A STUDY OF THE ROLE OF MICRONUTRIENT NUTRITION IN RELATION TO THE PATHOGENESIS OF HELMINTH DISEASE AND IMMUNE FUNCTION IN SHEEP

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by

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DEDICATION

I would like to dedicate this thesis to my parents, Mary and Daniel Ferguson and to my family, for their understanding, support and kindness during my period of study and also to the many friends that I have made during my time at Auchincruive. Thanks.

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ABSTRACT

Literature concerning cobalt, selenium, Vitamin E deficiencies and *Ostertagia circumcincta* infection was reviewed togther with the inter-relationship of these factors to immune function in lambs.

Two experiments were described. In the first, the inter-relationship between cobalt deficiency, *O. circumcincta* infection and immune function was examined. Sixty, nine to twelve week old Blueface Leicester cross Swaledale and Scottish Blackface lambs were assigned to three treatment groups dependent upon their maternal cobalt status thus: deficient lambs came from cobalt deficient ewes; depleted lambs came from cobalt sufficient ewes but were then fed a cobalt deficient diet; sufficient lambs also came from sufficient ewes by their status was maintained by weekly administration of a cobalt supplement. All lambs were fed a diet deficient in cobalt. The groups were then sub-divided into infected and non-infected, the latter receiving 2000 L3 *O. circumcincta* three times per week for 16 weeks.

Cobalt deficient lambs suffered extremely high mortality rates, regardless of infection, in comparison with a much lower rate among depleted lambs and none in the sufficient group. A possible link between hepatic encephalopathy and white liver disease was found. Neither liveweight effects nor anaemia attributable to poor performance associated with helminth infection were noted. However, cobalt deficient lambs had significantly reduced liveweight and anaemia in comparison with sufficient controls although some degree of anaemia was also evident in the latter group. Anaemia was found in depleted lambs, which matched sufficient controls in liveweight in the early part of the experiment

but showed liveweight impairment during the latter part presumably after exhaustion of maternally derived cobalt reserves.

Deficient lambs suffered more pathogenic *O. circumcincta* infection than their sufficient controls as measured by plasma pepsinogen concentrations but values found in all infected lambs were nonetheless greater than those in non-infected counterparts. Prolonged elevation of plasma pepsinogen concentration indicated a continued establishment of incoming larvae in infected groups which was confirmed by maintained circulatory eosinophilia particularly in cobalt deficient and depleted lambs. Depleted infected lambs revealed plasma pepsinogen concentrations similar to sufficient infected controls in the early part of the experiment but which rose substantially thereafter, again presumably after the exhausion of maternally derived cobalt reserves. Infected sufficient lambs had plasma pepsinogen values which were much lower than those found in depleted or deficient lambs. Similar effects were noted in worm egg count data. Total worm counts at sacrifice of four depleted and four sufficient lambs revealed significantly higher burdens in the depleted lambs. Absence of significant stunting, difference in adult:larval proportion and difference in male:female ratio in total worm counts suggested that immunity to infection was incomplete in those lambs. High mortality among cobalt deficient lambs prevented the collection of meaningful comparative data.

Significantly impaired immune function as measured by ability of neutrophils to kill ingested yeast cells was noted in cobalt deficient and depleted lambs in comparison to their sufficient controls. Attempts to investigate phagocytic impairment further were inconclusive. Serological response to *O. circumcincta* was relatively low in all lambs in

Experiment 1 and not thought to be indicative of immunity. Serological response to *Clostridium tetani* toxin revealed no significant difference in pre-vaccination titre in any group of lambs, thus suggesting no impairment in maternal passive transfer of those antibodies associated with cobalt deficiency. Cobalt sufficient lambs had post-vaccination titres which were significantly higher than both their own pre-vaccination levels and than the post-vaccination titres of deficient and depleted lambs. Deficient and depleted lambs had post-vaccination titres which were not significantly different from their pre-vaccination levels.

After sixteen weeks of infection, surviving lambs were treated with an anthelmintic containing selenium and cobalt and its effectiveness in removing parasite burden, remitting micronutrient deficiency and on growth rates examined. Results from this experiment are discussed and compared with relevant literature.

In the second experiment, the inter-relationship between selenium, Vitamin E deficiencies, *O. circumcincta* infection and immune function was examined. Fifty-two, twelve to sixteen week old Blueface Leicester cross Swaledale and Scottish Blackface lambs were assigned initially into two groups, one of which was supplemented with Vitamin E. The groups were then sub-divided into infected and non-infected sub-groups, the former being dosed with *O. circumcincta* at the rate previously mentioned. All lambs were fed a diet low in selenium and Vitamin E. Although their status fell, these lambs were never selenium deficient during the first part of the experiment. In the latter part however a proportion of the Vitamin E deficient lambs were supplemented with Vitamin E and allowed to become selenium deficient while the remainder

were supplemented with selenium and permitted to remain Vitamin E deficient. Supplementation with selenium reduced the amount of muscle damage associated with Vitamin E deficiency, as measured by creatine kinase activity, more rapidly than Vitamin E supplementation.

