that the OBT strains were a related group resembling but distinct from the B. alvei and B. circulans strains examined.

Comparison of the OBT Strains with B. thiaminolyticus (NCTC 10760).

B. thiaminolyticus (NCTC 10760) closely resembled the OBT strains both morphologically, in its response to the group II tests (Table 16) and in the production of thiaminase type I, but it differed from them in the following respects:- the colonies of B. thiaminolyticus (NCTC 10760) were non-adhesive and soft, and spore production was much less prolific than the least active spore producer of the OBT strains (No. 4).

It thus appeared that the OBT strains and B. thiaminolyticus (NCTC 10760) were members of a single group, with the species status of the organism known as B. thiaminolyticus which is still partially unresolved (Buchanan and Gibbons, 1974). In order to confirm the relationship between OBT strains and B. thiaminolyticus (NCTC 10760) the comparison of these organisms was extended to include certain other factors known to be of value in the taxonomy of the genus Bacillus.

Maximum Growth Temperature. The OBT strains grew well in nutrient broth at 50°C producing a turbid growth, with the exception of OBT/4 which produced a thin pellicle and no turbidity. B. thiaminolyticus (NCTC 10760) failed to grow above 45°C. The weak growth of OBT/4 at 50°C, and its less active spore production associated with less adhesive colonies, as compared with the other ovine strains, suggested that it lay between these and the NCTC strain in these respects.

Antibiotic Sensitivity. B. thiaminolyticus (NCTC 10760) was sensitive to the antibiotics tested with the exception of oxytetracycline, in which respect it resembled OBT/2, 3, 4 and 8 (Table 17). This is consistent with the variable sensitivity of the OBT strains to streptomycin and sulphafurazole, and the resistance of the majority to oxytetracycline (Table 17).

PAGE. Difficulty was experienced in producing comparable results between different PAGE batches for individual strains, and thus a separate batch was prepared for each comparison, which resulted in a weaker staining

reaction in this batch of gels (Compare Figures 50 and 51). There was a distinct similarity between OBT/1 and B. thiaminolyticus (NCTC 10760) which differed markedly from B. alvei (NCTC 6352) and B. circulans (NCTC 2610) which were examined at the same time (Figure 51).

Spore Ultrastructure. Spore structure has been used in taxonomic studies of the B. circulans complex (Gray and Hull, 1971), and thus OBT/1 and B. thiaminolyticus (NCTC 10760) spores were compared at the ultrastructural level, and found to have the following features in common:- the majority had an electron-dense, outer 'fibrous' coating of variable thickness, a laminated inner coat with 4 to 8 dense laminae, and the wall had 5 ridges on its outer surface running longitudinally (Figures 48 and 49). Though the sample of spores examined was limited, these findings suggested further resemblance between these 2 thiaminase-producing strains.

Discussion.

These investigations revealed considerable resemblance between the OBT strains and B. thiaminolyticus (NCTC 10760). Any differences observed between these organisms were considered to be minor and attributable to variation within a single group. The strain OBT/4, appeared to provide a link between the thiaminase-producing strains from sheep and NCTC strains which derived from human faeces. The similarity was further emphasised by the PAGE patterns, antibiotic sensitivity, spore structure and the properties of the thiaminases produced by these organisms. It was

thus concluded that the OBT strains should be classified with B. thiaminolyticus.

The Japanese Committee on the Problems of Thiamine Decomposing Bacteria, in 1951, considered that B. thiaminolyticus merited separate species status (Kimura, 1965). However, it was not accepted as a separate species by other taxonomists until 1974 when Buchanan and Gibbons considered it as a species requiring further taxonomic investigation. Using the criteria proposed by Wolf and Barker (1968) both the human and ovine strains of thiaminase-producing bacteria had characters intermediate between B. circulans and B. alvei. The absence of thiaminase production by the B. circulans complex, and the marked differences between the PAGE pattern of the B. circulans strain and that of the OBT strains, suggests that B. thiaminolyticus probably does not belong with the B. circulans complex.

It has been proposed that B. thiaminolyticus should be classified with B. alvei (Fijita et al., cited by Kimura, 1965; Hayashi and Nakayama, cited by Buchanan and Gibbons, 1974). This suggestion is supported by the partial similarity of the PAGE protein pattern of B. alvei to that of B. thiaminolyticus observed in this study. B. alvei does not produce thiaminase (Kimura, 1965; the present study), and has a characteristic colonial appearance (Wolf and Barker, 1968), which differs from that of B. thiaminolyticus. However, thiaminase is of doubtful taxonomic significance (Gordon, Haynes and Pang, 1973) and non-motile strains of B. alvei have been observed

(Clark, 1939, cited by Gordon, Haynes and Pang, 1973), which could result in the absence of characteristic satellite colonies of this species. It was therefore concluded from the present work that the term B. thiaminolyticus is a useful one in relation to thiaminase associated conditions, but its taxonomic acceptance as a distinct species should be deferred until more strains can be compared fully with B. alvei, as has been recommended by Gordon, Haynes and Pang (1973).

PART THREE.

ATTEMPTS TO ESTABLISH THE ROLE OF OF B. THIAMINOLYTICUS IN OVINE POLIOENCEPHALOMALACIA.

Introduction.

The thiaminase type I considered to be responsible for clinical PE (Edwin and Jackman, 1970), may be produced in some ovine cases by strains of B. thiaminolyticus (Section IV, Part One). Koch's postulates have proved invaluable for provision of criteria required to distinguish adventitious microbes from pathogenic ones (Davis et al., 1968). Koch's postulates state that if an organism is pathogenic it can be 1) regularly found in the lesions of the disease, 2) it can be isolated in pure culture on artificial media, 3) inoculation of the culture produces a similar disease in experimental animals and 4) the organism can be isolated from the lesions produced (Davis et al., 1968).

The diagnostic lesions of PE are in the brain, but these probably represent an indirect effect of thiaminase type I in the rumen contents (Edwin and Jackman, 1970). Thus for the application of Koch's postulates the "lesion" will be considered to be ruminal thiaminase activity. The first 2 postulates have been fulfilled, at least in part, as B. thiaminolyticus was isolated from affected rumen contents of sheep with the clinical disease, and grown in pure culture on artificial media (Section V, Part One). As an initial attempt to comply with the third postulate,

attempts were made to induce ruminal thiaminase activity, in the hope of producing clinical PE, by a) the administration to sheep of bacterial thiaminase produced *in-vitro*, or b) the administration of ovine strains of *B. thiaminolyticus* in pure culture.

Materials and Methods.

Experimental Animals.

The sheep used were 2 Dorset lambs (A and B), kept in a loose box, on a diet of hay, concentrate pellets and beet-pulp nuts, with water *ad lib*, and sheep No. C185, which had a ruminal fistula and was being maintained in a metabolism crate on hourly feeding (Table 8). The foodstuffs were examined every 3 to 4 days for evidence of thiaminase activity, and were found to be free of this enzyme throughout the period of study. Lamb A was a wether, and lamb B female, both being 4 months old at the commencement of the experimental period, with no ruminal or faecal thiaminase activity being detected during the 2 weeks prior to the experiments.

Preparation of Inocula.

Thiaminase Inoculum. This was a dialysed, ammonium sulphate precipitated thiaminase preparation (Section V, Part One) from a broth culture of OBT/4. The activity was assessed by assay of serial dilutions, just prior to administration, and found to be 447 Units /ml.

B. thiaminolyticus Inocula. OBT strains 4 and 13, were grown in pure culture in APT broth, the bacteria produced after 24 hours at 37°C harvested by centrifugation, resuspended in normal saline, and stored for up to 3 days at 4°C.

Prior to use the viable count of these dense suspensions was estimated using the method of Miles and Misra (1938).

Administration of Inocula.

Lambs A and B received inocula through a stomach tube, whilst the fistulated sheep was inoculated direct into the rumen via a plastic tube inserted into the fistula, to a point about mid-way between the fistula and the ventral ruminal wall. Bacterial cultures were administered via a sterile tube and funnel, and washed through with 100 ml of sterile saline.

Collection and Storage of Samples.

Faeces and rumen contents for thiaminase assays and bacteriological procedures were collected as described in Section IV, and used immediately or stored at -80°C for up to 2 weeks prior to use.

Bacterial Counting Procedures.

Serial 1:10 dilutions of rumen liquor in phosphate buffered saline pH 7·4 were prepared within 2 hours of collection, for viable counts. From each dilution 0·1 ml was spread onto each of 4 blood agar plates. Two plates were incubated aerobically and 2 anaerobically at 37°C for 72 hours, examined daily and observations made of the number and character of the organisms recovered in relation to thiaminase activity in the liquor under investigation.

Experimental Procedures and Results.

Administration of Thiaminase Type I.

Experiment A. The objective was to establish the amount of bacterial thiaminase required to maintain ruminal activity at a level comparable with that observed in cases

of PE, using a single large dose of the enzyme and following its rate of loss from the rumen. As it is not known if the faecal thiaminase found in sheep is of ruminal origin, faeces were also monitored to ascertain whether the enzyme passed through the gut.

Experimental Procedure: Lamb A, was given 145 ml of the thiaminase inoculum (total dose 64,815 units), which should have been detectable at a dilution of 1:1000, ie, in 145 litres of rumen liquor assuming even mixing and no destruction of enzyme activity or other cause of loss. Faeces and rumen contents were sampled for thiaminase estimations 30 minutes after inoculation, and thence daily for 3 weeks.

Results: No evidence of thiaminase activity was detected in any of the samples, including that taken 30 minutes after administration of the enzyme.

Conclusion: The ruminal fluid volume in lamb A was probably about 4 to 5 litres, and failure to recover activity in any of the samples suggested that the enzyme had been quickly destroyed, inactivated or lost from the rumen. The cause of this loss was not established, and as the procedure for preparing the enzyme was tedious this approach was no longer pursued and attempts were made to 'infect' sheep with OBT strains.

Administration of B. thiaminolyticus.

Experiment B/1. To establish whether the administration of a thiaminase-producing strain to a conventional sheep would result in ruminal thiaminase activity.

Experimental Procedure: Lamb B was used, having been free of thiaminase activity in the rumen and faeces during the preceding 5 weeks. An inoculum of 20 ml of B. thiaminolyticus strain OBT/4, with $3 \cdot 2 \times 10^9$ viable organisms per ml was administered by stomach tube and the lamb sampled for evidence of ruminal and faecal thiaminase activity 6 hours after the inoculation, thence daily for 7 days and then every 2 to 3 days for a further 3 weeks.

Results: No evidence of thiaminase activity was detected in any of the samples from this lamb.

Conclusion: OBT/4 failed to 'infect' this sheep, or had failed to produce thiaminase activity in gut contents during the period of study. This may have been due to conditions in the rumen being unsuitable for establishment of the infection. An experiment was then carried out to follow the fate of thiaminase-producing bacteria administered to sheep when conditions in the rumen might be "more favourable" to multiplication.

Experiment B/3. This was conducted in sheep C185, which was known to produce 'thiaminase peaks' in the rumen liquor (Section IV, Figure 42). The inoculum was prepared with OBT/13, which had previously been isolated from this animal during the height of a peak of ruminal thiaminase activity (Figure 42).

Rationale for the Experiment. OBT/13 was isolated by the nutrient enrichment procedure (Table 12) at the height of a peak (Figure 42) whilst similar procedures on a number of days when the ruminal thiaminase activity was low or absent in sheep C185, failed to yield the organism.

Therefore OBT/13 may have been responsible for the production of this peak of activity in the rumen. Based upon this speculation it was considered that the peak had occurred on this occasion possibly because the conditions in the rumen of sheep C185 prior to or during the peak, had favoured the growth of this thiaminase-producing organism. Thus in experiment B/2 an inoculum of this organism was given during a subsequent peak of thiaminase activity, in the hope that the conditions in the rumen at this time might favour its survival.

Experimental Procedure: The inoculum of OBT/13 (20 ml with $2 \cdot 1 \times 10^9$ viable organisms per ml) was introduced into the rumen of sheep C185 during a peak of thiaminase activity. Rumen liquor samples taken 2 minutes before, and 4 hours after inoculation and thence daily were subjected to bacterial counting procedures. Ruminal and faecal thiaminase activity was estimated concurrently.

Results: In the rumen contents taken from this sheep 2 minutes prior to inoculation no evidence of thiaminase producing bacteria was detected either anaerobically or aerobically. However, in liquor samples taken 4 hours after the inoculation and during the subsequent 3 days, a strain of OBT, apparently identical both culturally and morphologically to OBT/13 was observed in the mixed populations grown aerobically and anaerobically. Counts of these thiaminase-producing organisms on duplicate aerobically incubated plates gave closely similar results even after 1 - 2 weeks storage of samples at -80°C. These counts are presented in Table 18. The identities

of the organisms counted were confirmed by the examination of 8 colonies, selected from limiting dilutions of these organisms, for a) colonial texture and morphology on blood agar b) morphology of the organisms in Gram-stained smears from blood agar plates after 48 hours incubation at 37°C c) thiaminase activity in broth culture.

Table 18 reveals that there was loss of OBT/13 from the rumen of sheep C185 during the 3 days following its inoculation, but no marked increase in ruminal thiaminase activity followed administration of the organism. Again the administration of a thiaminase-producing strain had failed to stimulate production of this enzyme in the rumen. No thiaminase activity was detected in the faeces of this animal.

Discussion.

In PE thiaminase type I is believed to have a noxious effect upon the sheep and may thus be classed as a toxin (Stedmans' Medical Dictionary, 1966). An important step in the determination of toxicity by bacterial products is provided by the induction of symptoms resembling those of the disease attributed to the bacterium, by the administration of toxin in the absence of the organism (van Heyningen, 1955). In the present study, however, administered thiaminase rapidly disappeared, with little apparent effect on the animal. Thus toxicity of B. thiaminolyticus thiaminase in sheep has yet to be established.

Attempts to infect sheep with B. thiaminolyticus also met with little success, as this did not promote ruminal thiaminase production. It has been shown by other workers

that animals vary considerably in their resistance to thiaminase-producing bacteria (Hamada, cited by Evans et al., 1958), with sheep being particularly resistant to them. Polyneuritic symptoms, attributed to thiamine deficiency, have been induced in hens by the oral administration of B. thiaminolyticus (Matsukawa et al., 1955), whilst similar experiments with cattle (Evans et al., 1958) and sheep (Hamada, cited by Evans et al., 1958) failed to produce any evidence of clinical disturbance. Further work is required therefore to establish why species differ in their susceptibility to thiaminase-producing bacteria, and why ruminants appear to be so resistant to experimental infection with these organisms.

Sporing organisms depend for their success on the accumulation of spores in the environment and few have distinctive habitats (Buchanan and Gibbons, 1974). As the OBT strains are prolific spore formers, sheep may be constantly exposed to infection with multiplication of these bacteria in the rumen being dependent upon alterations of ruminal conditions. This may explain the failure of simple infection experiments, such as those carried out in the present study. Before the role of B. thiaminolyticus in ovine PE can be established it will be necessary to determine the factors in the ruminal microenvironment which control the ability of these bacteria to grow and produce the enzyme in an active form. The next part of the present work describes studies of some factors which may influence the success of thiaminase-producing organisms in the rumen.

Since the commencement of this investigation, strains

of Clostridium sporogenes which produce thiaminase type I activity, have been isolated from animals affected with PE (Shreeve and Edwin, 1974) including sheep (Shreeve, personal communication, 1975). Strains of Cl. sporogenes from sheep, kindly supplied to the author by Mrs. J.E. Shreeve, were included in the subsequent study.

PART FOUR.

THE INHIBITION OF B. THIAMINOLYTICUS AND Cl. SPOROGENES BY OTHER BACTERIAL SPECIES AND VOLATILE FATTY ACIDS.

Introduction.

During concurrent investigations, briefly described in Appendix No.4, it was found that B. thiaminolyticus (NCTC 10760) was inhibited on certain media by the thiamine dependent organism Lactobacillus viridescens (ATCC 12706). Subsequently it was observed that strains of both OBT and Clostridium sporogenes may be inhibited by a range of other bacterial species. The reason for this inhibition, along with attempts to establish its relevance to PE, are presented here.

Materials and Methods.

Bacterial Strains.

B. thiaminolyticus (NCTC 10760), OBT/1, 2, 4 and 9, and 2 strains of Cl. sporogenes were used. The bacteria studied for their ability to inhibit these strains are listed in Table 19.

Media.

APT and Reinforced Clostridial Medium (RCM) (Oxoid CM 119), broths were used, and solid media included blood agar, nutrient agar, and the above broths with 1·3% agar. Solid media were prepared in 15 ml amounts and poured into 4" diameter petri-dishes.

Inhibition Tests using a range of Bacterial Species.

On Solid Media. Lawns of B. thiaminolyticus or Cl. sporogenes were prepared by spreading 0·1 ml of a 24 hour broth culture over the surface of the solid media. Bacterial counts carried out on such broths (Miles and Misra, 1938), indicated that they contained 1 to 5 x 10^8 B. thiaminolyticus per ml of APT broth, or 2 to 8 x 10^9 Cl. sporogenes per ml of RCM broth. Organisms to be tested for their ability to inhibit the thiaminase-producing strains were applied to lawns by loop inoculation of an area approximately 5 mm in diameter, and the plates were incubated for 24 to 48 hours, the atmosphere and temperature being selected as follows:- where B. thiaminolyticus was tested the atmosphere was that required by the inhibiting strain; in the case of Cl. sporogenes they were incubated anaerobically; any plates inoculated with L. viridescens were incubated at 32°C with 10% CO_2. Plates were poured at 56°C and dried open for half an hour at 37°C; excessive drying produced irregular lawns of the test organisms.

Tests were also carried out in which inhibitory bacteria were removed prior to lawn application. The bacteria being tested in this way for inhibitory activity were patch inoculated as described above, after 24 hours incubation at 37°C the growth was removed with a glass slide and the plate sterilised with chloroform vapour (Gillies, 1964). Subsequently a lawn of thiaminase-producing organisms was applied to the plate, reincubated and examined for inhibition zones.

Effect of pH on inhibition: Inhibition tests were repeated using APT and RCM agar buffered to pH 6·0, 6·5, 7·0 or 7·5 using 0·05 M Sorensens phosphate buffer. The pH was checked with a Pye Unicam pH meter, and corrected where necessary with 1N NaOH.

Inhibition Tests in Broth. Streptococcus bovis and Streptococcus faecalis (Table 19), were grown in APT or RCM broths, for 48 hours at 37°C. The bacteria were removed from these broths by centrifugation at 18,000 g for 1 hour, and the supernates examined for inhibitory activity. These broth supernates will be referred to as "converted broths."

Inhibition at a Range of Dilutions and a Standard pH: The "converted broths" were mixed with an equal volume of 0·1 M Sorensens phosphate buffer at pH 6·0 and readjusted to pH 6·0 with 1N NaOH. Doubling dilutions of these preparations were made in APT or RCM broths, buffered to pH 6·0 in 10 ml aliquots. These diluted broths were autoclaved for 15 minutes at 15 lbs per square inch pressure, and after cooling each was inoculated with 0·1 ml of a 24 hour broth culture of a thiaminase-producing strain. The inhibitory activity of the "converted broth" was measured by its ability to delay or prevent the growth of the test strain. The turbidity of each dilution after 24 hours incubation at 37°C was measured using a nephelometer and an estimation of the concentration of bacteria, obtained from standard curves (Figure 52). These curves were prepared by making doubling dilutions from a broth of known viable count (Miles and Misra, 1938), the optical density for each dilution being recorded.

Inhibition at a Standard Dilution and Range of pH: Doubling dilutions of "converted broths" were prepared at pH 6·0, 6·5 or 7·0 in APT broth, in 10 ml aliquots. These were autoclaved, received a standard inoculum of a thiaminase producing strain and the inhibition measured as described above.

Effect of Dialysis on Inhibition of Broth: "Converted broths" were dialysed against 0·05 M Sorensens phosphate buffer at pH 6·0 for 48 hours at 4°C. These preparations were then compared with undialysed "converted broth" at pH 6·0 for the ability to inhibit thiaminase-producing strains.

Inhibition Tests Using Volatile Fatty Acids.

Results obtained from the present study indicated that the inhibition of thiaminase-producing bacteria may have been attributable to the production of VFAs by inhibitory strains. The VFAs, lactic, acetic and propionic were thus examined for their ability to inhibit the growth of B. thiaminolyticus and Cl. sporogenes in broth a) at a range of dilutions with a standard pH, and b) at a range of pH with a standard dilution. Tests were conducted in the same manner as described for "converted broths", with 1% VFA used in place of converted broth.

Effect of Succinic Acid on Inhibition by Bacteria Using Solid Media.

It has been shown that the inhibition of Salmonella enteritidis by certain volatile fatty acids may be prevented by the addition of 0·1 M succinic acid to the medium (Bohnhoff, et al, 1961). Inhibition tests were carried out

with known inhibitory strains (Table 19), on solid media at pH 6·5, with 0·1 M succinic added, and buffered with phosphate buffer.

Throughout, all broth cultures were checked for purity by plating on solid media and examining Gram-stained smears of growth produced after incubation for 48 hours at 37°C.

Results.

Unless otherwise stated, the results obtained for B. thiaminolyticus and Cl sporogenes strains were similar.

Inhibition Tests on Solid Media.

Of 21 bacterial species tested, 16 were found to produce a zone of inhibition in lawns of thiaminase-producing strains on APT or RCM agar (Figure 53 and Table 19). B. thiaminolyticus was inhibited under both aerobic and anaerobic conditions by the facultative anaerobes used. The Campylobacter strains failed to inhibit in-spite of producing a strong growth on test plates incubated under microaerophilic conditions (Lawson and Rowland, 1974). Repeated tests gave consistent results. No visible surface growth was present from the edge of the inhibitory plaque to a distance of 1 to 15 mm outwards (Figure 53). At the edge of the zone there was an area of reduced growth, in which individual colonies were apparent (Figure 53). Removal of the inhibitory colonies prior to sterilisation and application of the lawn revealed that the inhibitory activity remained in the medium and was resistant to chloroform.

On blood agar or nutrient agar no inhibition was produced by any of the strains. To establish whether a constituent of APT agar absent from nutrient and blood agar, was required to produce this inhibition the individual components of APT broth were added to nutrient agar. It was found that inhibition occurred on nutrient agar if 1% dextrose was included, but not with any other constituents of APT broth, in the absence of dextrose. No inhibition was produced if 0·1% dextrose was used.

Effect of pH on Solid Media. Inhibitory bacteria produced a distinct zone of inhibition at pH 6·0 and 6·5, and a very narrow zone or no inhibition at pH 7·0 and 7·5 in lawns of thiaminase-producing strains.

Effect of Succinic Acid. The inclusion of 0·1 M succinic acid in solid media containing 1% dextrose, prevented inhibition.

Inhibition in Broth.

"Converted broth" inhibited the growth of thiaminase-producing organisms at pH 6·0 this effect being proportional to the concentration of the converted broth (Figure 54). It was found that the inhibitory activity of a standard dilution of converted broth decreased with increased pH (Table 20).

Dialysis of converted broth resulted in loss of inhibitory activity.

From these results it was concluded that the inhibitory activity of the tested bacteria, on thiaminase-producing strains, was attributable to the production of a substance in the presence of glucose with the following characteristics:-

it is heat stable, dialysable, resistant to chloroform, and more active at low pH. Volatile fatty acids have all these properties, are produced by many bacteria in the presence of a suitable carbohydrate source, and in the present work glucose would be such a source. Thus lactic, acetic and propionic acid were studied for inhibitory activity on the thiaminase-producing strains.

Inhibitory Activity of Acetic, Propionic and Lactic Acids.

Doubling dilutions of the VFAs buffered in broth at pH 6·0 revealed that they inhibited the thiaminase-producing strains, and that this inhibition decreased with increasing dilution in a similar fashion to "converted broths" (Figure 54). At pH 6·0 in APT broth, 0·5% acetic, lactic and propionic acids produced almost complete inhibition of the growth of B. thiaminolyticus, whilst with increasing pH this concentration of these acids was decreasingly inhibitory (Table 20) and thus closely resembled the effects obtained with "converted broths." These findings suggest that the inhibitory activity of the bacteria tested was due to the manufacture of VFAs by these organisms.

Discussion.

This study has demonstrated that the growth of B. thiaminolyticus and Cl. sporogenes may be inhibited by the presence in cultures of other bacterial species. The inhibitory organisms used would be likely to produce one or more VFAs in the presence of an adequate source of fermentable carbohydrate (Davis et al., 1968), whilst the majority of non-inhibitory species tested do not metabolise glucose (Breed, Murray and Smith, 1957).

In broth cultures the inhibition produced by acetic, lactic and propionic acids, closely resembled that produced by broth supernates of inhibitory bacteria. These findings suggest that the "in-vitro" inhibition of B. thiaminolyticus and Cl. sporogenes may be due to the manufacture of VFAs by certain bacteria.

Matsukawa et al., (cited by Kimura, 1965) proposed that the absence of thiaminase bacilli and thiaminase disease in milk-fed infants may be due to the antagonistic action of Lacto-bacillus (Bifidobacterium) bifidum on the thiaminase producing bacteria, B. thiaminolyticus and Clostridium sporogenes. Kimura and Sakakibara (cited by Kimura, 1965) were however unable to confirm this phenomenon, when conducting tests in vitro. These workers used an adequate source of fermentable carbohydrate, but the pH of media used was 7·2. The inhibition produced in the present investigation was found to be pH dependent, and no inhibition by B. bifidus or other bacteria occurred unless the pH was below 7·0. Thus the pH of the media used by Kimura and Sakakibara could account for their failure to demonstrate inhibition, and the consequent conflict of opinion between these workers and Matsukawa et al.

Acetic and propionic acids may be present in the rumen at the appropriate pH, and at concentrations (Hungate, 1966) which in-vitro would inhibit both B. thiaminolyticus and Cl. sporogenes. If these organisms are involved in the production of the ruminal thiaminase implicated in the cause of PE, it is conceivable that the disease may be preceded by alteration of ruminal VFA concentration, pH or

other factors affecting the inhibitory activity of these acids.

The inhibition of thiaminase-producing strains by bacteria was antagonised by the presence of succinic acid in the media, and thus resembled the inhibition of Salmonella enteritidis by acetic and butyric acids (Bohnhoff, et al, 1961). These workers proposed that certain VFAs and pH may control the ability of S. enteritidis to survive and multiply in the colonic contents of the mouse. Thus these factors may modify the resistance of the mouse to this organism. Similar mechanisms may determine the ability of thiaminase-producing organisms to multiply in the rumen of sheep.

Members of the genus Bacillus and Cl. sporogenes are also inhibited by certain long-chain fatty acids (Galbraith et al,,1971), which may be present in the rumen (Cuthbertson, 1969). It is thus possible that the action and interaction of a range of factors, including fatty acids, control the production of thiaminase type I by bacteria in the rumen.

The inhibition of thiaminase-producing bacteria, by other bacterial species, may represent an important factor in the isolation of these organisms from mixed cultures. Rumen contents and faeces contain many bacteria capable of producing large quantities of VFAs from a suitable carbohydrate source (Hungate, 1966). Therefore, in these isolation procedures, the use of media containing such carbohydrates would be inadvisable.

General Conclusions from Section Five.

This work has indicated that thiaminase type I in the rumen contents and faeces of sheep may be produced by strains of B. thiaminolyticus. Concurrent investigations by other workers (Shreeve and Edwin, 1974), resulted in the isolation of strains of Cl. sporogenes from sheep affected with PE. The latter organisms produce thiaminase type I with similar substrate specificities to those of the enzyme of B. thiaminolyticus, and could thus be associated with the aetiology of PE, (Shreeve and Edwin, 1974). In the present study, not all the samples which contained enzyme yielded strains of B. thiaminolyticus, and this may be due to the difficulty experienced in their isolation. Alternatively, the thiaminase in such samples may have been produced by Cl. sporogenes or in some other way.

Attempts to produce PE by the administration of bacterial thiaminase, or strains of B. thiaminolyticus, failed. However, it has been demonstrated recently that the administration of Bracken thiaminase to sheep induces a condition which resembles natural PE clinically, biochemically and neuropathologically (Evans et al, 1975). The thiaminase of Bracken (Pteridium aquilinum) is of type I and has similar co-factor specificities to the thiaminases of B. thiaminolyticus and Cl. sporogenes. Therefore the work of Evans et al, (1975) not only strongly supports the implication of thiaminase type I in PE, but suggests that the thiaminase produced by B. thiaminolyticus or Cl. sporogenes would probably be capable of inducing the condition if they could be maintained for long enough and at a sufficient concentration in the rumen.

Both B. thiaminolyticus and Cl. sporogenes were inhibited in a similar manner by other bacterial species and volatile fatty acids. Thus it was concluded that a variety of influences on rumen function might provoke thiaminase production from either of these bacteria, with the final outcome being PE irrespective of which causative organism was responsible.

SECTION VI.

GENERAL DISCUSSION.

The characteristic brain lesions of Polioencephalomalacia do not by themselves permit a specific aetiological diagnosis. It is believed that many cases of clinical PE are induced by the action of thiaminase type I in the rumen contents of affected animals (Edwin and Jackman, 1970; Edwin and Jackman, 1973; Roberts and Boyd, 1974), and that the presence of this enzyme within the rumen has diagnostic significance (Edwin and Jackman, 1974). However, similar clinical and pathological changes to 'PE' may be induced by other mechanisms, such as Clostridium welchii type D toxin in sheep (Jubb and Kennedy, 1970) or lead poisoning in cattle (Christian and Tryphonas, 1971; Wells et al., 1975). Thus the first problem encountered in the present study was the differentiation of 'PE' from other similar neurological disorders of sheep. This necessitated an assessment of the uniformity of the pathological changes of the syndrome in relation to alimentary thiaminase.

There was found to be a good correlation between the presence of cerebro-cortical malacia and ruminal thiaminase type I activity, whilst the clinically normal 'control' animals used in the initial study showed no evidence of the enzyme. These findings are consistent with those of other workers (Edwin and Jackman, 1973; Roberts and Boyd, 1974) and thus supported the implication of thiaminase type I in the cause of PE (Edwin and Jackman, 1970).

It has been suggested that PE should be classed as a 'thiaminase disease' (Evans et al., 1975), however in this work of 21 cases of PE diagnosed on the basis of brain pathology, 2 had no ruminal thiaminase activity. Thus factors other than impaired thiamine metabolism could have been responsible for the brain lesions in these cases. It would probably avoid confusion if the term PE were in future confined, when-ever possible, to those cases caused by the action of thiaminase type I.

It has been suggested by Edwin and Jackman (1974) that the detection of "significant concentrations" of thiaminase in the rumen may be "accepted as strong supporting evidence towards a tentative diagnosis" of PE. In view of the extent of the distribution of this enzyme in the clinically normal sheep examined in the present study, the value of thiaminase estimations alone would appear to be doubtful. Thus whilst the absence of thiaminase could indicate another cause, its presence may impede the detection of factors responsible for similar diseases. It is considered that where possible the diagnosis of PE within a flock, assuming its classification as a 'thiaminase disease' (Evans et al., 1975), should be based upon the following criteria when ever possible:-

a) a consistent clinical history.

b) the presence of appropriate brain lesions in affected sheep.

c) the presence of thiaminase type I in the rumen contents of affected animals.

d) biochemical evidence of impaired thiamine metabolism associated with a favourable response to thiamine therapy in sheep during the early stages of the condition.

e) the exclusion of any other known causes of similar brain lesions.

Considerations of the action of naturally occurring thiaminase type I on ruminant metabolism will be influenced by the assay method adopted, and insensitive procedures could fail to detect small but potentially significant quantities of the enzyme. The isotope method first described by Edwin and Jackman (1970) has been used by a number of workers in studies of PE (Edwin and Jackman, 1974; Roberts and Boyd, 1974; and the present study). However, different concentrations of nicotinic acid have been used in the substrate solutions described by individual workers, this ranging from 0·29 (Edwin and Jackman, 1974) to 12·4 mMolar solution (Roberts and Boyd, 1974). In the present study the concentration of this co-factor was adjusted in an attempt to maximise the sensitivity of the assay without using a gross excess. The concentration selected (4·06 mM) appeared to yield reliable results, and was apparently an improvement on that recommended by Edwin and Jackman (1970).

In addition to concentration, the nature of the co-factor used in the assay, may also be important. This is demonstrated by the increased thiazole release observed when equimolar pyridine replaced nicotinic acid in the presence of a standard enzyme concentration. The nature of the

co-factor, or co-factors of importance in natural PE is unknown, but if such compounds are even more active than pyridine in the thiaminase type I mediated base-exchange reaction, the suitability of nicotinic acid in the assay procedure is doubtful, especially if only small amounts of enzyme are present. The ideal co-factor for this assay is probably that which gives the greatest sensitivity but further work is required on this aspect.

Standardisation of the 'thiaminase units' with careful statement of the conditions employed for the assay, would help to clarify comparison of results reported by different workers. It is preferable also that these should comply with the international requirements for enzyme units (International Union of Biochemistry, 1964). The expression of activity in less exact units makes assessment of results difficult.

The study of amprolium poisoning in this work demonstrated that the encephalopathy produced by this compound closely resembles that of natural PE. The mechanisms responsible for the bone-marrow damage, and the apparent anti-mitotic activity in the bone-marrow, which were also induced by amprolium, are unknown. It is tempting to draw an analogy with Bracken poisoning of cattle in which similar bone-marrow lesions occur in association with thiaminase type I (Naftalin and Cushnie, 1954) but a relationship between the mechanisms involved in these conditions has yet to be confirmed.

Diarrhoea may be a feature of natural PE (Little, 1969) and it is present in other conditions in which impaired

thiamine metabolism is implicated, including thiamine deficiency of lambs (Draper and Johnson, 1951), and oxythiamine poisoning of calves (Markson et al., 1972). The cause of the diarrhoea in amprolium poisoned lambs was not established. One of the principal causes of diarrhoea is retardation of water absorption from the lumen of the gut (Phillips, 1972). Water uptake in the gut is dependent upon the active transport of solutes across its wall, this process requiring energy (Phillips, 1972). It is therefore conceivable that impaired thiamine metabolism due to a range of causes, could result in defective fluid transport across the gut wall as a consequence of impaired energy metabolism. This is speculative but could be a working hypothesis for investigating the pathogenesis of the diarrhoea.

In the flock studies the greatest number of PE cases occurred soon after sheep had received oral anthelmintic treatment. The anthelmintics used were ones which are known to react with thiamine in the reaction catalysed by thiaminase type I (Roberts and Boyd, 1974), and could therefore possibly have yielded reaction products with antithiamine activity, and consequently contributed to the severity of the outbreaks studied. This would appear to be an area of potential economic importance in the sheep industry, and certainly merits further investigation.

The presence of thiaminase type I in fistulated sheep could be of importance to research workers using such animals. Thiamine has numerous and diverse functions in mammalian metabolism, both as a co-enzyme and as a metabolite

(Edwin and Lewis, 1971). Furthermore, many rumen micro-organisms are influenced by the availability of this vitamin (Hungate, 1966). Thus the metabolism of sheep may be influenced directly by the production of thiamine antimetabolites in the rumen, or indirectly as a consequence of changing ruminal microbial populations, as the result of the activity of alimentary thiaminase type I. This enzyme should thus be taken into consideration when conducting experiments in sheep.

In conclusion the probable sequence of events which precedes the onset of clinical PE includes some ruminal change which allows the manufacture of active thiaminase type I by bacteria, and the availability of a suitable co-factor for the production of thiamine antimetabolites. This situation, after an unknown period of time, induces a state of impaired thiamine metabolism in the animal, to which the brain appears to be the most susceptible. Further work to reduce the incidence of PE would probably be best directed towards attempts to establish which co-factors lead to the development of this disease, and the managemental changes which might be adopted to minimise ruminal thiaminase type I activity.

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APPENDICES.

Appendix No. 1

Davidson's Fluid.

From 'Studies of the Visual System of the Rat' by S. McCormick. PhD Thesis. University of Edinburgh, 1973. Volume 1, page 80.

20	parts	40% formaldehyde.
10	"	glacial acetic acid.
30	"	95% I.M.S.
30	"	distilled water.

Appendix No. 2

Calculation of the conversion factor used for thiaminase estimations.

For the purpose of these calculations it was necessary to determine the efficiency of extraction, by ethyl acetate, of thiazole from the reaction mixture. Repeated extractions were carried out with several thiazole concentrations and under the conditions defined for the assay procedure. The mean of 8 such extractions indicated that 73·0375 (SD $\pm$ 2·4254) % of thiazole entered the ethyl acetate phase.

The following data were also used:

1) 1 nCi ≡ 37 DPS.

2) Molecular weight of thiamine as the hydrochloride is 337·27 ($C_{12}H_{17}N_4OSCl.\ HCl$).

3) The thiaminase type I-catalysed reaction between thiamine and nicotinic acid yields one thiazole molecule per thiamine molecule cleaved.

4) The substrate solution contains 100 μg (296·4983 nmole) thiamine and 50 nCi per ml.

One unit of thiaminase activity yields 1×10^{-1} nmoles thiazole ml^{-1} $minute^{-1}$. Thus after correction for the fact that the efficiency of extraction is 73·0375%, 0·25 ml of enzyme preparation is used in the assay, only half of the ethyl acetate extract is used for counting, and the incubation is for 1 hour.

$$1 \text{ DPS} = \frac{296 \cdot 4983 \times 10}{37 \times 50} \times \frac{100}{73 \cdot 0375} \times \frac{4 \times 2}{60} \text{ units per ml of enzyme preparation.}$$

$$= 0 \cdot 292579 \text{ units.}$$

Thus in the calculation of units of thiaminase activity from 'DPS counts' the conversion factor $= \frac{1}{0 \cdot 292579}$

$$= 3 \cdot 417879$$

The conversion factor adopted for this work was 3·42.

Appendix No. 3

Bacillus circulans strains supplied by Dr. J. Wolf, Leeds, University.

NRS Strains.

290	384	399	716	924	1169	1177	1341	1352
295	387	625	727	977	1170	1218	1342	1356
313	388	639	728	1029A	1171	1275	1343	
359	390	666	826	1061	1172	1280	1344	
375	395	677	831	1136	1173	1281	1346	
381	396	678	838A	1145	1174	1282	1347	
382	397	679	839A	1167	1175	1337	1349	
383	398	715	841	1168	1176	1340	1351	

LS Strains.

2	10	25	64	82	92	98	114
3	11	27	67	83	93	102	117
4	15	30	72	84	94	104	118
8	17	31	79	85	95	106	120
9	22	41	80	86	97	107	122
							126

CN Strains.

1609	2201	2610	2629	2719	4076	4078	4080	4082
1761	2526	2621	2718	2755	4077	4079	4081	4083

Note: These represent a selection of the strains listed by Wolf and Chowdhury (1971).

Appendix No. 4

Detection of the inhibitory activity of *Lactobacillus viridescens* on *Bacillus thiaminolyticus*.

During these investigations an attempt was made to devise a simple plate test for the identification of thiaminase-producing bacteria. This test was to be based upon the use of *Lactobacillus viridescens* (ATCC 12706) which has an unusually high requirement for thiamine, and is employed in a microbiological method for the assay of this vitamin (Deibel, R.H., Evans, J.B. and Niven, C.F., 1957, Journal of Bacteriology, 74, 818). Lawns of *L. viridescens* were prepared on a range of media, and then *Bacillus thiaminolyticus* (NCTC 10760), was applied to a small area of the lawn in the hope of producing a zone of inhibition of the thiamine dependent strain as a consequence of the activity of thiaminase during incubation. However, *B. thiaminolyticus* failed to grow on these lawns. As a result of curiosity the 'test' was set up in reverse, and this revealed a wide zone of inhibition of the thiaminase-producing strain, by the thiamine-dependent lactobacillus.

Appendix No. 5

AN ULTRASTRUCTURAL STUDY OF OVINE POLIO-ENCEPHALOMALACIA

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PLATES XXXVII–XLII

THE terms " polio-encephalomalacia " (PE) and " cerebrocortical necrosis " are considered synonymous (Little, 1969; Edwin and Lewis, 1971). Polio-encephalomalacia was first described in sheep and cattle in the USA (Jenson, Griner and Adams, 1956) and a similar, if not identical condition was reported in Britain 3 yr later (Terlecki and Markson, 1959). This acute neurological disease affects several ruminant species and has an almost world-wide distribution (Little).

The pathology has been described by several workers (Jenson, *et al.*; Hartley and Kater, 1959; Terlecki and Markson, 1961; Zlotnik, Nisbet and Campbell, 1963), but no details of the ultrastructure of these changes have been reported. The present work provides such information and correlates these findings with those observed by light microscopy.

MATERIALS AND METHODS

Three sheep showing characteristic signs of polio-encephalomalacia (Terlecki and Markson, 1961) and two normal control sheep were studied (table).

Samples of rumen liquor from cases A and B and from both controls were examined for thiaminase-I activity by the method of Edwin and Jackman (1970). In cases A and B the diagnosis was supported by raised levels of thiaminase-I activity.

Intravenous heparin was administered at the rate of 0·5 mg per kg body weight and then the animals were anaesthetised with sodium pentobarbitone.

The brain was fixed by perfusion as follows. The animal was laid on its back, the common carotid arteries were exposed by blunt dissection through a long cutaneous incision and a 2-in. (50-mm) segment of one artery was gently clamped, and a metal cannula inserted and tied in with braided nylon. The clamps were removed from the vessel and the perfusing fluid, consisting of 3 per cent. glutaraldehyde in sodium cacodylate buffer at *p*H 7·4, was connected and allowed to run in via a drop chamber from a height of 100 cm above the cannula. The jugular vein on that side was incised and a cannula quickly inserted in the other carotid artery; the perfusing fluid was then run freely into both carotids and the other jugular vein was incised. The branches of these vessels that did not supply the brain were clamped off quickly to avoid wastage. Four litres of fluid were used in each case. The head was left on throughout this procedure.