A higher mortality rate was experienced in Vitamin E deficient lambs, regardless of infection, than their supplemented equivalents but no mortality was noted in selenium deficient lambs. No production deficit was noted due to Vitamin E deficiency but was evident in selenium deficient lambs. A production deficit due to *O. circumcincta* infection was present in all infected groups but as not statistically significant. Selenium deficient lambs had the highest faecal worm egg counts, plasma pepsinogen concentrations and total worm burdens at slaughter. Sufficient controls had the lowest worm egg count and total worm counts while Vitamin E deficient lambs were intermediate. Little evidence of stunting of parasites or significant difference in male:female ratio or proportion of adults:larvae was found and thus in conjunction with the maintained circulatory eosinophilia suggests that immunity to *O. circumcincta* infection was incomplete in this experiment. No anaemia due to infection or selenium or Vitamin E deficiency was noted in Experiment 2.

Significantly impaired immune function as measured by the ability of neutrophils to kill ingested yeast cells and the serological response to *O. circumcincta* antigen was noted in both selenium and Vitamin E deficient groups. Selenium deficient lambs showed the greatest impairment of neutrophil function and lowest titres to *O. circumcincta* while Vitamin E deficient lambs were intermediate. Attempts to investigate phagocytosis in selenium and Vitamin E deficient lambs furnished only inconclusive results. Serological response to *C. tetani* revealed no

difference in magnitude of response attributable to Vitamin E deficiency. Selenium deficient lambs were not assayed in this test. After sixteen weeks of infection, surviving lambs were treated with an anthelmintic containing selenium and cobalt and its effectiveness assessed in terms of remitting micronutrient deficiency, removal of parasite burden and subsequent effects on growth. Results from this experiment are discussed and compared with other relevant studies.

When the results of Experiments 1 and 2 were compared with each other and to other relevant studies, it was clear that cobalt deficiency was the most detrimental of the deficiencies induced in the present study in terms of immunity to disease, production effects, anaemia and response to helminth infection. Whether these were true effects of cobalt deficiency or conditioned by a secondary protein deficit associated with inappetance remains to be elucidated. Notwithstanding the fact that the deficiency established in lambs in Experiment 1 was more protracted by virtue of the cobalt deficient state of their dams, selenium deficient lambs in Experiment 2 showed much less severe effects and Vitamin E deficient lambs were the least affected. In the latter two cases it appeared that direct micronutrient effects occurred but further studies were throught to be necessary before precise roles for selenium and Vitamin E in helminth and other infection in sheep could be defined.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 HISTORICAL PERSPECTIVE

The importance of trace element deficiency and parasitic infection has been individually recognised for many years.

Hogg, "The Ettrick Shepherd", wrote in the Shepherd's Guide (1807) specifically of "a distemper, which is most severe upon young sheep, but is confined mostly to some districts in the West of Scotland, where the land is very coarse." He was speaking of a condition variously called at that time "pining, daising and vinkish" and now known as cobalt deficiency.

Although there are fewer historical records, selenium deficiency was also likely to have been present, if undiagnosed, from the time of Hogg and before. As late as 1937 and up until 1956 however, selenium was still only identified by Moxon (1937) in terms of its toxic effects in producing two diseases in animals namely "Blind staggers" and "Alkali disease" now known to be chronic and acute selenium poisoning respectively.

It was not until 1957 that Schwarz and Foltz discovered an essential physiological role for selenium in preventing liver necrosis in rats. Subsequent examination has shown further roles for selenium in animal nutrition principally the prevention of nutritional muscular dystrophy (by protecting intracellular structures against oxidative damage).

Ostertagia circumcincta was speciated as relatively recently as 1894 by Stadelmann but it too may well have existed, if similarly

undiagnosed at the time of Hogg. Infestation by parasitic nematodes probably existed in prehistoric times.

Hogg (1807) by remarkably astute observation was both able to speculate on the causes of diseases and to recommend suitable corrective action. He wrote of a 'rot' (or 'poke') which infected sheep and stated that "sudden fall in condition is the sole cause of the rot".

Further reports detailed findings consistent with liver fluke infestation but by failing to differentiate between what we now know to be symptoms he was probably unable to separate various forms of infection. Two types of rot were reported by Hogg, namely "Black Rot" and "Hunger Rot" corresponding to the intake of 'foul' food and too little food respectively.