The brain was removed 1 hr later and cut coronally into ¼-in.-thick slices. Alternate blocks of cerebral cortex were transferred to fresh perfusion fluid, and from the remaining tissue selected material was given 2 hours' post-fixation in du Bosq fixative at 60°C, and embedded in paraffin wax; 6-μm-thick sections were stained with haematoxylin and eosin (HE), and by the periodic acid-Schiff (PAS) and ninhydrin-Schiff methods, and examined by

Received 20 Apr. 1972; *accepted* 28 Sept. 1972.

light microscopy. On the basis of these observations, areas from the tissue stored in perfusion fluid were selected for Araldite embedding; small blocks were cut and immersed for 2 hr in fresh fluid, washed for 24 hr in cacodylate buffer at *p*H 7·4, post-fixed in 1 per cent. osmium

TABLE

Summary of the three test and two control animals studied

Animal and breed	Age (yr)	Sex	Duration of signs of disease (hr)
Case A (Cheviot)	$\frac{9}{12}$	F	12
Case B (Suffolk)	$\frac{6}{12}$	Castrated M	30
Case C (Blackface)	5	F	48
Control A (Dorset Horn)	$1\frac{4}{12}$	F	0
Control B (Blackface)	8	F	0

tetroxide and embedded in Araldite. Ultrathin sections were cut with glass knives on LKB and Reichert Ultrotomes and stained with lead citrate; 1-μm sections were stained at 60°C by Giemsa's method. Ultrathin sections were examined and photographed in a Siemens Elmiskop I electron microscope.

RESULTS

Characteristic lesions of PE were present in the cerebral cortex of all three clinically affected sheep; there was a wide range of severity both within and between cases. The earliest evidence of malacia was focal, laminar or diffuse spongy change, with moderate to severe neuronal necrosis in the middle of affected areas. The transition to necrosis of almost all cortical elements appeared to follow a fairly uniform pattern.

Early spongy change

This represented the predominant lesion in case A, was minimal in case B and absent in case C.

Light microscopy. The lesions contained numerous glial cells with large rounded nuclei, watery cytoplasm and swollen processes (fig. 1). In paraffin-wax-embedded sections the neuropil had a foamy texture (fig. 2), which was associated with numerous clear irregular spaces in the neuropil in Araldite-embedded material. Neurones and blood vessels were surrounded by clear spaces (fig. 2), which were found to be divided by membranes in Araldite sections and thus appeared as a series of vacuoles (fig. 1). Partial or total occlusion of capillary lumina, due to apparent compression, was observed in some areas. Degenerating neurones showed progressive shrinkage and angularity with increased cytoplasmic eosinophilia and moderate to severe nuclear pyknosis. Many neurones, each surrounded by a large clear space, showed no evidence of degeneration.

Electron microscopy. Consistent features of oedematous cortex were swollen watery astrocytes and satellite cells, many of which showed an apparent increase in nuclear volume. Organelles were dispersed in the electron-lucent cytoplasm. Mitochondria were of two types—either small and dense or hypertrophied. The latter, which predominated, were 2–3 times larger than those of control material, with a clear matrix and a small number of well-preserved cristae. Smooth and rough endoplasmic reticulum (ER) were generally well preserved, as were the glial fibrils and dense bodies in astrocytes.

Clusters of osmiophilic granules were observed, concentrated largely in the swollen processes of astrocytes, which ramified in the surrounding neuropil. These granules were 80–100 nm in diameter, consisted of subunits 15–18 nm in diameter and were considered to be glycogen. Oligodendrocytes were generally well preserved in moderately oedematous cortex.

The clear spaces observed in the neuropil in Araldite sections were readily identified and found to be membrane-bounded (fig. 3). In areas of moderate oedema about 40 per cent. contained glial fibrils and occasionally mitochondria, ER and glycogen granules, and were considered to be astrocytic. Approximately 55 per cent. could not be definitely classified, but the majority of those had irregular profiles, contained little or no evidence of microtubules or neurofilaments and no synaptic vesicles, but often showed glycogen granules and were probably astrocytic. Of the remaining 5 per cent. the majority contained synaptic vesicles and so were considered to be axon terminals.

In areas of severe oedema many axons showed increased density of the axoplasm and myelinated axons were intermittently separated from their myelin sheaths by an electron-lucent space. The only consistent change observed in dendrites was clumping of microtubules.

Hypertrophied mitochondria were evident in both normal and swollen glial and neuronal elements throughout the oedematous neuropil. Many had ruptured to produce crescentic or irregular profiles.

Oedematous neuropil showed a marked increase in the numbers of glycogen granules, and counts in representative fields were increased 5–6 times compared with controls. About 75 per cent. of these granules were within swollen astrocytic processes.

The perineuronal vacuoles described in Araldite sections were easily identified and found to be membrane-bounded. They were not easily classified, as many contained only bizarre membranous remnants and clumps of glycogen granules (fig. 4). They appeared to be cell-processes, but they did not show any evidence of synaptic junctions or vesicles. The less severely distended processes occasionally contained mitochondria, rough and smooth ER and clumps of glial fibrils in addition to glycogen granules (fig. 5). This suggests that the perineuronal vacuoles were also astrocytic. Between these distended processes normal axon-terminals with axo-somatic synapses were present (fig. 4, inset).

The perivascular spaces observed by light microscopy were found to be membrane-bounded; many contained mitochondria and rough ER and occasionally clumps of glial fibrils and glycogen. They were considered to be astrocyte end-feet (fig. 6).

Many neurones surrounded by large swollen processes showed no evidence of degeneration. Those undergoing necrosis followed a fairly uniform pattern of disruptive change in the perikaryon. Distension of Golgi saccules was the primary feature. Stacks of saccules had lost their compact arrangement (figs. 4 and 5) and ultimately appeared as an array of bizarre membranous fragments and vesicles.

In neurones with severe distension of Golgi saccules, Nissl ribosomes had lost their polysomal configuration to attain an amorphous appearance. Mitochondria were progressively distended and many were ruptured. Neurones were apparently compressed and showed variable electron-density, those most affected appearing as electron-dense angular bodies. (fig. 7).

Severe malacia

These lesions were seen predominantly in cases B and C, and were sharply demarcated from the adjacent cortex. They were not enclosed by a zone of spongy change.

Light microscopy. There was necrosis of almost all cortical elements except blood vessels. Necrotic neurones were scattered throughout the neuropil, which had a coarse, foamy appearance with irregular microcavitation. Adjacent to many blood vessels, which were prominent because of endothelial swelling and occasional mitotic figures, was a narrow zone of amorphous eosinophilic material. This material was PAS- and ninhydrin-Schiff-positive and diastase-resistant. Mononuclear inflammatory cells, principally monocytes and lymphocytes, were predominantly found near the meningeal surface. Many swollen neurones and glia were present in a narrow band of tissue abutting on the degenerate cortex.

Electron microscopy. Adjacent to necrotic neurones the outlines of swollen processes could often be detected (fig. 8). Swollen endothelial cells were seen to bulge into the lumen of many vessels (fig. 9) with apparent occlusion of smaller capillaries. The only consistent change observed in severely swollen endothelium was a reduced number of pinocytic vesicles (fig. 10).

The PAS-positive material resembled that in poorly perfused blood vessels and was considered to be precipitated serum-protein (fig. 10).

Neurones and glia adjacent to malacic cortex were swollen and watery, with organelles dispersed in the clear hyaloplasm (fig. 8). Neuronal and glial processes were often ruptured to produce an apparent increase in the extracellular space (fig. 8). There was only minimal evidence of an accumulation of electron-dense material in the true extracellular compartment in the tissue closely apposed to the malacic cortex.

Control material

Cortex from control animals showed a typical compact morphology by light microscopy (fig. 11) and by electron microscopy (figs. 12 and 13). There was no evidence of swollen astrocytes or satellite cells. Mitochondrial preservation was generally good, and about 10 per cent. showed sac-like distensions, characterised by the absence of cristae from the expanded portion of the wall.

The remainder were compact with a dense maxtrix (figs. 12 and 13). All other structures had an apparently normal appearance.

Discussion

The earliest evidence of polio-encephalomalacia (PE) observed by light microscopy was a foamy appearance of the neuropil with distended perineuronal and perivascular spaces and swollen glial cells. These changes represent consistent features in animals showing clinical symptoms for less than 24 hr (Terlecki and Markson, 1961) and were the predominant lesion in case A of the present study. It has been proposed by Markson and Terlecki (1968) that the basic lesion is necrosis of neurones with associated perineuronal and pericapillary oedema. The present study indicates that neuronal necrosis is secondary to an oedema of the intracellular compartment, as many neurones in oedematous tissue showed no evidence of degenerative change even at the ultrastructural level.

The oedema, which may be classified as cytopathic (Klatzo, 1967), involved principally astrocytes and satellite cells. Swollen watery astrocytes have been described in a wide range of conditions, including the effects of poor fixation (Lee and Bakay, 1966). The excellent correlation between the morphology of oedematous tissue in paraffin- wax- and Araldite-embedded sections and the absence of watery astrocytes in control material indicates that they are not artefacts, but evidence of early oedema in PE. The greater susceptibility of the cell processes to swelling, as compared with the cell body, may be due to surface-volume relation (Birks, 1965, cited by Cornog, Gonatas and Feierman, 1967).

Cells may swell because of defects of their volume-control mechanism or in response to alteration of their external environment (Ginn, Shelburne and Trump, 1968). Support for the suggestion that a defect of cell-volume regulation is responsible for the oedema in PE is provided by work done on rats treated with ouabain (Cornog *et al.*). The changes described in astrocytes in the cerebral cortex of these animals resembled closely those observed in the early oedematous process of PE. These workers attributed glial swellihg in impaired fluid control as a result of inhibition of Na^+-, K^+-activated, membrane-bound ATPase, which is believed to be related to, if not identical with the electrolyte transport mechanism (Ginn *et al.*).

Cellular volume-control depends upon energy for the active transport of electrolytes and it has been proposed that any factor that reduces the level of ATP may thus produce oedema (Sato *et al.*, 1969). There is considerable evidence that an aberration of thiamine metabolism plays a major role in the pathogenesis of PE (Little, 1969; Edwin and Lewis, 1971). This has been attributed to high levels of thiaminase-I activity, and the synthesis of thiamine antimetabolites in the rumen (Edwin and Jackman, 1970). The early oedema of PE may therefore be the result of reduced ATP-production following a defect of carbohydrate metabolism in the astrocyte. This hypothesis is supported by the suggestion that glial-cell swelling represents the earliest morphological change in thiamine-deficiency neuropathy of rats (Collins, 1967; Robertson, Wasan and Skinner, 1968).

It has been proposed, however, that astroglial swelling may occur as a response to extensive neuronal depolarisation as a result of the considerable release of K^+ and its retention by astrocytes (de Robertis, Alberici and Arnaiz, 1969). Thiamine *per se* is believed to have a role, independent of its co-enzyme function, in nerve impulse conduction and sodium transport (Cooper, Roth and Mohandas, 1963; Itokawa and Cooper, 1969, cited by Edwin and

Lewis). Maintenance of neuronal membrane potential is also energy-dependent. Thus thiamine may play a dual role, direct and indirect, in neuronal membrane physiology. Thus alternative mechanisms may be responsible for astroglial swelling. The oedema may therefore be secondary to a primary neuronal defect at the biochemical level.

That glial swelling represents the first morphological change in thiamine-deficiency neuropathy of rats, has been disputed by Tellez and Terry (1968). These investigators described primary alterations in axons and presynaptic terminals and proposed that earlier workers had killed their animals either too late or too early. This argument has yet to be resolved; examination of cases of PE in an even earlier clinical or a preclinical state may provide further information.

Pill (1967) argued that compression of arterioles may lead to tissue anoxia with consequent cortical necrosis. Vascular compression was a feature in areas of early spongy change. Arterioles were patent, but many capillary lumina were almost completely obliterated in some areas by swollen astrocyte end-feet.

Terlecki, Baldwin and Bell (1966) stated that the nature and distribution of the brain lesions in PE have all the features usually attributed to anoxia and that they do not differ essentially from those they observed in cases of surgical ischaemia. However, these workers described areas of spongy texture in which all neurones were abnormal. In PE in areas of oedema many neurones showed no evidence of degeneration. Furthermore, in ultrastructural studies of the effects of hypoxia and anoxia on the CNS, synaptic lesions and mitochondrial swelling represented the earliest change in neurones, followed by swelling of ER and Golgi apparatus (Hager, Hirschberger and Scholz, 1960; Webster and Ames, 1965). These changes differ from those observed in the early malacia of PE, but the value of a direct comparison is difficult to assess due to differences of technique and experimental subject.

In ouabain-treated rat cerebral cortex some neurones surrounded by swollen processes were apparently compressed and showed loss of polysomal configuration of ribosomes with pallor of mitochondrial matrices (Cornog *et al.*). These changes and those observed in neurones in early PE may thus be secondary to oedema of adjacent structures.

The clusters of osmiophilic granules present in increased numbers in areas of early malacia were considered to be glycogen on the basis of staining characteristic and morphology (Revel, Napolitano and Fawcett, 1960; Revel, 1964). Similar accumulation of glycogen, described within glial cells in thiamine-deficient rat cerebellum, were attributed to thiamine deficiency and the consequent impairment of carbohydrate metabolism (Collins and Converse, 1970). A similar proposal may be made for the increased glycogen in PE.

However, glycogen accumulation within glial cells has also been attributed to a lowered rate of glucose utilisation by neurones with piling-up of unused glucose in the surrounding astrocytes (Klatzo *et al.*, 1970). Thus impaired neuronal thiamine metabolism or neuronal damage due to local ischaemia following capillary compression, may account for the presence of increased glycogen in PE.

Swollen mitochondria with clear matrices have been described in a number of conditions including anoxia (Webster and Ames) and thiamine deficiency (Suzuki, 1967). In thiamine-deficient rat myocardium mitochondrial hypertrophy was attributed, at least in part, to a disturbance of energy production.

It is tempting to propose that glial-cell swelling, glycogen accumulation and mitochondrial hypertrophy observed in the present study were all directly associated with the primary defect of thiamine metabolism and the consequent disturbance of carbohydrate utilisation.

The amorphous material observed adjacent to many vessels, considered to

ULTRASTRUCTURE OF OVINE POLIO-ENCEPHALOMALACIA

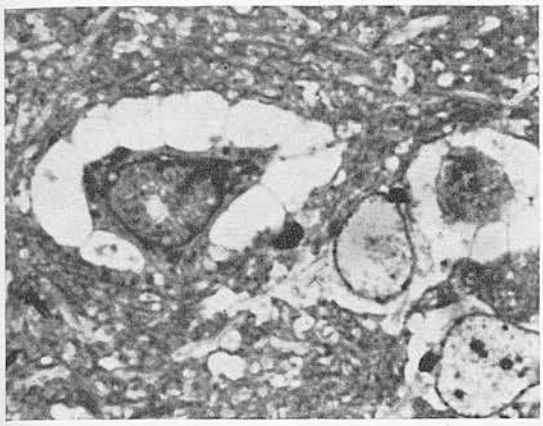

FIG. 1.—Case A. Parietal cortex. Area of early malacia. Irregular clear spaces in neuropil, perineuronal space divided by membranes, apparent compression of the neurone and swollen process of glial cell with large clear nucleus. Giemsa. ×1120.

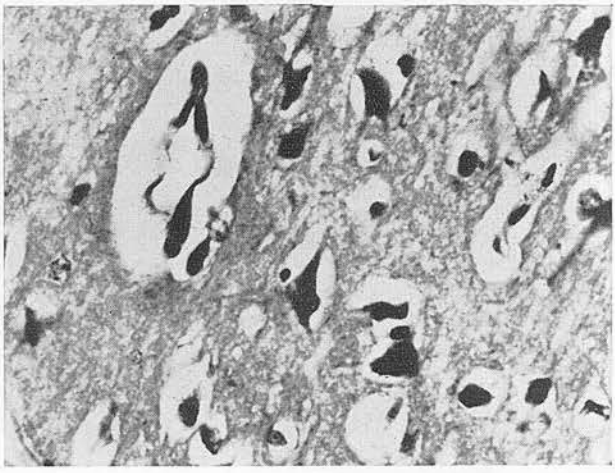

FIG. 2.—Case A. Internal pyramidal layer of parietal cerebral cortex. Area of early malacia. Foamy texture of the neuropil and distinct perivascular and perineuronal spaces. Haematoxylin and eosin (HE). ×480.

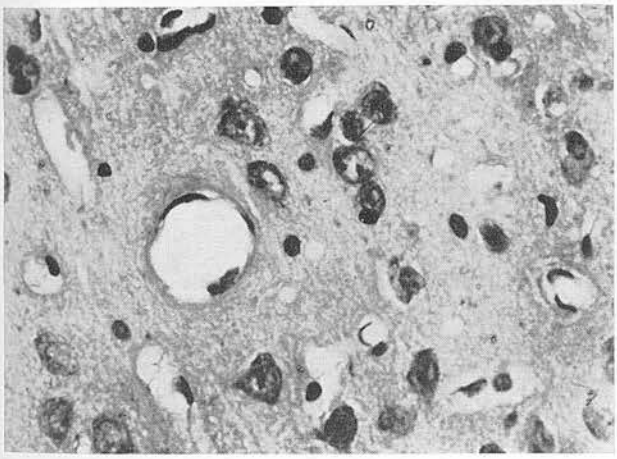

FIG. 11. — Control A. Internal pyramidal layer of parietal cerebral cortex. Compact neuropil and little evidence of perivascular and perineuronal spaces. HE. ×480.

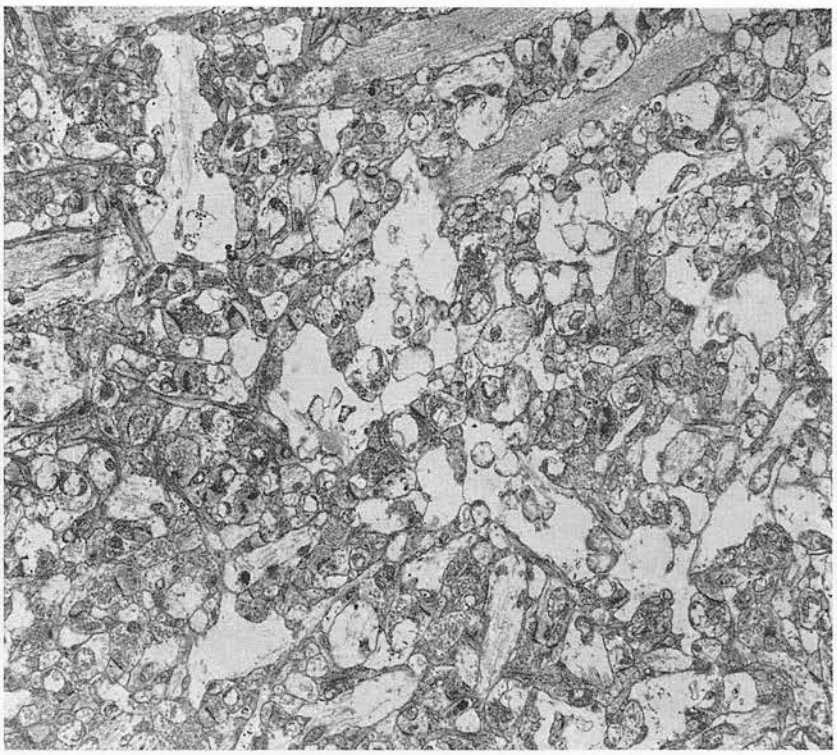

FIG. 3.—Electron micrograph (EM). Case A. Internal pyramidal layer of parietal cerebral cortex. Area of early malacia. Swollen watery processes in neuropil, many with glial fibrils and occasional clusters of granules. ×5400.

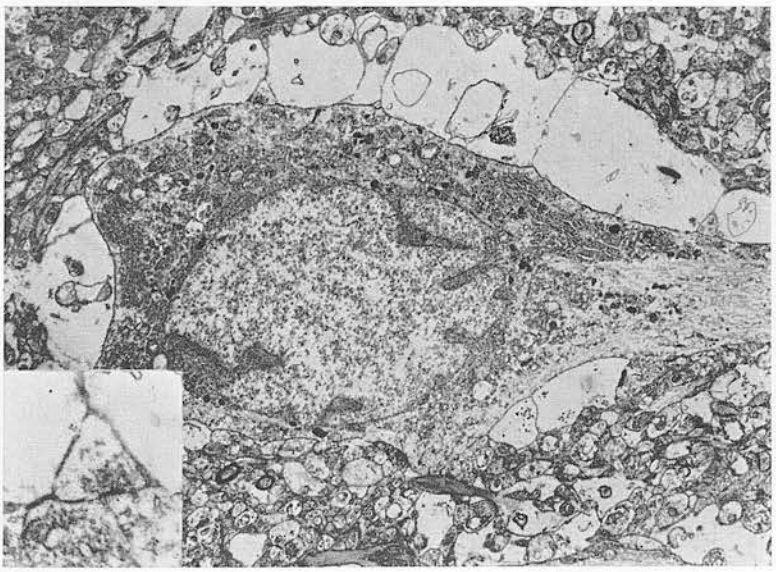

FIG. 4.—Case A. Internal pyramidal layer of parietal cortex in area of early malacia. Swollen juxtaneuronal processes containing glycogen granules and bizarre membraneous profiles. EM. ×4050. Inset—well-preserved axon-terminal and synapse. EM. ×18,000.

ULTRASTRUCTURE OF OVINE POLIO-ENCEPHALOMALACIA

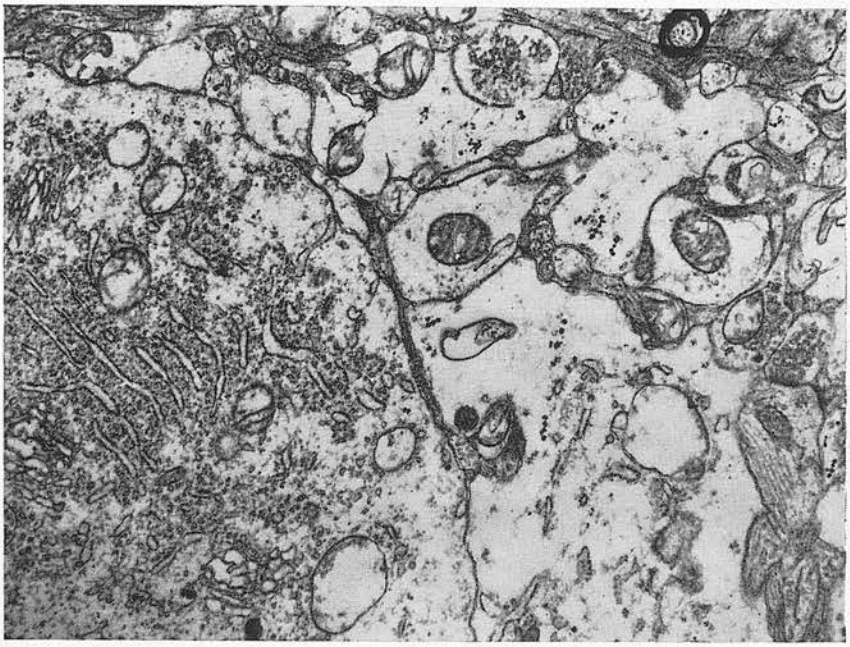

FIG. 5.—Case A. Internal pyramidal layer of parietal cerebral cortex. Area of early malacia. Glial cell process, adjacent to a neurone, shows early swelling. EM. ×15,000.

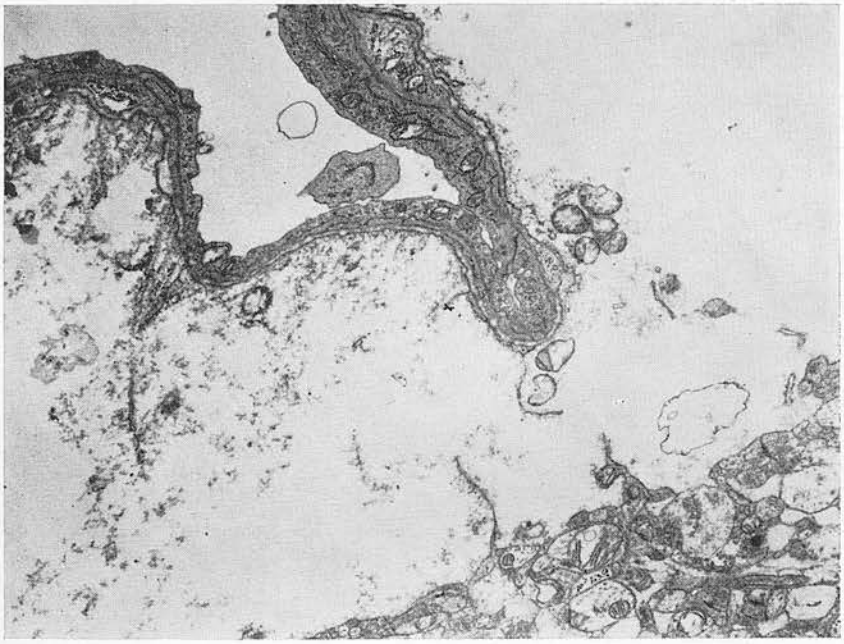

FIG. 6.—Case A. Internal pyramidal layer of parietal cortex. Area of early malacia. Severely swollen perivascular astrocyte end-feet. EM. ×9000.

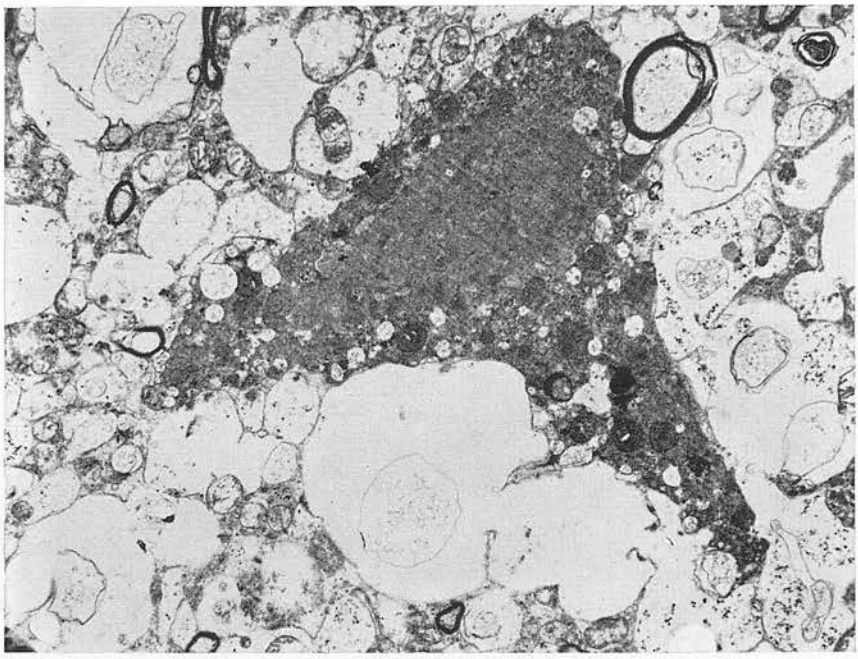

FIG. 7.—Case C. Parietal cerebral cortex. Area of severe malacia. Necrotic neurone with barely perceptible nucleus. EM. ×6000.

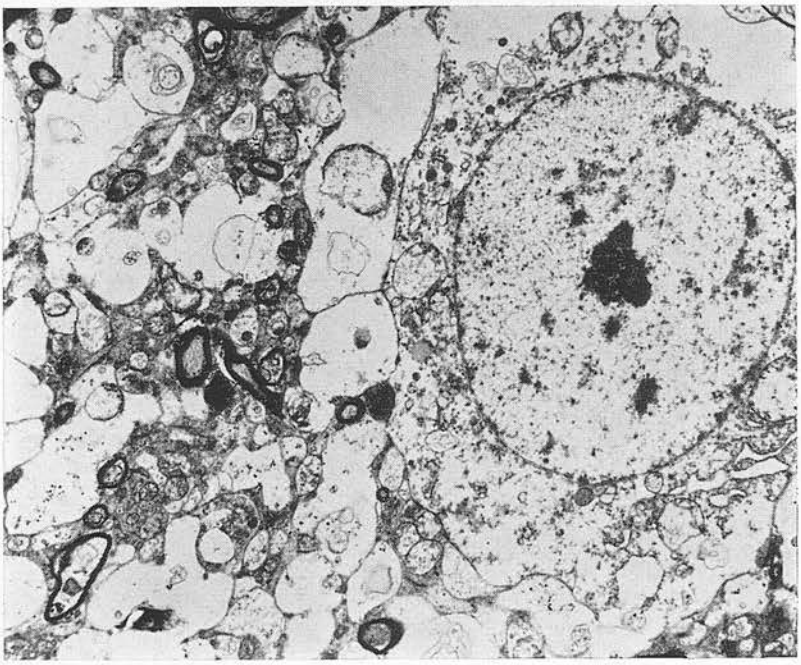

FIG. 8.—Case C. Periphery of malacic cortex. Swelling of glial and neuronal processes in the neuropil. Neurone showing moderate swelling, distension of ER and dispersal of remaining organelles. EM. ×6000.

ULTRASTRUCTURE OF OVINE POLIO-ENCEPHALOMALACIA

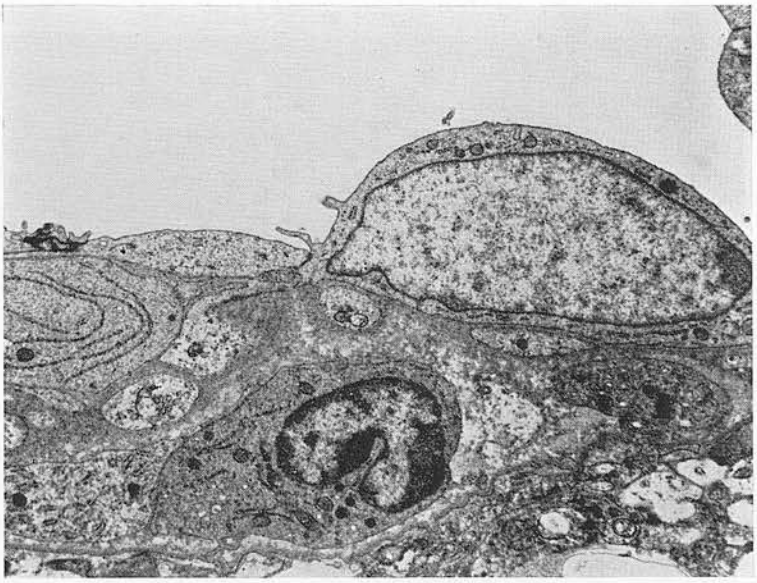

FIG. 9.—Case C. Parietal cerebral cortex, area of severe malacia. Swollen endothelial cell bulging into the vascular lumen. EM. ×9000.

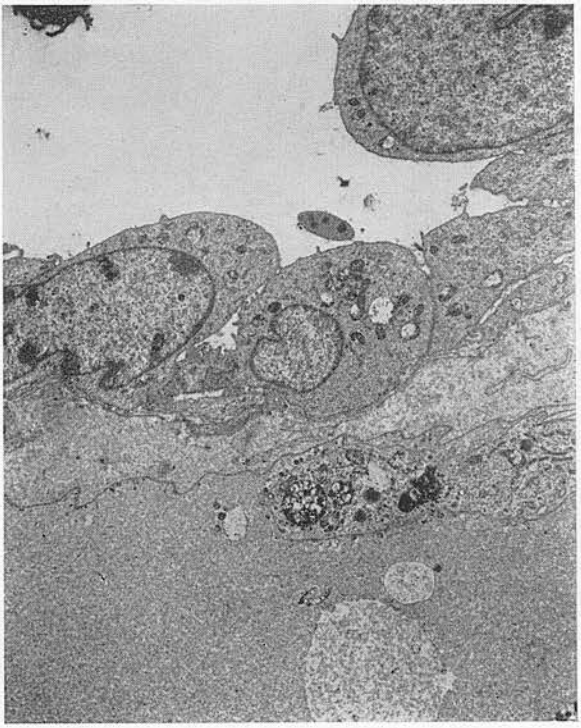

FIG. 10.—Case C. Molecular layer of severely malacic cerebral cortex. Endothelial swelling and apparent leakage of serum protein. EM. ×6000.

ULTRASTRUCTURE OF OVINE POLIO-ENCEPHALOMALACIA

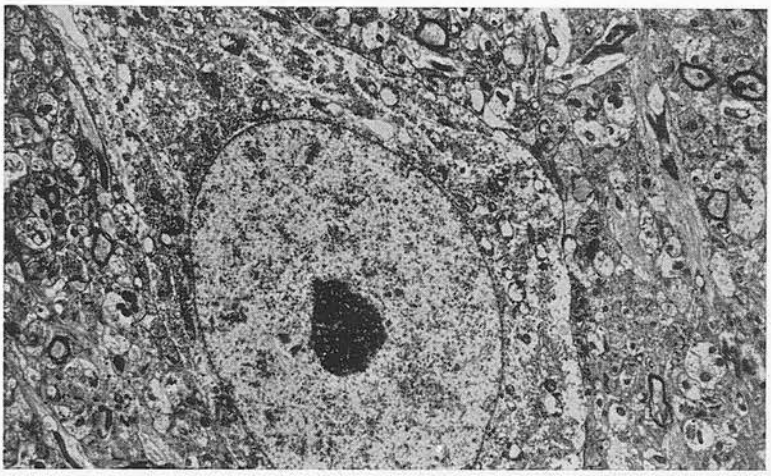

FIG. 12.—Control A. Internal pyramidal layer of parietal cerebral cortex. Compact neuropil, normal neuronal morphology. EM. ×4500.

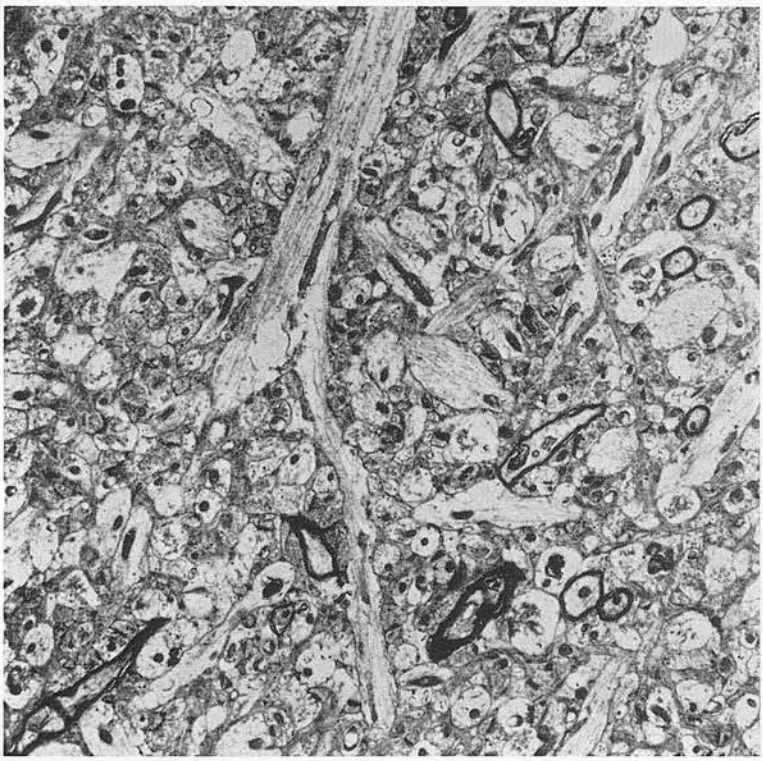

FIG. 13.—Control A. Internal pyramidal layer of parietal cerebral cortex. Neuropil shows normal compact morphology. Mitochondria generally well preserved with a dense matrix. EM. ×6000.

be serum protein on the basis of morphology and staining characteristics (Blakemore, 1969) suggests that vascular breakdown has occurred with the production of a state of vasogenic oedema (Klatzo). Many cases show severe brain swelling (Markson and Terlecki) and an increased CSF pressure has been recorded in bovine PE (Howard and Fawcett, 1966). Increased intracerebral pressure may be due to the disturbance of fluid exchange mechanisms associated with vasogenic oedema, but a correlation between the degree of swelling and the extent of vascular breakdown has yet to be established.

Summary

Cerebrocortical lesions in three cases of ovine polio-encephalomalacia were examined by light and electron microscopy. The earliest morphological evidence of the malacic process appeared to be spongy change consisting of perineuronal and perivascular spaces with a foamy texture of the ground-substance. At an ultrastructural level this appeared as oedema of the intracellular compartment principally involving astrocytes and satellite cells. Increased numbers of glycogen granules and hypertrophied mitochondria were observed in oedematous cortex. Neuronal degeneration appeared to be secondary and followed a uniform pattern with primary distension and disruption of Golgi saccules and loss of the polysomal configuration of Nissl ribosomes.

Older lesions were sharply demarcated from adjacent cortex by a zone of swollen neurones and glia. Endothelial swelling, leakage of serum-protein from blood vessels, and infiltration by mononuclear inflammatory cells were features of later lesions.

The significance of these findings, and their relevance to the implications of an aberration of thiamine metabolism in the pathogenesis of the disease, are discussed.

I am grateful to Dr R. M. Barlow and Mr J. S. Gilmour for helpful discussion, to Dr N. F. Suttle for carrying out thiaminase assays, to Mr W. Smith, Mr E. W. Gray and Mrs Pat Gray for their considerable help and patience in the preparation of electron micrographs, to Miss Sheila Wright for capable technical assistance and to Mr D. G. Watson, Mr B. J. Easter and Mr T. S. Bain for preparation of light micrographs.

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Appendix No. 6

AMPROLIUM POISONING OF PRERUMINANT LAMBS. AN ULTRASTRUCTURAL STUDY OF THE CEREBRAL MALACIA AND THE NATURE OF THE INFLAMMATORY RESPONSE

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Moredun Research Institute, Gilmerton, Edinburgh

PLATES CXXXVIII–CXLIV

IT has been proposed that the earliest morphological evidence of cerebrocortical malacia in cases of polioencephalomalacia (PE) is cytopathic oedema of astrocytes and satellite cells (Morgan, 1973). Lesions resembling natural PE have been produced experimentally in calves (Pill, *et al.*, 1966; Little, 1969; Markson, *et al.*, 1972) and sheep (Sinha, 1971; Loew and Dunlop, 1972) by administration of Amprolium* (1-(4 amino-2-n propyl-5 pyriminidymethyl)-2-picolinium chloride hydrochloride). This experiment has been conducted in order to study the morphogenesis of cerebrocortical lesions induced by Amprolium in preruminant lambs. In this work the terms polioencephalomalacia (PE) and cerebrocortical necrosis (CCN) are considered synonymous.

MATERIALS AND METHODS

Twenty-one Suffolk × Dorset lambs having received colostrum for 24 hr were reared on a proprietary milk substitute diet. The diet, " Nutrilamb "† milk replacer, was prepared to give a final level of 0·25 mg thiamine per litre of milk, and fed *ad libitum* from an automatic feeder. The lambs were housed on slats in loose boxes and the air temperature range was 8°–15°C. On arrival each animal received 11 mg/kg oral terramycin/day for 3 days and then phthalysulphathiazole was administered orally at the rate of 0·3 g/kg throughout the remainder of the experiment. The animals were split into two groups in adjacent loose boxes (table). The 12 test animals were given 280 mg/kg body weight Amprolium/day, orally, in aqueous solution. Of the nine control animals, four received 1 mg thiamine/day orally throughout the experiment and five were untreated. All animals were weighed at weekly intervals throughout the experiment. With two exceptions the lambs were killed at 2, 8, 18 or 36 hr after the onset of neurological signs.

Intravenous heparin was administered (0·5 mg/kg body weight) and the lambs were anaesthetised with sodium pentobarbitone. The brain was perfused via cannulae inserted in the common carotid arteries. Three litres of perfusate was run in from a height of 100 cm above the cannulae via a drop chamber, without a saline rinse. The perfusate used was a mixture of 4 per cent. formaldehyde and 1 per cent. glutaraldehyde in 0·1M phosphate buffer at *p*H 7·4. The brain was removed 1 hr later, and blocks 1 × 3 × 3 mm, for electron microscopy, were immersed in fresh perfusate for 24 hr, washed in phosphate buffer containing

Received 1 May 1973; *accepted* 13 July 1973.

* Amprolium—Merck, Sharp & Dohme Ltd.

† Nutrilamb—S.A.I. Ltd.

10 per cent. sucrose for 18 hr, fixed in 1 per cent. osmium tetroxide in phosphate buffer and embedded in Araldite. One μm Araldite sections were stained with Giemsa at 60°C. Paraffin sections of material that was immersed in fresh perfusate for 3 wk were stained with haematoxylin and eosin, periodic acid-Schiff, Bests carmine and ninhydrin-Schiff methods.

Results

Details of sex and time of experiment are shown in the table.

All the lambs grew well for the first 2 wk but in the 3rd wk the food consumption of the Amprolium-treated group decreased and the average weight gain of the group declined. The nine control animals grew well throughout

Table

Details of 12 test animals

No.	Sex	Total time of expt (days)	Approx. time elapsed from first CNS signs to killing (hr)	Haemorrhages present in the brain parenchyma	Brain swelling apparent macroscopically
1	F	27	36	−	+
2	F	26	36	+	+
3	F	28	36	+	+
4	F	24	18	+	+
5	M	27	18	−	−
6	M	26	18	−	−
7	F	23	8	−	−
8	M	25	8	+	+
9	F	26	2	−	−
10	F	25	2	+	+
11	M	30	...	−	−
12	F	30	...	−	−

and no significant difference in weight gain was recorded between those given a thiamine supplement and the untreated group. During the 4th wk 10 of the Amprolium-treated animals developed a characteristic clinical syndrome. Initially there was diarrhoea and anorexia which lasted for 24-36 hr. Clinical neurological signs developed within 1–2 days of the onset of diarrhoea. The ears drooped, and the lambs became lethargic, then ataxic and finally recumbent with nystagmus and intermittent convulsions, terminating in extensor spasms and opisthotonus. In recumbent animals convulsions could be precipitated by gentle dorsal flexion of the neck. Recumbent animals developed a slow laboured respiration and tachycardia. The clinical course from early lethargy to *extremis* lasted approximately 36 hr.

Due to this consistent clinical syndrome, it was possible to sample serially throughout the period of neurological disturbance (table).

Gross pathology. All the test animals showed petechiae and ecchymoses in most organs. In animals killed *in extremis* haemorrhages tended to be diffuse in tissue such as myocardium, bladder wall, subcutaneous loose connective tissue and intestinal serosa. Ten animals showed areas of cerebrocortical malacia of variable extent and of similar appearance to that observed in

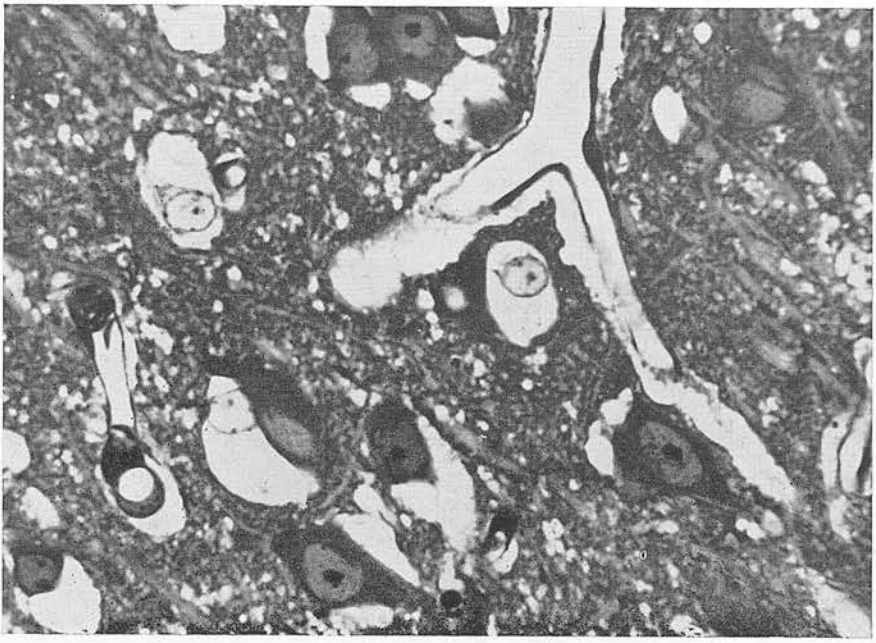

FIG. 1.—Case 9. Parietal cerebral cortex. Area of early malacia. Astroglial swelling, foamy vacuolation of the neuropil and perivascular spaces. Giemsa. ×180.

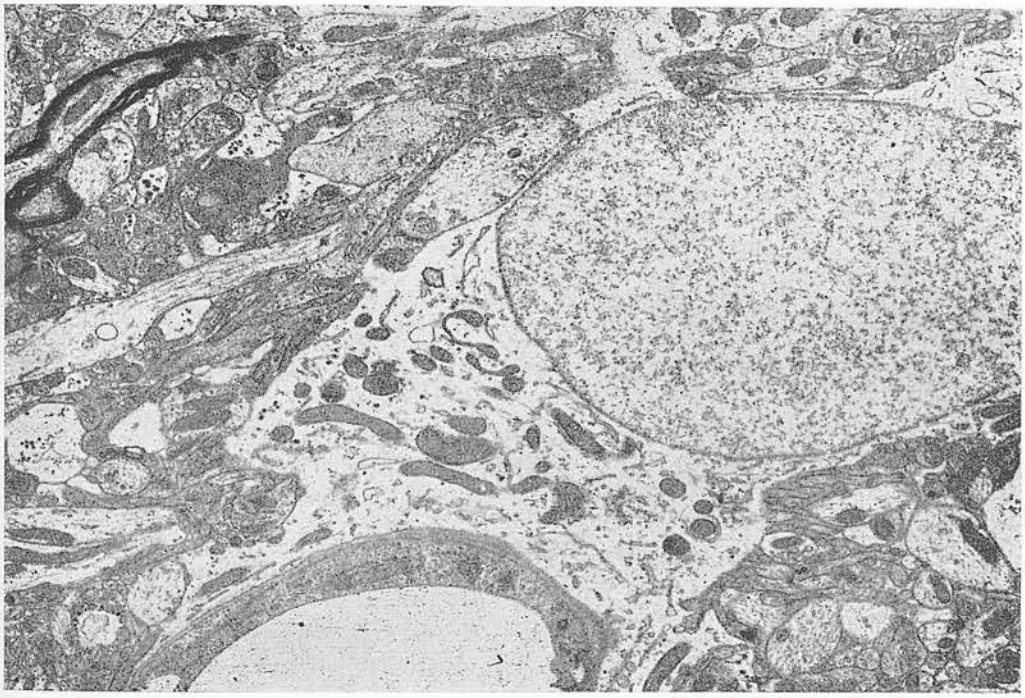

FIG. 2.—Case 9. Parietal cerebral cortex. Area ot early malacia. Moderately distended astroglial cytoplasm with swollen nucleus. Many swollen astroglial processes in neuropil contain glycogen granules. Electron micrograph (EM). ×9000.

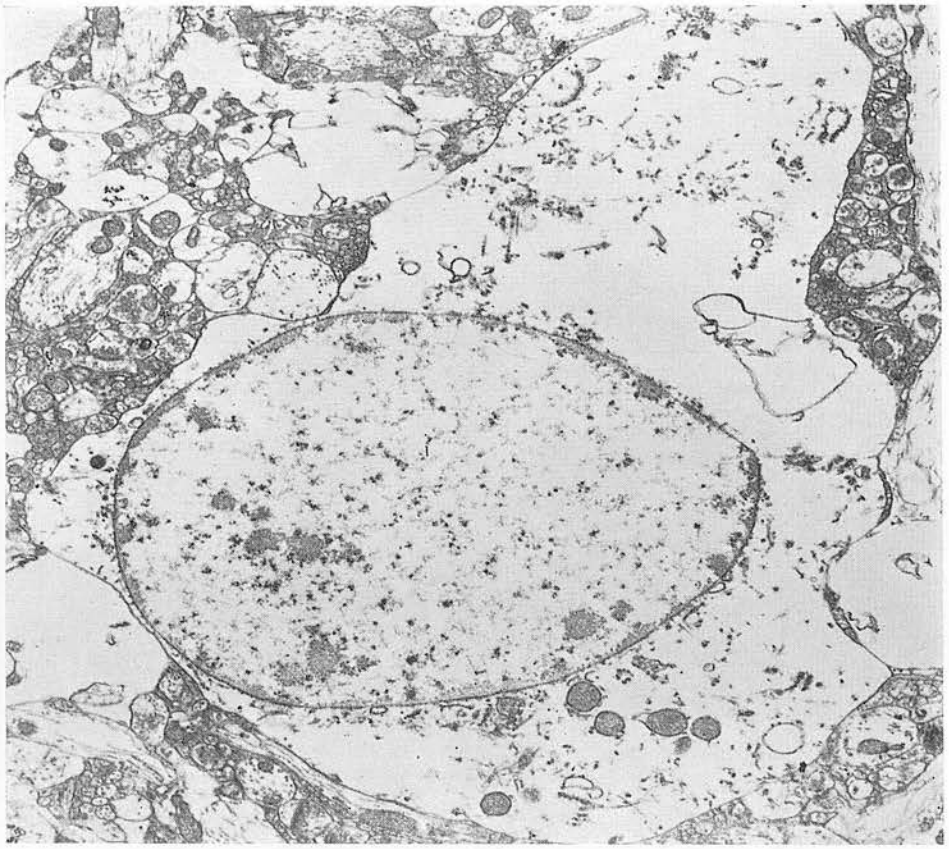

FIG. 3.—Case 8. Parietal cerebral cortex. Area of early malacia. Moderate clumping of organelles in severely distended watery astrocyte hyaloplasm. Aggregation of chromatin in distended nucleus. EM. ×9000.

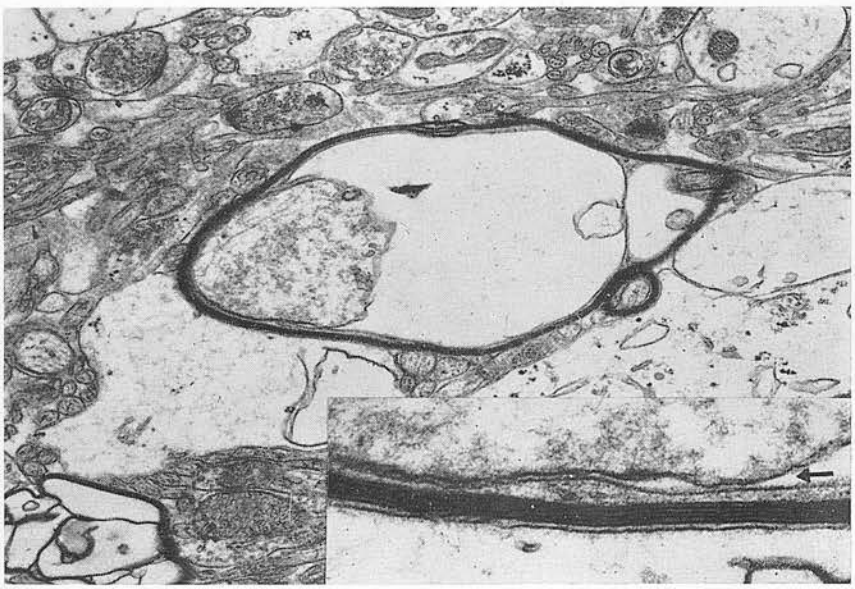

FIG. 4.—Case 9. Parietal cerebral cortex adjacent to white matter. Distended myelin sheath. EM. ×15,000

Inset—To show separation of oligodendrocyte and axon (arrow). EM. ×60,000.

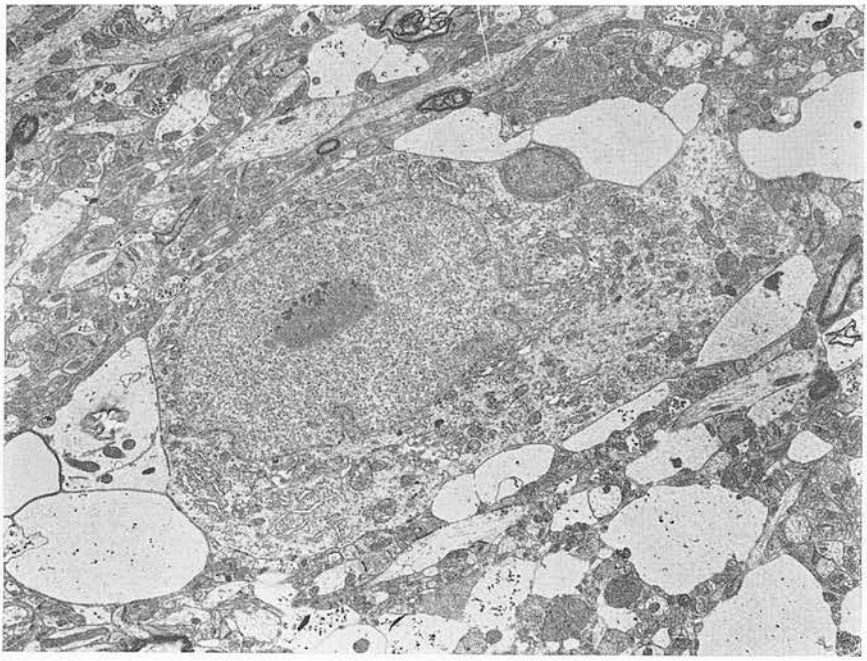

FIG. 5.—Case 7. Neurone in area of early malacia showing minimal distension of mitochondria and golgi saccules. EM. ×4500.

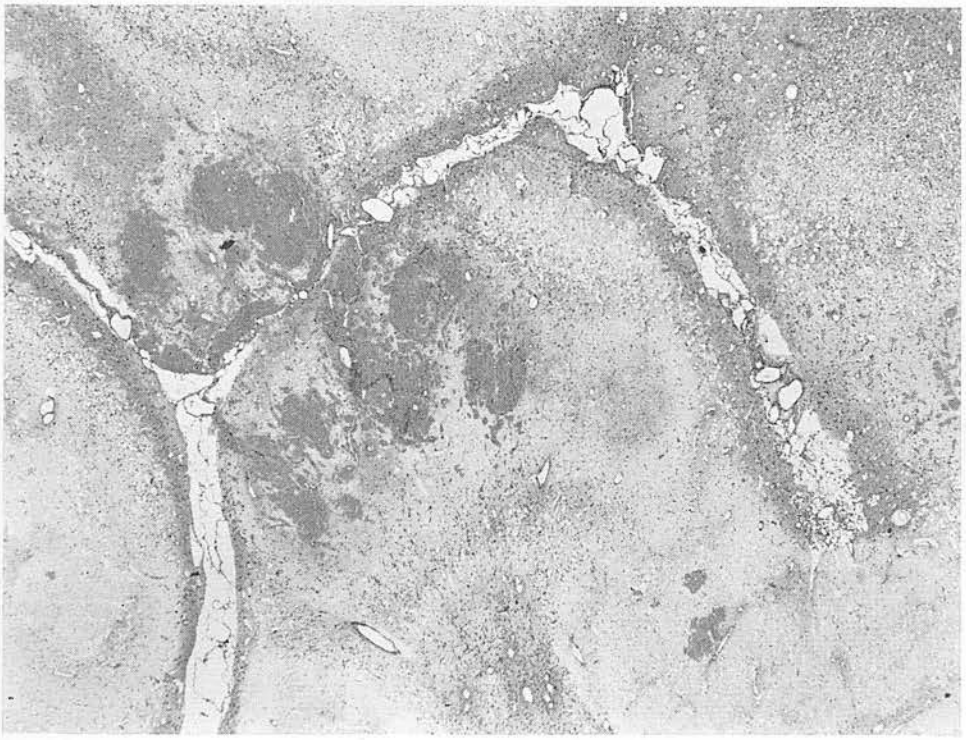

FIG. 6.—Case 2. Parietal cerebral cortex. Extensive area of malacia, diffuse spongy change and haemorrhages. Haematoxylin and eosin (HE). ×15.

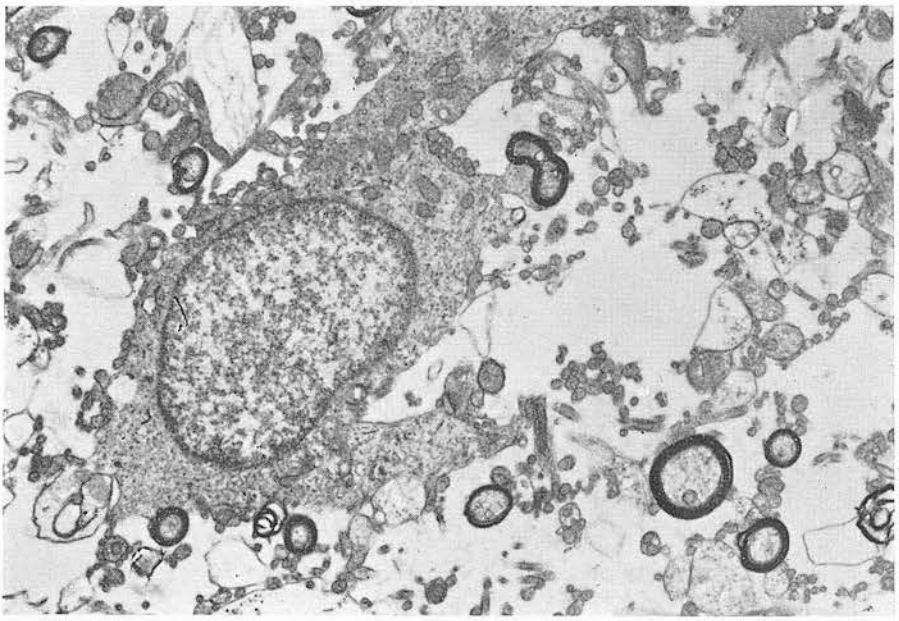

FIG. 7.—Case 2. Gyral white matter core adjacent to severely malacic cortex. Glial cell and neuronal processes bathed in electron-lucent oedema fluid. EM. ×9000.

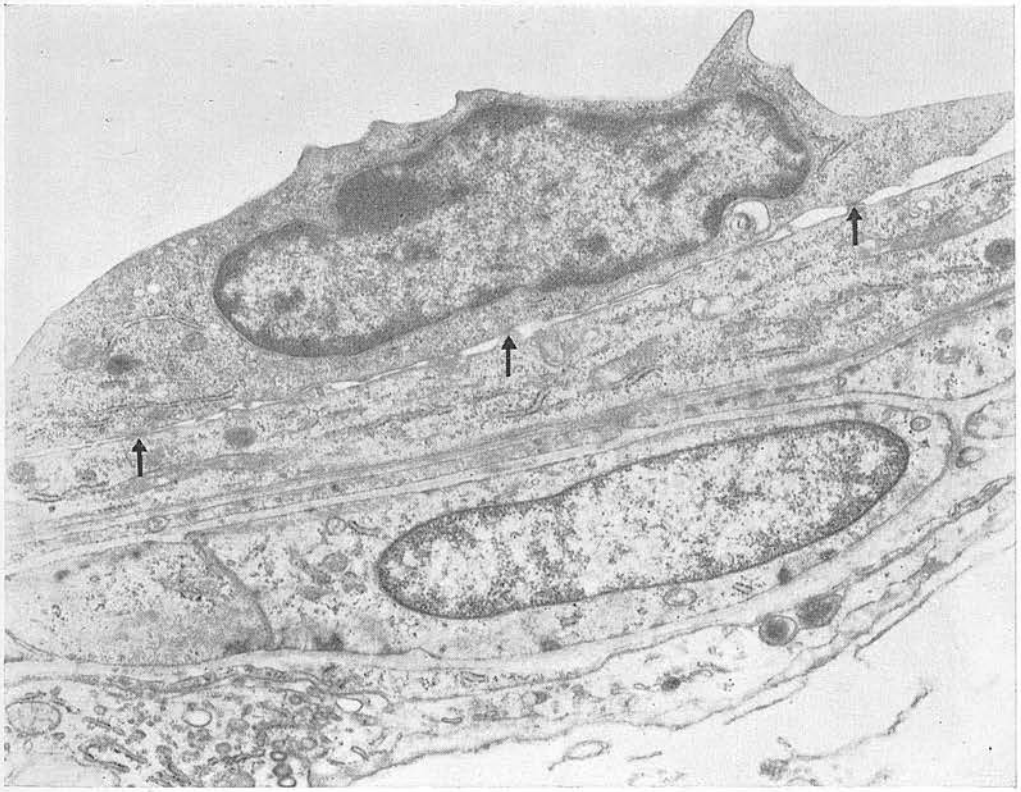

FIG. 8.—Case 10. Mononuclear inflammatory cell adherent to endothelial luminal plasmalemma. Points of close contact (arrows). EM. ×7500.

AMPROLIUM POISONING

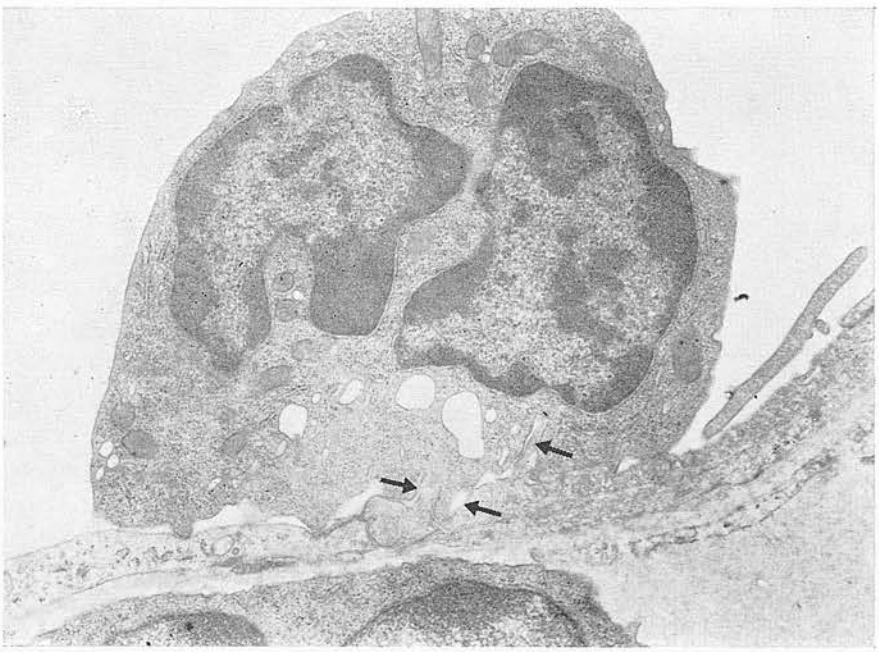

FIG. 9.—Case 10. Mutual interdigitation of processes by endothelial and inflammatory cells (arrows). EM. ×15,000.

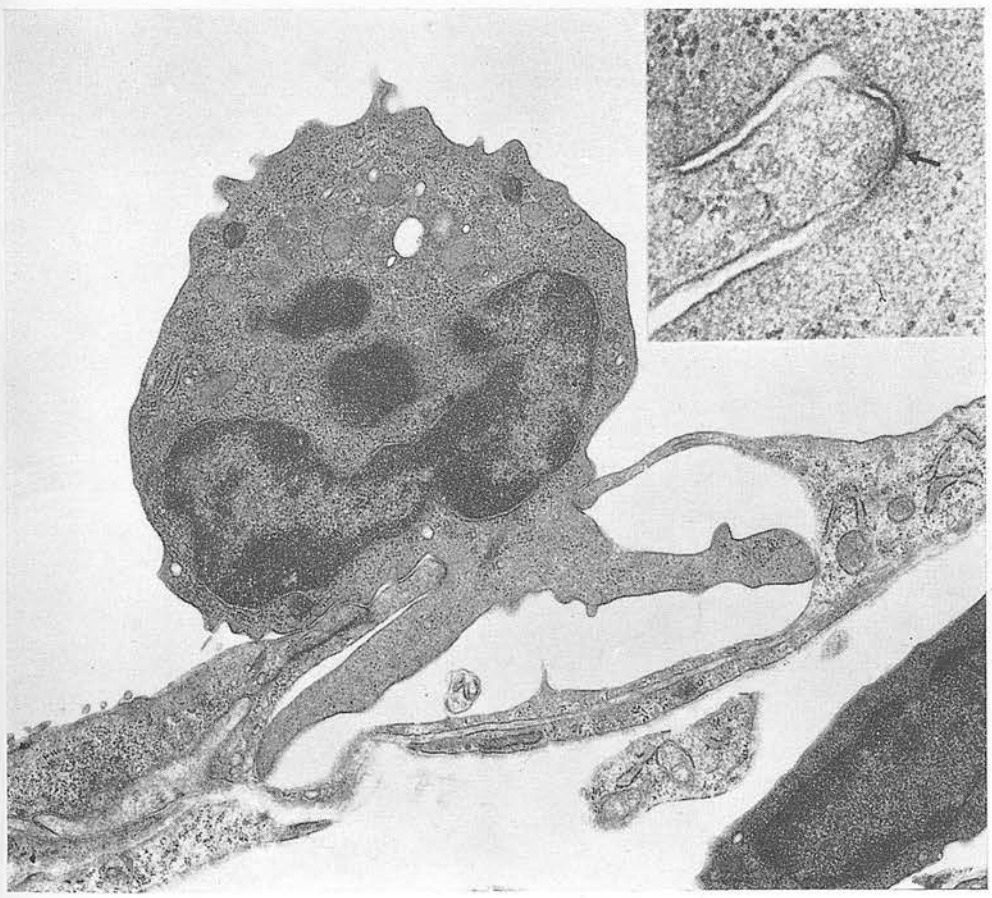

FIG. 10.—Case 10. Migration of inflammatory cell into endothelial "vacuole" EM. ×15,000. Inset—Close contact between endothelial and inflammatory cells preventing escape of blood (arrow). EM. ×66,000.

AMPROLIUM POISONING

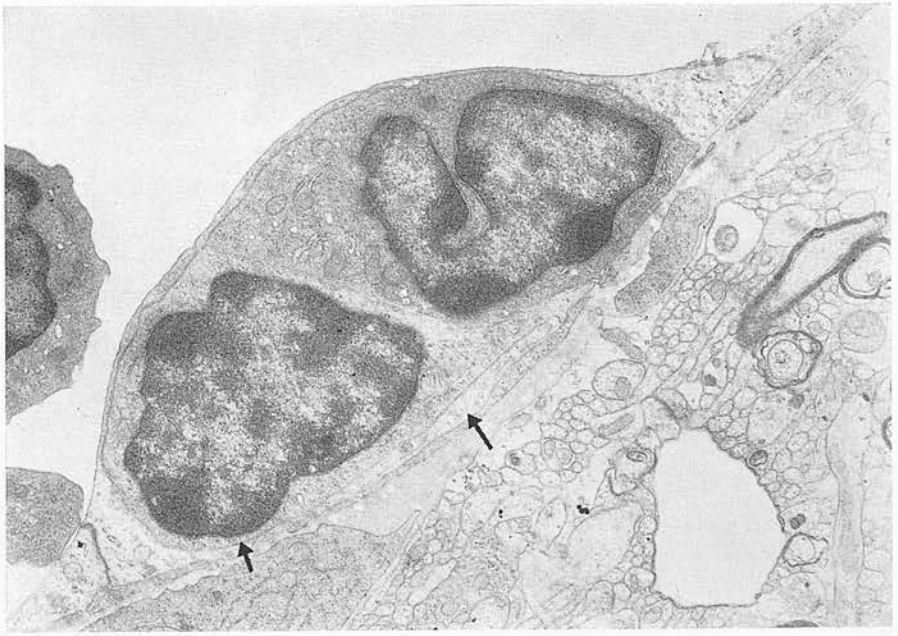

FIG. 11.—Case 10. Inflammatory cells separated from endothelial basement membrane by incomplete endothelial tongue (arrows). EM. ×10,000.

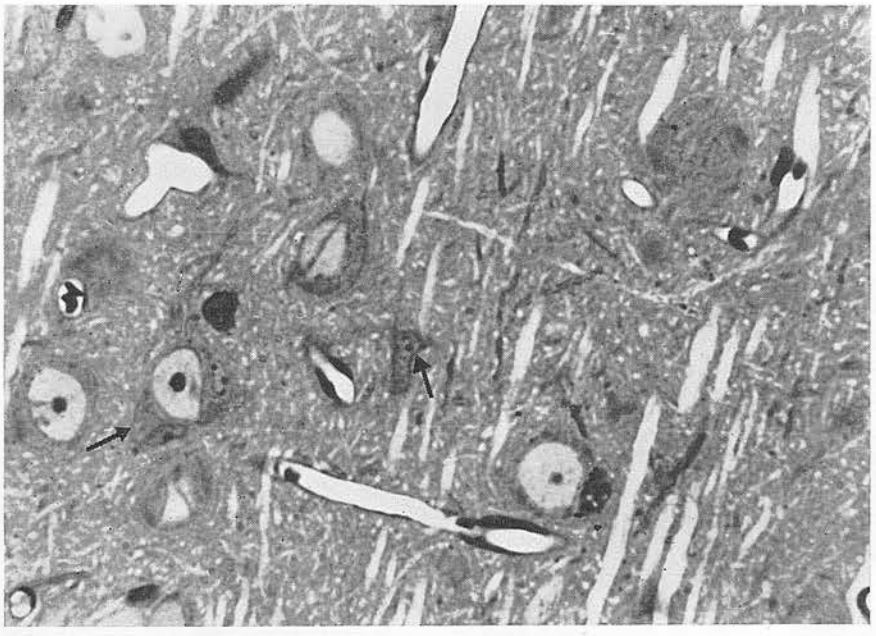

FIG. 12.—Control No. 8. Parietal cerebral cortex. Normal morphology. Arrows indicate astroglial nuclei. Note the compact nature of neuropil and absence of perivascular spaces. Giemsa. ×180.

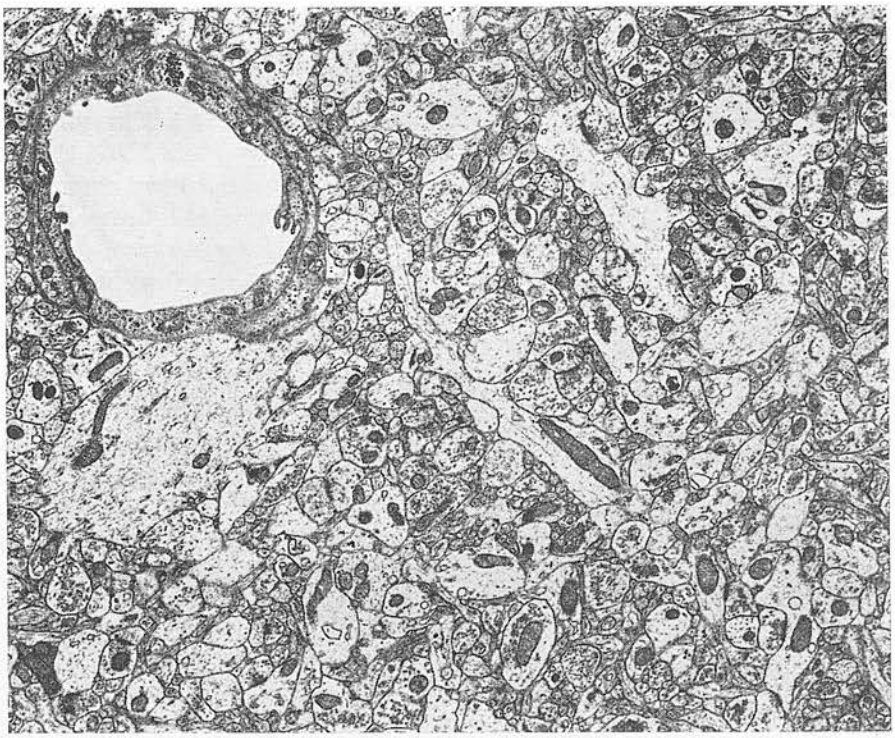

FIG. 13.—Control No. 5. Normal vascular and neuropil morphology. Note the absence of swollen astroglial processes. EM. ×9000.

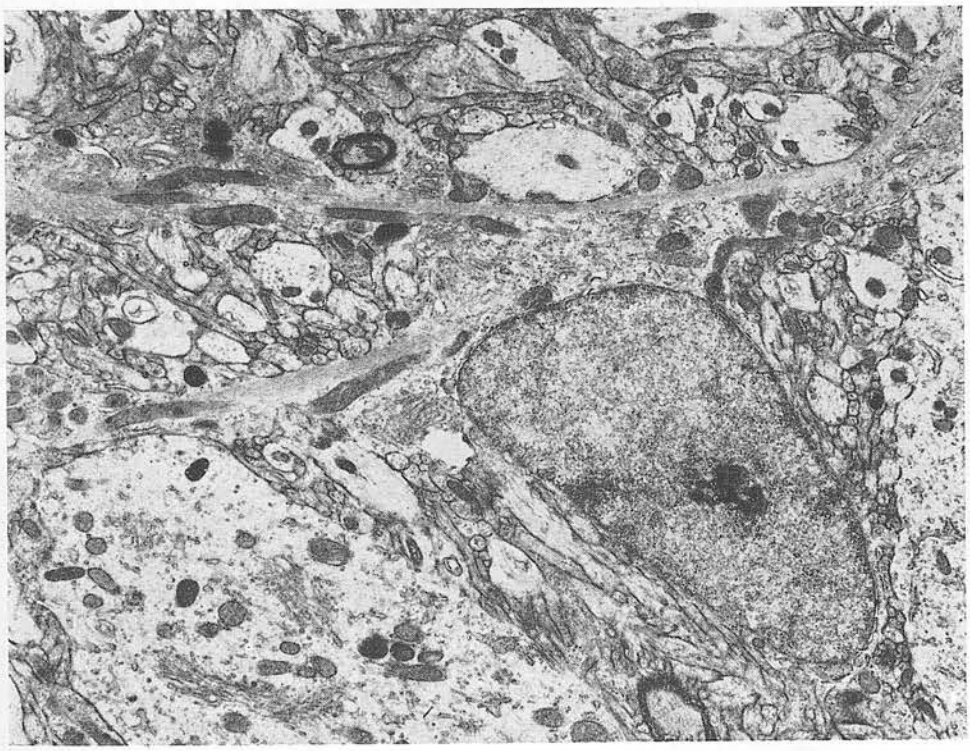

FIG. 14.—Control No. 8. Parietal cerebral cortex. Astrocyte. Compact cytoplasm with fibrils and other organelles. Moderately electron-dense nucleus. EM. ×9000.

natural PE. Haemorrhages of varying severity were present in the dorsal cerebral meninges and in the brain parenchyma in five cases. Gross evidence of brain swelling, including compression of cerebrocortical gyri, tentorial herniation of the occipital poles, and cerebellar coning was present in six cases. Animals nos. 5 and 6 were killed prior to the development of symptoms of CNS disturbance but following the onset of diarrhoea, in the hope of finding very early lesions. These two cases had small haemorrhages in many organs but no gross neuropathological changes were observed. These observations are summarised in the table.

Microscopic findings. Histological examination of tissues other than brain revealed consistent changes only in heart-muscle and duodenum. In the myocardium many dark-staining myocardial cells were observed. In the duodenum there were areas of flattened distorted villi with evidence of degeneration of the mucosal epithelium and severe distension of the underlying Brunner's glands.

Lesions in the brain were confined to the cerebral cortex except in those with lambs severe cerebellar distortion in which there were petechial haemorrhages and malacic foci in the herniated cerebellar uvula.

The description of the cerebrocortical changes is divided into three sections:

1. *The early malacia*

The earliest evidence of cerebrocortical malacia was spongy change of a focal or laminar nature. This type of change was most extensive in animals killed at 2–8 hr after the onset of symptoms and of minimal extent in those killed at 18 hr. This spongy change was characterised by perineuronal and perivascular spaces with a foamy appearance of the neuropil (fig. 1). Laminar lesions did not appear to be located in any particular cortical laminae and would often pass across a sulcus to continue in the opposing gyrus. Malacic areas were generally present on both sides of a sulcus with one lesion often appearing slightly more advanced than the opposing one.

Electron microscopy revealed that the perivascular and perineuronal spaces and the foamy appearance of the neuropil were attributable to watery swelling of astroglial cells (fig. 2). This astroglial change appeared to follow a uniform sequence, with perivascular end-feet being the first and in some areas the only structures to be affected. The processes in the neuropil and adjacent to neurones were the next to swell followed by distension of the perikaryon and nucleus (fig. 2 and 3). Severely distended astroglia then showed evidence of degeneration with clumping of organelles in the watery hyaloplasm and granular aggregation of chromatin principally at the nuclear membrane.

Many swollen astroglial cells and their processes contained clusters of glycogen granules (fig. 2 and 4). Counts of representative areas in experimental animals revealed a near three-fold increase in the number of granules as compared with the controls.

In areas of moderately severe astroglial swelling the blood vessels were well perfused and there was no evidence of capillary or arteriolar compression by

distended astroglial end-feet. Most neurones and oligodendroglia in areas of early malacia had a normal appearance by light and electron microscopy and there was no evidence of any increase in the extracellular space.

Those neurones undergoing degenerative change showed changes of two types. The first which predominated, resembled that described in polioencephalomalacia (Morgan, 1973) with distension of golgi saccules and mitochondrial swelling (fig. 5). The second type was characterised by distension of saccules of the endoplasmic reticulum and golgi apparatus. In areas of early malacia adjacent to subcortical white matter many myelin sheaths were distended by an accumulation of electron-lucent fluid in the periaxonal space (fig. 4 and fig. 4 inset) around apparently normal axons. A few swollen myelin sheaths also showed separation of myelin lamellae at the intraperiod line. This periaxonal and intramyelinic oedema contributed significantly to the malacia in these areas.

2. *Further development of the malacia*

In lambs killed 18–36 hr after the onset of clinical signs, the pathology had progressed to coagulative neuronal necrosis, distension and fragmentation of glia, changes closely resembling those of natural PE (Morgan, 1973). The most notable difference between PE and Amprolium poisoning was the greater haemorrhage in several Amprolium-poisoned lambs (fig. 6). Haemorrhage was present in five animals as shown in the table and resulted in extensive loss of serum protein from many blood vessels into the brain parenchyma. In the remaining lambs no haemorrhage was observed but isolated vessels in lamb no. 1 showed small amounts of extravasated serum proteins in electron micrographs.

In some areas of severe malacia there was extracellular oedema of the underlying gyral white matter core with very little evidence of the swelling of the myelin sheaths which were bathed in clear oedema fluid (fig. 7).

3. *The inflammatory response*

In all animals killed at 2 and 8 hr after the onset of clinical symptoms there was a mixed population of polymorphononuclear leucocytes (PMNs) monocytes and macrophages in which the first predominated. At 18 hr there were many more macrophages and the majority of PMNs were apparently degenerating, often in large clumps. In the three animals killed at 36 hr there was extensive infiltration by macrophages.

Both PMNs and monocytes may enter the brain parenchyma by transendothelial migration since leucocytes were not seen to pass between endothelial cells. This migration may involve the active participation of both endothelial and inflammatory cells and seemed to proceed through the following sequence of events: (*a*) adherence of inflammatory cells to endothelium (fig. 8) with intermittent points of close contact of their plasmalemmae; (*b*) mutual interdigitation of cytoplasmic processes (fig. 9); (*c*) migration of the inflammatory cell into an endothelial vacuole (fig. 10) with close contact between the cells at the point of entry (fig. 10 inset) and consequent closure of luminal

endothelial cytoplasm over inflammatory cell; (*d*) retraction of contraluminal endothelial cytoplasmic sheet (fig. 11); (*e*) migration of inflammatory cell through endothelial basement membrane. Throughout this process the endothelial cells appeared to act as a " double-door " which prevented leakage of blood.

Monocytes were readily identified within vascular lumenae (fig. 8) but were not distinguishable from vascular pericytes in the control lambs.

Controls. No significant pathological changes were observed in the nine controls. The brains appeared normal by light and electron microscopy (figs. 12, 13 and 14). Astroglial cytoplasm was barely perceptible by light microscopy (fig. 12) but could be readily identified in electron micrographs (fig. 14).

Discussion

Pill *et al.* (1966) demonstrated that the administration of Amprolium to a preruminant calf produced lesions in the brain indistinguishable from PE at the light microscopic level. Since then several workers have examined these changes by light microscopy in calves (Markson, Terlecki and Lewis, 1966; Little, 1969; Markson *et al.*, 1972) and sheep (Sinha, 1971; Loew *et al.*, 1972). The similarity of lesions produced by Amprolium and those of PE when observed by light microscopy has been used as evidence for the hypothesis that PE is the result of thiamine antimetabolite production in the rumen (Edwin and Jackman, 1970) as Amprolium is a thiamine antagonist.

This experiment was designed with the intention of producing encephalomalacia by Amprolium administration for a standard period of time in order to investigate the morphogenesis of the brain lesions by light and electron microscopy.

The effects of Amprolium on the brain are prevented by simultaneous administration of thiamine (Markson *et al.*, 1972). Thiamine intake was therefore kept at a low and approximately constant level by rearing preruminant lambs on a semi-synthetic diet of moderately low thiamine content and administering antibiotics to reduce intestinal bacterial synthesis of thiamine. The fact that this diet contained adequate thiamine for normal growth and development was established by the observation that thiamine supplemented and unsupplemented controls grew at about the same rate. Slatted floors were used to reduce coprophagy.

The success of this system was demonstrated by the development of cerebrocortical malacia in 10 out of the 12 Amprolium-treated group after 25–28 days. The consistent nature of the clinical syndrome also made possible a serial killing over the clinical period.

The earliest evidence of cerebrocortical malacia due to Amprolium poisoning was found to be oedema of astrocytes leading to the development of perineuronal and perivascular spaces and foamy neuropil in paraffin sections. Swollen watery astrocytes have been described in a number of conditions including poor fixation. The excellent correlation between the morphology of oedematous tissue in paraffin and Araldite sections and the absence of watery

astrocytes in control material is an indication that they are not artefacts but evidence of early oedema. This oedema was identical to that described as the primary morphological change in ovine PE (Morgan, 1973).

Accumulation of glycogen in astrocytes was observed in both conditions and further development of the cortical malacia was also very similar; degeneration of neurones and other parenchymal elements followed the primary oedema in both cases. The striking resemblance of these two encephalomalacias at the ultrastructural level thus provides further evidence of similarity.

Evidence that the primary astroglial oedema has rapid onset is provided by the absence of lesions in the brains of lambs nos. 5 and 6. All other test animals showed diarrhoea for 24–36 hr prior to the occurrence of CNS symptoms. Lambs nos. 5 and 6 were slaughtered 24 hr after the onset of diarrhoea in the hope of finding early lesions. The absence of lesions in the brains of these two lambs, if they are representative of their fellows, indicates that the oedema develops between 24 and 36 hr from the commencement of diarrhoea.

Morgan (1973) proposed that astroglial swelling in PE may result from defects of cell volume regulation due to impaired energy metabolism within the astrocyte or as a response to change in the extracellular fluid bathing astrocytes. It was also suggested that the inhibition of thiamine in neuronal membrane metabolism might account for the astroglial oedema. These arguments may be applied equally to the primary oedema of Amprolium poisoning.

Alteration of the composition of the extracellular fluid with consequent astroglial swelling may also be the result of vascular leakage. Evidence of vascular breakdown was present in six Amprolium-treated lambs. The possibility that vascular leakage, not apparent by routine electron-microscopic examination, was present in areas of early malacia cannot be excluded. Further evidence that the oedema may have a vascular origin is provided by the observation that perivascular astroglial processes were the first to swell.

Acute thiamine deficiency in man, mink, foxes, cats (Innes and Saunders, 1962) and rats (Manz and Robertson, 1972) is also associated with haemorrhages in the brain. These haemorrhages however, are bilaterally symmetrical and largely confined to the brain-stem, and thus in both respects differ from the lesions in the brains of Amprolium-poisoned lambs. The use of horseradish peroxidase as a vascular tracer has provided evidence to suggest that the early oedema in the rat thiamine deficiency encephalopathy occurs in the absence of change in vascular permeability (Manz and Robertson, 1972). This may also be the case in the early oedema of Amprolium poisoning, but in the absence of further investigation, the pathogenesis of the early astroglial swelling remains unknown.

Morgan (1973) proposed that brain swelling in natural cases of PE may be due to vascular breakdown. In this experiment a relationship was observed between brain swelling and haemorrhage in brain parenchyma. There was one exception in that test animal no. 1 had brain swelling, as shown in the table, but no haemorrhage. In this case however electron-microscopic examination revealed that serum proteins had leaked from some vessels in the malacic cortex. Thus in both PE and Amprolium poisoning cerebral swelling may be

the result of severe disturbance of fluid balance in the brain due to extravasation of blood-proteins.

The vascular breakdown observed in the Amprolium-poisoned lambs was very extensive involving most organs including the brain. This degree of haemorrhage has not been reported in natural PE and the mechanisms responsible merit further investigation.

A number of changes were observed in this experiment which contrast with those described in natural PE and by other workers studying Amprolium toxicity. Firstly, the distribution of lesions in the lambs in this experiment was predominantly in the cerebral cortex with the only other change being in herniated cerebellar tissue. The cerebellar lesions were attributed to pressure as a result of cerebral swelling. Thus in the lambs in this experiment the lesion distribution differed significantly from that described in Amprolium poisoning of adult sheep (Sinha, 1971) in which they occurred in the cerebrum, brain-stem and cerebellum. In natural PE of adult sheep lesions also occur frequently in the hippocampus, brain-stem and cerebellum (Terlecki and Markson, 1961; Morgan, 1973). This may reflect a variation between the young preruminant and ruminating sheep of tissue susceptibility to toxicity by thiamine antimetabolities.

The involvement of myelin sheaths in the early malacia was striking and has yet to be described in natural PE. The oedema fluid appeared to accumulate principally in the periaxonal space and less frequently within the myelin sheath in the intraperiod line. Thiamine is present in the axonal membrane (Tanaka and Cooper, 1968) and may have a role in this site independent of its coenzyme function in nerve impulse conduction and sodium transport. Thus inhibition of thiamine by Amprolium in the axonal membrane may account for the periaxonal accumulation of fluid observed in this condition.

Morgan (1973) described hypertrophic mitochondria in the early malacia of natural PE. These were not observed in Amprolium poisoning, but this may reflect a difference of processing procedure.

Transendothelial migration of lymphocytes (Baringer and Griffith; Astrom, Webster and Arnason cited by Doherty *et. al.*, 1971) and plasma cells (Doherty *et al.*, 1971) into the CNS has been reported. This work suggests that monocytes and PMNs may also enter the brain by this route in considerable numbers. The process appeared to involve the active participation of both endothelial and inflammatory cells, but cautious interpretation is necessary (Schoefl, 1972).

This study also demonstrated the characteristic nature of the inflammatory response, initially by polymorphonuclear leucocytes which were later replaced by a population of macrophages. In this experiment the clinical picture was used as a base-line for a serial killing procedure, the direct correlation between the inflammatory-cell type and time of kill indicates the success of this system.

Summary

Preruminant lambs reared on a semi-synthetic milk substitute diet, were given Amprolium orally for 3–4 wk before succumbing to a consistent clinical syndrome of diarrhoea followed by a state of lethargy which proceeded to

ataxia and convulsions. Post-mortem examination revealed lesions of cerebrocortical malacia and haemorrhages in most organs. The morphogenesis of the brain lesions was studied by light and electron microscopy.

The earliest evidence of malacia was the formation of perineuronal and perivascular spaces and a foamy transformation of the neuropil. This change was attributed to oedema of astrocytes. Neuronal degeneration appeared after the oedema. These changes closely resemble those of natural polioencephalomalacia, and it is suggested that the findings support the hypothesis that Amprolium toxicity and PE have a common aetiology in thiamine inhibition. In white matter adjacent to areas of early malacia many myelin sheaths were separated from their axons by large electron-lucent spaces. Brain swelling observed in six cases was apparently related to vascular breakdown. The inflammatory response initially involved polymorphonuclear leucocytes which were later replaced by macrophages. Inflammatory cells may enter the brain by transendothelial migration. This process may involve the active participation of both endothelial and inflammatory cells.

I am grateful to Dr R. M. Barlow and Mr J. S. Gilmour for helpful discussion. Mr W. Smith, Mr E. W. Gray and Mrs Pat Gray and Mr C. R. Hood, for the preparation of electron micrographs, Miss Sheila Wright for capable technical assistance and Mr D. G. Watson and Mr B. J. Easter for preparation of light micrographs.

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14—PATHOLOGY

AMPROLIUM POISONING OF PRERUMINANT LAMBS: AN INVESTIGATION OF THE ENCEPHALOPATHY AND THE HAEMORRHAGIC AND DIARRHOEIC SYNDROMES

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Received 10 June 1974; *accepted* 23 July 1974.

PLATE XXV

INTRODUCTION

IN a study of the encephalopathy produced in preruminant lambs by the administration of amprolium (Merck, Sharp & Dohme Ltd) (1-(4 amino-2-n propyl-5 pyrimidinylmethyl)-2-picolinium chloride hydrochloride), Morgan (1974) reported that the lambs developed severe diarrhoea and extensive systemic haemorrhages in addition to the brain lesions. This paper describes an experiment conducted to investigate the mechanisms responsible for these changes.

MATERIALS AND METHODS

Experimental regimen. Twenty-four Suffolk × Dorset lambs, having received colostrum for 24 hr, were reared indoors with an air temperature range of 8°–14°C. Nutrilamb (SAI Ltd) milk replacer, prepared to contain 0·25 mg thiamine per litre of milk, was fed *ad libitum* from an automatic feeder. Each lamb received 11 mg/kg body weight of oral phthalylsulphathiazole daily throughout the experiment in order to reduce intestinal bacterial synthesis of thiamine. The lambs were separated into two groups of 12, one experimental (group E) and one control (group C), available twins being replaced one in each group (table I). Lambs in group E were, in addition, dosed orally each day with 280 mg/kg body weight of amprolium in aqueous solution, the 12 group C lambs being untreated controls. Three amprolium-treated and three control lambs were killed on days 7, 14 and 21 of the experiment. The remaining lambs from group E were killed as soon as they exhibited evidence of CNS disturbance, along with their appropriate controls. These killing groups are numbered 1–4 and the lambs in each group identified as a, b and c as shown in table I. Individual lambs are referred to according to their position in this table.

Blood samples were taken weekly for the Lee-White test of clotting time (Archer, 1965) from the lambs in groups E4 and C4. The clot-retraction was expressed as the percentage volume of serum produced from 4·5 ml of clotted blood after 24 hr at 37°C. Blood was also taken into citrated tubes for the Quick one-stage prothrombin test, using both human thromboplastin (Symplastin (W. R. Warner Ltd, Eastleigh, Hants.)), and sheep-brain thromboplastin (Archer, 1965), and into EDTA for platelet counts (Archer, 1965).

Post-mortem procedure. With the exception of lamb E4a, which died, each lamb was anaesthetised with sodium-pentobarbitone and a cysternal sample of cerebrospinal fluid (CSF) withdrawn via a 21 gauge × 1½ in. hypodermic needle. The animal was then exsanguinated, the small intestine immediately removed, and a 5-cm segment of intestine cut from the proximal and distal ends of the anterior third. These segments were cut in half transversely, opened along the mesenteric attachment and one piece placed in 4 per cent. glutaraldehyde in 0·1M

phosphate buffer, *p*H 7·4 and the other portion into cold neutral Baker's formol. The remainder of the first third of the intestine was stored on ice. Faeces samples were taken from the rectum and stored in plastic pots at −20°C.

The bone marrow of the left femur was rapidly exposed by sawing through the bone at the level of the minor trochanter. A small block was excised with a scalpel, cut into 1-mm cubes and immersed in 4 per cent. glutaraldehyde in 0·1M phosphate buffer and the adjacent 1-cm core of marrow immersed in 10 per cent. formol-saline. Bone marrow smears from the femur and the left sixth rib were made on to glass slides, air dried and fixed in aceton-free methylated spirit. A 1-cm square segment of the ventral aspect of the urinary bladder was immersed in 4 per cent. glutaraldehyde and another in 10 per cent. formol saline.

TABLE I

Experimental regimen

Amprolium-treated (group E)			Twins	Controls (group C)		
Lamb no.	Sex	Day of kill		Lamb no.	Sex	Day of kill
E1a	F	7	−	C1a	F	7
E1b	F	7	+	C1b	M	7
E1c	M	7	−	C1c	M	7
E2a	F	14	+	C2a	M	14
E2b	F	14	+	C2b	M	14
E2c	M	14	−	C2c	M	14
E3a	M	21	+	C3a	F	21
E3b	M	21	+	C3b	M	21
E3c	F	21	−	C3c	F	21
E4a	F	24(D)	+	C4a	F	33
E4b	M	31	+	C4b	M	31
E4c	F	33	+	C4c	M	33

The day of kill is from the first day of amprolium administration.
D = Died.

The brain was removed rapidly and cut into 3-mm-thick, coronal slices, alternate pieces being placed in 10 per cent. formol saline, whilst the remaining tissue was placed in polythene bags and " quenched " in a mixture of dry ice and isopentane for storage at −80°C.

Histological processing procedures. The samples in 10 per cent. formol saline were processed to processed to paraffin wax, 6-μm sections were cut and stained with haematoxylin and eosin, and Heidenhain's iron haematoxylin. The material in 4 per cent. glutaraldehyde was fixed for 24 hr, washed in phosphate buffer and embedded in Araldite. One μm Araldite sections were cut on a Porter-Blum MT1 ultratome and stained with Giemsa at 60°C. The samples in cold neutral Baker's formol were fixed for 24 hr and subjected to " Gomori Calcium Method " and the " Azo-dye Coupling Method " for alkaline phosphatase (Bancroft, 1967) using 15-μm frozen sections. Bone marrow smears were stained with Leishman's stain and used for the differential counting procedure. " Total counts " in bone marrow sections from the femur were estimated by counting the number of cells in a 100-μm-sided square, using an eyepiece graticule. For this purpose areas were selected in the stroma, adjacent to the periphery of the marrow, in which fat cells did not impinge on the field to be counted. Using randomised slides, five fields were counted in both 6-μm paraffin sections stained with haematoxylin and eosin, and 1-μm Araldite sections stained with Giemsa, and a mean value was calculated for each lamb. The total numbers of mitotic figures were similarly determined using a 100-μm-sided square, except that paraffin sections were stained with Heidenhain's iron haematoxylin to facilitate recognition of mitotic figures.

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Enzyme assays. Five equally spaced 10-cm segments were cut from the first third of the small intestine, and the mucosa removed by scraping lightly with a glass slide. These mucosal samples were pooled and homogenised in distilled water with a glass Camlab homogeniser (Camlab Ltd, Cambridge). The homogenates were extracted at 4°C for 1 hr, made up to volume and centrifuged at 10,000*g* at 4°C for 15 min. and the clear supernate stored at −20°C for assay. Lactase activity was determined by the method of Dahlqvist (1964), using a substrate concentration of 28 mM in O.I.M. maleate buffer *p*H 5·4, and alkaline phosphatase activity assayed by the method of Salomon *et al.* (1964) using a substrate concentration of 6 mM disodium phenyl phosphate in 0·2M tris buffer *p*H 9·6. The total nitrogen content of the homogenates was determined on a Techicon Auto-analyzer following method sheet N14b (Protein = nitrogen × 6·25). Enzyme activity is expressed as milli-units per mg extractable protein (1 unit is equivalent to the hydrolysis or liberation of 1 μ. mole/minute at 37°C).

Homogenates were prepared from the dorso-medial parietal cortex of thawed brain slices after separation from the underlying white matter with a scalpel. Cerebellar samples were taken from the core of the cerebellum just dorsal to the *corpus medullare.* Weighed samples were homogenised in 0·1M tris-HCl buffer, *p*H 7·6, centrifuged at 10,000*g* for 30 min. at 4°C and made up to volume. Transketolase (TK) activity was determined by measuring the rate of formation of sedoheptulose-7-phosphate from ribose-5-phosphate in glycyl-glycine buffer *p*H 7·8 for 30 min. at 37°C as described by Novello and McLean (1968). The sedoheptulose-7-phosphate produced was assayed by the cysteine/sulphuric acid method of Dische (1953). TK activity is expressed as units per gram fat-free dry weight (1 unit is defined as the amount of enzyme catalysing the formation of 1 μ. mole of product per hour at 37°C).

Other procedures. The levels of Na^+ and K^+ in the CSF were estimated with an I.L. 343 Flame Photometer. Faecal *p*H was measured by immersing the electrode of a Pye Unicam *p*H meter in faeces mixed with an equal weight of distilled water.

Results

Control lambs (group C)

The control lambs remained healthy throughout the experiment and post-mortem examinations revealed no evidence of pathological change. They gave a normal response to the blood-tests (fig. 1). The mean TK activity was 132 (SD±16·6) units per gram fat free dry weight for cerebral cortex and 141 (SD±34·9) units per gram fat free dry weight for cerebellum. The bone marrow appeared normal in histological (figs. 2 and 3) and the differential, "total" and mitotic counts of the bone marrow are recorded in table II. There was no evidence of pathological change in the alimentary tract and the mean intestinal alkaline phosphatase and lactase activities were 1192 (SD±392) and 127 (SD±21) milli-units respectively.

Amprolium-treated lambs (group E)

These animals were clinically normal for the first 14 days of amprolium administration and the post-mortem examinations of the first two killing groups (table I) revealed no macroscopic evidence of pathological change. Between days 15 and 20 several amprolium-treated lambs developed loose faeces and at post-mortem examination on day 21, moderately extensive haemorrhages were found in lambs E3b and E3c.

On day 23 lamb E4a developed very severe, greenish mucoid diarrhoea and died on the morning of the 24th day with conjunctival ecchymoses and slight

epistaxis. This lamb was found to have petechiae and ecchymoses in the intestinal tract, subcutaneous loose connective tissues, skeletal muscles and bone marrow and diffuse haemorrhages in the diaphragm, epicardium, endocardium and myocardium, urinary bladder submucosa and the retro-orbital

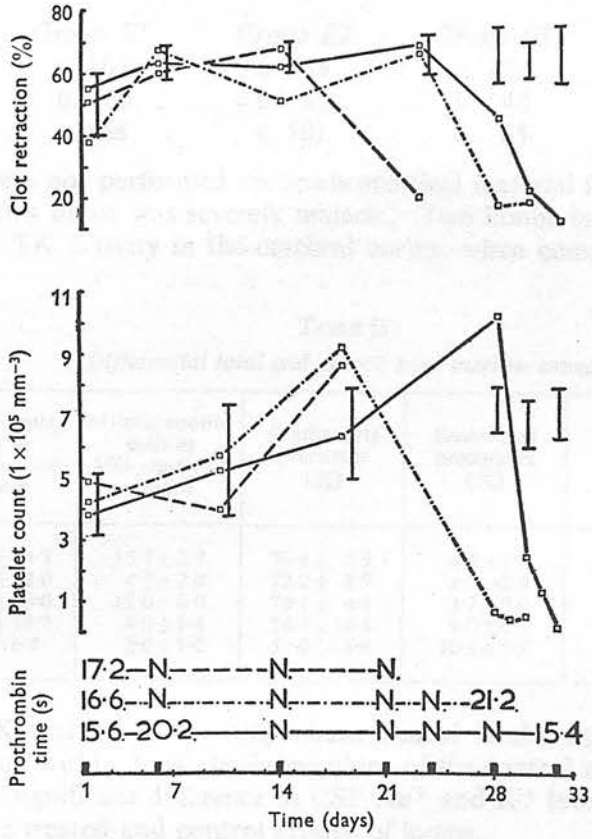

FIG. 1.—Percentage clot retraction, blood platelet count, and prothrombin time of lambs in Group E4. The ranges of values for their controls are represented by the vertical lines and the prothrombin times of these lambs was within the range of 13·0–19·5 s. Key: □---□ = E4a, □-·-·-□ = E4b □——□ = E4c, N = no clot formed, I = control range.

tissues. These changes closely resembled those observed in the previous experiment (Morgan, 1974), in that the haemorrhages predominated in actively mobile tissues. Haemorrhages were also present in the dorsal cerebral meninges and there was gross evidence of brain swelling and cerebrocortical softening.

On days 31 and 33 lambs E4b and E4c respectively were killed after the onset of ataxia following the appearance of severe diarrhoea during the preceding 2–3 days. Post-mortem examination revealed very similar changes to those described in lamb E4a. In the lambs with haemorrhages there was an excess of pale-yellow fluid in the pericardial sac.

Brain pathology. Histopathological examination of the brain revealed the characteristic lesions of amprolium poisoning (Morgan, 1974) in lambs E4a, E4b and E4c. In all three lambs there was severe cerebrocortical malacia, but

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only lamb E4a showed evidence to the cerebellum and this was attributed to cerebellar coning. There was no evidence of neuropathological changes in the other experimental lambs.

The cerebrocortical TK activity (units per gram fat free dry weight) of the amprolium-treated lambs is shown below:

Group E1	*Group E2*	*Group E3*
a. 163	a. 188	a. 101
b. 169	b. 92	b. 48
c. 158	c. 101	c. 65

TK assays were not performed on cerebrocortical material from the lambs in group E4 as this tissue was severely malacic. Two lambs in group E3 had significantly low TK activity in the cerebral cortex, when compared with the control values.

TABLE II

Differential total and mitotic bone marrow counts

Killing group	Total counts, cells in 100-μm-sided square	Mitotic counts, cells in 100-μm-sided square	Erythrocyte precursor (%)	Eosinophil precursors (%)	Neutrophil precursors (%)	Basophils, lymphocytes, and others (%)
C1–4	192·9±24·7	15·7±2·7	76·8± 5·9	4·2±1·2	15·4± 3·5	3·5±0·6
E1	161·0±52·0	4·7±2·0	72·2± 8·9	3·0±2·8	20·5± 9·0	4·3±1·3
E2	171·0±26·0	12·0±6·0	76·1± 4·4	3·7±0·9	15·7± 5·1	4·3±0·3
E3	112·0±54·7	4·0±1·4	54·3±19·4	9·0±4·0	33·5±16·5	3·1±0·6
E4	55·0±16·4	2·0±1·0	52·0± 6·4	10·8±5·3	35·0± 9·5	2·1±1·9

Cerebellar TK activity of the amprolium treated lambs, apart from E1b (219 units) were all within 2 standard deviations of the control mean.

There was no significant difference in CSF Na^+ and K^+ levels or Na^+/K^+ ratio, between the treated and control groups of lambs.

Intestinal pathology. In lamb E2b there were areas of flattened distorted villi with degeneration of mucosal epithelium and distension of the underlying Brünner's glands in the duodenum with intestinal alkaline phosphatase and lactase activities of 372 and 63 milli-units per mg mucosal protein respectively. Histochemical examination also revealed a severe reduction of the alkaline phosphatase activity in areas with flattened villi, in both the Gomori and Diazo preparations. These histological and biochemical changes were not seen in any other amprolium-treated lambs.

The *p*H of the watery and mucoid faeces taken *post mortem* from lambs E4b and E4c was 9·0 whilst that of their controls C4b and C4c was 7·2 and 7·1 respectively. There were no other consistent differences of faecal *p*H between the lambs of groups E and C.

Vascular histopathology. Examination of blood vessels in areas of haemorrhage in the intestine and bladder in both paraffin- and Araldite-embedded material failed to reveal any evidence of vascular damage.

Haematological tests. The first evidence of haematological disturbance was the failure of plasmas from lambs E4a, E4b and E4c to produce a clot in the Quick prothrombin test, using both the human and sheep thromboplastin (fig. 1). A normal response to this test was produced by these plasmas when 0·1 ml of plasma from the appropriate control was included in the incubation mixture. Plasmas from lambs E3a, E3b and E3c gave a normal response to the Quick test on days 14 and 19, with a clot being produced in the same range of time as the controls (13–19·5 s).

On day 23, lamb E4a showed impaired clot retraction and lambs E4b and E4c developed a similar defect associated with thrombocytopenia, during the 1–2 days prior to the onset of ataxia (fig. 1). The Lee-White test however revealed no differences between groups E and C, in which clotting-times varied from 1 to 6 min.

Bone marrow pathology. In all five lambs with haemorrhages the histological examination revealed marked bone marrow depopulation (figs. 2 and 4). The majority of the remaining myeloid cells were mature eosinophils, polymorphonuclear leucocytes (PMNs), megakaryocytes and macrophages. Most of these cells appeared normal except for the megakaryocytes, many of which showed a range of degenerative changes from moderate vacuolation of the peripheral cytoplasm to complete necrosis (figs. 3 and 5). In paraffin sections stained with haematoxylin and eosin the degenerate megakaryocytes showed increased cytoplasmic eosinophilia whilst in Araldite sections they were seen to contain perinuclear " lakes " of amorphous material (fig. 5).

Differential cell counts of bone marrow smears revealed that in the depopulated marrow of lambs in groups E3 and E4, there was a decreased proportion of erythrocyte precursors, and a concurrent increase in the proportion of eosinophil and neutrophil precursors in comparison with the control group of lambs. The depopulation was confirmed by the " total " bone marrow cell counts, and the mitotic counts revealed that there was a considerable reduction in the number of dividing cells in the bone marrow of lambs in the amprolium-treated group, with only lambs E2a and E2c having mitotic counts within the range of the control groups (table II).

Discussion

Amprolium is a thiamine antagonist and is known to enter the brain (Markson *et al.*, 1972). Inhibition of transketolase (TK) activity provides a very sensitive indicator of thiamine inadequacy (Brin, 1962). It has been shown that TK activity in rats decreases initially in the sites in which brain-lesions of thiamine-deficiency predominate (Dreyfus, 1965). In this experiment we found that two lambs which had developed severe diarrhoea and systemic haemorhages after 3 wk of amprolium administration, had no histological evidence of brain lesions but had a low cerebrocortical TK activity. This observation supports the proposal that impaired thiamine metabolism in the cerevral cortex precedes the onset of the brain lesions.

The cerebellar TK activity of the amprolium-treated lambs, including those with severe cerebrocortical malacia, was apparently unaffected. Only one

amprolium-treated lamb had lesions in the cerebellum, and these were attributed to cerebellar distortion as a consequence of brain swelling. This restriction of primary brain lesions to the cerebral cortex may indicate regional differences of susceptibility to thiamine deficiency or antagonism as has been suggested for the rat (Dreyfus, 1965).

The earliest evidence of cerebrocortical malacia in preruminant lambs poisoned with amprolium appears to be oedema of astrocytes (Morgan, 1974). This oedema may be due to raised extracellular K^+ following the inhibition of thiamine either as a coenzyme in neuronal energy-metabolism or as a metabolite in neural membranes. However, there was no significant difference between amprolium-treated and control lambs in the CSF K^+ or the K^+/Na^+ ratio in the present experiment. As the CSF is probably in direct communication with the brain extracellular space (Brightman, 1968) it seems unlikely that the extensive depolarisation of neuronal membranes is the cause of this astrooglial swelling.

Morgan (1974) reported that in the amprolium-treated lambs, all of which had severe diarrhoea, there was mucosal flattening and distension of Brünner's glands in the duodenum. In this present experiment these changes were observed in only one lamb, which had received amprolium for 2 wk, and was showing no signs of diarrhoea. This finding represents a marked difference between these two experiments, which in every other respect were apparently very similar. However, there was no evidence of pathological change in the duodenum of any of the control lambs in this or the previous experiment (Morgan, 1974), and the association of the mucosal flattening and distension of Brünner's glands with amprolium poisoning is unknown.

The diarrhoea may be attributable to the anti-thiamine activity of amprolium, as it is also a feature of experimental thiamine deficiency in lambs (Draper and Johnson, 1951). Amprolium does not produce diarrhoea in adult sheep (Sinha, 1971; Loew and Dunlop, 1972) which suggests that age may be a factor in the response of the alimentary tract to amprolium poisoning.

The in-vitro clotting times were normal throughout the experiment and thus the Lee-White test appears to be of little value for the diagnosis of this type of haemostatic defect. The significance of plasma clot failure in the Quick test in three amprolium-treated lambs is unknown especially as they were clinically normal at the time when this defect was apparent.

The onset of haemorrhages was associated with a marked thrombocytopenia, attributable to severe degeneration of bone marrow megakaryocytes. The functions of blood platelets include the maintenance of normal haemostasis by adhesion and aggregation in injured blood vessels (Born, 1970). The haemorrhages may have been the consequence of an insufficiency of platelets for the repair of minute holes in capillary walls incurred as a result of physiological trauma. This hypothesis is supported by the observation that the majority of haemorrhages occurred in mobile tissues in which the capillaries might be more susceptible to damage. Extensive examination of blood vessels failed to show any morphological evidence of vascular damage, indicating that the leakage points may be very small.

In addition to the megakaryocyte degeneration, the bone marrow of lambs with haemorrhages was depleted of many myeloid elements and the differential counts showed that erythrocyte precursors were the most severely affected. Since the remaining cells in the marrow of the amprolium-treated lambs showed little evidence of degeneration, it seems likely that the marrow depopulation was due to decreased production of cells rather than increased cell destruction. This proposal is supported by the finding that the number of myeloid cells containing mitotic figures was considerably reduced even in lambs which had received amprolium for only 7 days. Thus amprolium may inhibit myeloid cell division or be highly toxic to the myeloblasts, but its mode of action upon the bone marrow has yet to be established.

The neuropathology of amprolium toxicity closely resembles that of Polioencephalomalacia (Morgan, 1974), however in the natural disease multiple haemorrhages and bone marrow damage have not been reported. Thus conclusions about the nature of PE drawn from the results of experiments using amprolium should be treated with caution. Furthermore workers using amprolium have referred to the neurological syndrome it produces as PE (Loew and Dunlop, 1972) and cerebrocortical necrosis (Markson *et al.*, 1972), whereas the term " amprolium poisoning encephalopathy " is more correct and its use would avoid confusion.

Summary

An experiment was conducted to investigate the mechanisms responsible for the brain lesions, diarrhoea and haemorrhages produced by amprolium poisoning of preruminant lambs. The encephalopathy was preceded by a reduction of cerebrocortical transketolase activity. Diarrhoea was not associated with histological evidence of pathological change in the small intestine, and in lambs with severe diarrhoea the small intestinal levels of alkaline phosphatase and lactase were unaffected. Haemorrhages were associated with a thrombocytopenia which was attributed to degeneration of bone marrow megakaryocytes. The bone marrow of the majority of the lambs which had received amprolium for 3 wk or more was severely depopulated, erythrocyte precursors being the most severely affected. The marrow depopulation was attributed to decreased myeloid cell production, as the majority of the remaining cells showed little evidence of degeneration and the number of mitotic figures in the marrow of amprolium-treated lambs was considerably reduced as compared with the controls.

We thank Dr R. M. Barlow, Dr A. Field and Mr J. S. Gilmour for helpful discussion, Miss Sheila Wright, Mr R. B. Graham and Mr B. J. Easter for technical assistance and Mr R. Brown and the staff of the R(D)SVS Clinical Laboratory for haematological work.

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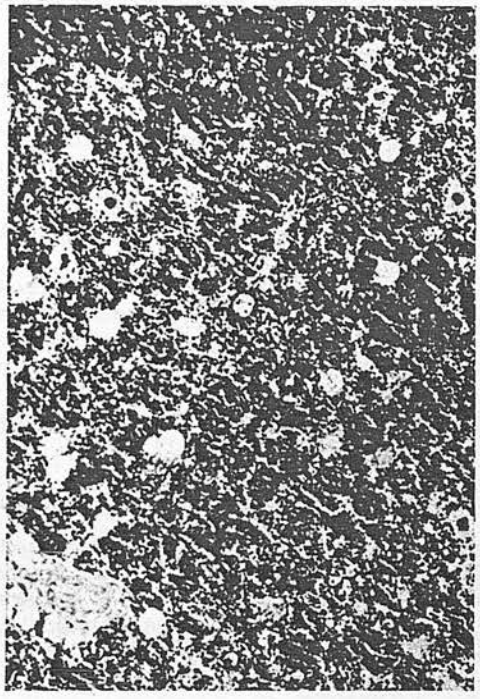

FIG. 2.—Bone marrow from control lamb C4c. Compare with fig. 5. Haematoxylin and eosin (HE). ×90.

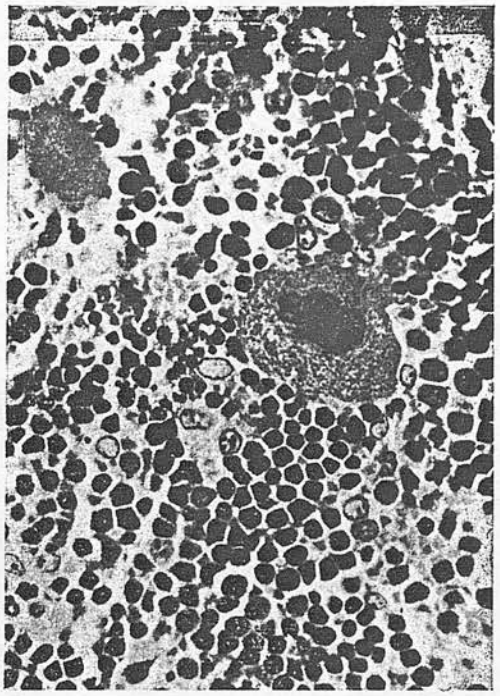

FIG. 3.—Bone marrow from control lamb C4c, showing normal megakaryocyte morphology. Araldite section stained with Giemsa. ×450.

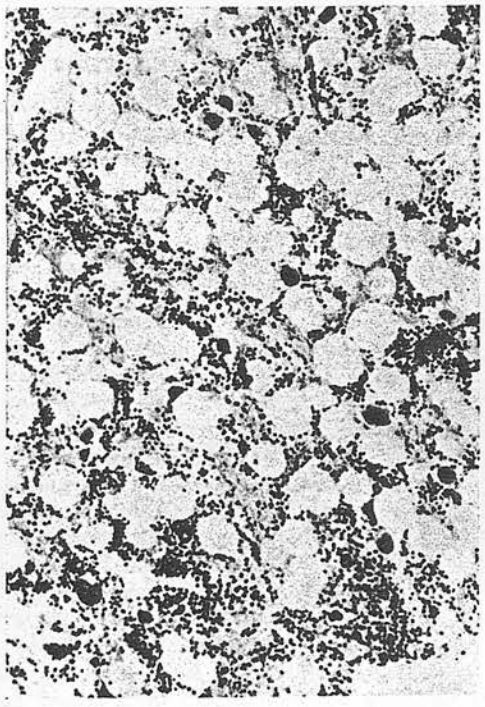

FIG. 4.—Bone marrow from femur of lamb E4c showing increased fat and depopulation of the stroma. HE. ×90.

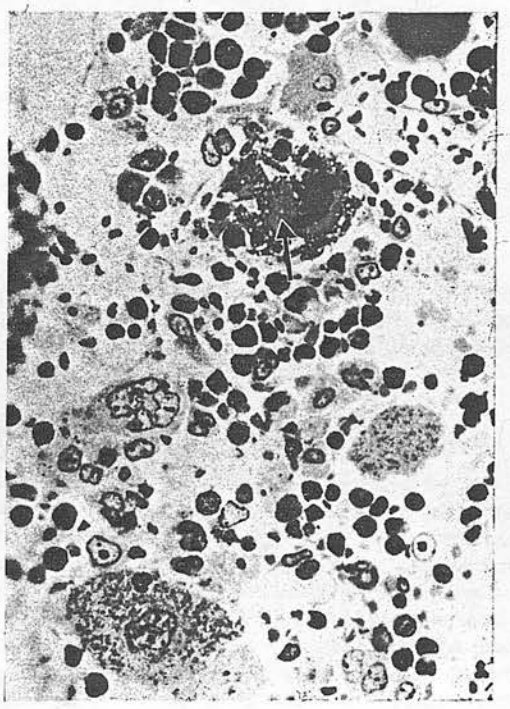

FIG. 5.—Bone marrow from femur of lamb E4c showing degenerate megakaryocyte with perinuclear amorphous material (arrow). Giemsa. ×450.

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Appendix No. 8

Thiaminase type 1-producing bacilli and ovine polioencephalomalacia

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Vet. Rec. (1974). **95.** *361-363*

Thiaminase type 1-producing bacilli were isolated from sheep, several of which had died from polioencephalomalacia. The thiaminase type 1 was apparently identical to the thiaminase which has been implicated in the aetiology of this disease. It is postulated that the thiaminases present in the rumen contents and faeces of sheep may be of bacterial origin.

Introduction

EDWIN and Jackman (1970) found a type 1 thiaminase in the alimentary contents of sheep and cattle and suggested that the presence of this enzyme may be an important factor in the pathogenesis of polioencephalomalacia (PE), which is also known as cerebrocortical necrosis. The source of the thiaminase was not, however, demonstrated by these workers.

This communication records the isolation of thiaminase type 1-producing bacteria, belonging to the genus Bacillus, from sheep rumen contents and faeces which contained type 1 thiaminase.

Materials and methods

Faeces samples from each of 170 Greyface ewes, one of which had recently died of PE; the rumen contents of this animal, and those from a further four sheep that had died of PE on four separate premises, were assayed for thiaminase activity using the method of Edwin and Jackman (1970). Thirty-six of the faeces samples and all five rumen contents exhibited marked thiaminase activity. The five rumen contents and the 22 faeces samples with the highest activity were examined for the source of the thiaminase. The method used was based upon that described by Shimazono and Katsura (1965) for the isolation of thiaminase-producing bacteria.

Approximately 1 g of each sample was suspended in 10 ml of nutrient broth, heated at 80°C for 20 minutes to destroy non-sporing bacteria, and incubated at 37°C for 48 hours. The broths were then tested for thiaminase activity and where this was present they were plated on to solid media. After 24 to 72 hours' incubation at 37°C, representatives of each of the colony types present were selected, purified and tested for their ability to produce thiaminase in broth culture. The majority of bacteria examined in this manner failed to produce thiaminase but direct broth subcultures from the original heated broths were found to give rise to variable and often high thiaminase activity, as did repeated subcultures from broth to broth.

At each subculture the broths with high thiaminase activity were plated on to solid media and colonies were examined for the ability to produce thiaminase after purification. This procedure resulted in the isolation, after a variable number of broth to broth subcultures, of thiaminase-producing organisms from three of the 22 faeces and all five rumen contents. These organisms were purified by replating on media containing 2 per cent agar, individual colonies picked and replated before the organisms were characterised. All of the eight isolates capable of producing thiaminase were found to have the following characteristics.

They grow as smooth, flat, membranous spreading colonies on horse-blood agar, and adhere to this medium. They are aerobic, weakly Gram-positive, slender rods which spore readily after 48 hours, producing oval thick-walled spores, centrally placed in swollen sporangia. These characteristics are consistent with those of the genus Bacillus and suggest that these organisms belong within group II of this genus (Wolf and Barker 1968). These thiaminase producing strains were compared with *B circulans* (NCTC 2610), *B polymyxa* (NCTC 10343), *B alvei* (NCTC 6352), *B macerans* (NCTC 6355) and *B thiaminolyticus* (NCTC 10760). The latter organism has been poorly characterised in comparison with other members of the genus and is not listed in the current taxonomy (Breed and others 1957). Our isolates were found to differ from each other in a few of the tests applied (Wolf and Barker 1968) but they corresponded most closely with *B thiaminolyticus* (Table 1).

The difficulty experienced in isolating the thiaminase-producing bacteria from alimentary contents was due to other bacteria retarding their growth on solid media and possibly because of the small number of spores germinating from heated broth cultures. They were readily recognised, however, by the characteristic adherence of the colonies to blood agar and the prolific sporulation observed after 24 to 48 hours. An attempt was also made to isolate these organisms, without success after two broth to broth subcultures, from the "thiaminase-free" rumen contents of 10 normal sheep. The value of these results as "negative controls" is doubtful, however, as we have yet to establish the ideal conditions required for the isolation of the thiaminase-producing bacteria.

Results

The thiaminases produced by our isolates were compared with the thiaminases present in the faeces and rumen liquor from which the bacilli were obtained. They were all found io be type 1 thiaminases (Shimazono and Katsura 1965), with the same substrate specificities in the range of substrates tested. Nicotinic acid, pyridine and aniline were found to react readily with thiamine in the reaction catalysed by these enzymes and cysteine, para-amino benzoic acid, meso-amino benzoic acid, hydroxyproline, lysine and phenylalanine failed to react. The thiaminases produced by our Bacillus isolates were found to mediate in the base-exchange reaction described by Edwin and Jackman, with the production of pyrimidinyl-nicotinic acid from thiamine and nicotinic acid. The pyrimidinyl-nicotinic acid was identified on paper chromatograms using the methods described by Edwin and Jackman (1970) and Shimazono and Katsura (1965).

This study has thus confirmed the observation of Edwin

TABLE 1: Details of characteristics of Bacillus strains examined

Name and origin of strain	Thiaminase producing bacilli (from sheep)	*Bacillus thiaminolyticus* (NCTC)	*Bacillus alvei* (NCTC)	*Bacillus circulans* (NCTC)	*Bacillus macerans* (NCTC)	*Bacillus polymyxa* (NCTC)
Number of strains examined	8	1	1	1	1	1
Spreading membranous colonies	+	+	—	—	—	—
Spore rapidly and readily	+	—	—	—	—	—
Hydrolyse starch	±	+	+	+	+	+
Oxidase	+	+	+	—	+	—
Catalase	±	—	—	+	+	+
Produce gas in glucose broth	∓	—	—	—	—	+
Thiaminase type 1 produced in broth	+	+	—	—	—	—

Key + = positive
— = negative
± = most strains positive
∓ = most strains negative

and Jackman (1970) that thiaminase type 1 is present in the rumen contents of sheep which have died of PE. This enzyme was also found to be present in a proportion of faeces samples from clinically normal ewes. Furthermore, organisms of the genus Bacillus which produce thiaminases similar to those implicated in the aetiology of PE were present in all the rumen contents examined from sheep affected with this disease and in a proportion of faeces samples which contained the enzyme.

Discussion

Since the completion of that part of our investigations it has been reported by Shreeve and Edwin (1974) that strains of *Clostridium sporogenes*, which produce thiaminase type 1, have been isolated from "animals" (the species of which was not stated) affected with PE. There is evidence, therefore, that the alimentary thiaminases, associated with outbreaks of PE may be produced by either Clostridia or members of the genus Bacillus. We have conducted a preliminary study to test the proposal that the thiaminase production from rumen samples of different species could be favoured by either aerobic or anaerobic conditions of growth.

TABLE 2: Thiaminase activity produced in broths inoculated with rumen contents from animals affected with polioencephalomalacia

Animal (species)	Number examined	Thiaminase activity produced	
		Cooked meat broth	Aerated broth
Calf (bovine)	2	+	—
Goat	1	+	—
Sheep	5	—	+

Key + = high activity produced
— = low or no activity produced

Samples of rumen contents from a goat, two calves and those of the five sheep from which we had isolated thiaminase-producing bacilli, were inoculated, in duplicate, into aerated nutrient broth and cooked meat broth to encourage the growth of aerobic and anaerobic bacteria respectively. These broths were incubated at 37°C for 48 hours, and then assayed for thiaminase activity (Table 2).

These findings suggest that there may be a correlation between the "host" species and the nature of the source of the thiaminase, with aerobes predominating in certain outbreaks in sheep and anaerobes in calves and the goat. This intriguing distinction requires investigation in a large number of animals, however, and further work is necessary to determine the distribution of these, and possibly other, thiaminase producing bacteria in ruminants and their environment and their exact aetiological significance.

Acknowledgments.—We thank the many people whose assistance made this work possible and especially Miss P. Wooding, Dr B. S. W. Smith, Dr J. S. Slater and Mr C. C. Renwick.

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RÉSUMÉ.—Ce rapport décrit comment on a pu isoler, sur des ovins dont plusiers avaient succombé à une polio-encéphalite, des bacilles quit fabriquent la thiaminase type 1 qui jouait un rôle dans l'étiologie de la maladie. On suppose que les thiaminases présentes dans le contenu du rumen et les excréments des moutons seraient d'origine bactérienne.

ZUSAMMENFASSUNG.—Diese Mitteilung beschreibt die Isolierung von Schafen, von denen mehrere an Polioenzephalomalazie verendet waren, von Bazillen, die Thiaminase des Typs 1 hervorrufen, die anscheinend mit der Thiaminase identisch ist, die in die Atiologie dieser Krankheit einbegriffen ist. Es wird vorausgesetzt, dass die in im Panseninhalt und Kot der Schafe vorhandenen Thiaminasen bakteriologischen Ursprungs sein können.

Printed by H. R. GRUBB LTD., 15 Imperial Way, Purley Way, Croydon, CR9 4PY.