Hogg described languor and listless inactivity in affected sheep. 'Thin' blood and a detailed description of pale mucous membranes ultimately in tender skin and 'leaness about the brisket'. These symptoms could indicate any one of a number of infestations and thus describe undiagnosed parasitic infection even at that time.

It is possible that where sheep are grazing a mineral deficient area of parasite - trace element interaction occurs. It is important therefore that the role of trace element nutrition in the pathogenesis of helminth and other infections should be better understood.

The studies presented in this thesis examine the effects of selected micro-nutrient deficiencies on helminth infection and immune function in sheep.

1.2 THE HISTORY OF COBALT AND VITAMIN B_{12}

Elemental cobalt was named by German copper miners working in the Harz Mountains after evil spirits or gnomes (Kobold in German) took pleasure in exposing ores which resembled copper but which yielded none when roasted. Cobalt was shown by Brandt in 1735 to be a new metal and was more fully characterised by Bergmann in 1780 (MacPherson, 1982).

Cobalt deficiency may have been recognised as early as 1807 by Hogg who surmised that the cause of deficiency was related to grazing and soil type and was thus dietary in origin but he was unable to determine the exact cause of the disease.

The worldwide nature of cobalt deficiency is reflected in the variety of names by which the condition is known viz; "Pine", "Vinkish", "Vanquish" and "Daising" are few of the names used in Scotland by Hogg and others. In Australia where cobalt deficiency is widespread the condition is called "Coast Disease" or referred to as "Coastiness" in sheep and "Bush Sickness" or "Wasting Disease" in cattle. Cobalt deficiency is also known as "Nakuruitis" in East Africa and "Salt Sickness" in Florida.

Early speculation as to the cause of this disease came from Orr and Holm (1931) and Aston (1932) who suggested that symptoms of

deficiency could be cured in cattle by the administration of iron ores and salts. This led to a belief that iron deficiency was the cause of the disease.

However the first evidence against this theory was presented by Filmer and Underwood in 1934 who, investigating a wasting disease of cattle, enzootic marasmus, found no relation between the size of a dose effective in curing the disease and the amount of iron supplied in that dose.

Fractionation studies published by Underwood and Filmer (1935) on the ferric mineral Limonite ($Fe_2O_3.H_2O$) suggested that 'the potency of the mineral rested in its cobalt content'. Marston (1935) and Lines (1935) were the first to note improvement in clinically affected sheep on dosing with cobalt salts. This was the first indication that cobalt deficiency was important in understanding these diseases. However Marston *et al* (1938) showed that copper as well as cobalt was needed to cure true Coastal Disease.

Shortly afterwards, a systematic approach by Underwood and Filmer (1935) showed animals suffering from "Denmark Wasting Disease" (another local name for the condition), could be cured by dosing with small amounts of cobalt salts. Askew and Dixon (1936) reported that Bush Sickness was amenable to the same treatment.

In Scotland early work describing the incidence of a condition later defined as cobalt deficiency was reported by McGowan and Smith (1922). Corner and Smith (1938) and Corner (1939) described cobalt deficiency in the Scottish Borders and suggested possible

oral cobalt supplementation rates likely to correct deficiency. Dunlop (1947) described the prevalence of the condition in Ayrshire.

Progress in understanding the mode of action of cobalt in preventing deficiency in ruminants was slow until 1948 when Smith discovered that an anti-pernicious anaemia factor subsequently called Vitamin B_{12}, was a compound which contained 4% cobalt. This discovery was made simultaneously with the American group led by Folkers and published by Rickes *et al* (1948). It was not long until the precise structure of Vitamin B_{12} was elucidated by four research teams led by Folkers (USA), and Smith, Hodgkin and Sir Alexander Todd (UK).

Smith *et al* (1951) were able to show that lambs given parenteral injections of Vitamin B_{12} showed remission of all signs of cobalt deficiency. Confirmation of these findings was furnished by Anderson and Andrews (1952).

The fact that cobalt was converted to Vitamin B_{12} by rumen micro-organisms was first reported by Kercher and Smith (1956) and subsequently by Smith and Loosli in 1957. The concept of storage of Vitamin B_{12} in the liver was first published by Andrews *et al* in 1958. Early experiments by Dryden *et al* (1962) found that different forms of Vitamin B_{12} were produced, some physiologically active, others not. The next step was to elucidate the role of cobalt and Vitamin B_{12} in preventing deficiency diseases.

1.3 METABOLIC FUNCTION OF COBALT AND VITAMIN B_{12}

Dryden *et al* (1962) were the first to ascertain that 'several strains of rumen bacteria each produced a mixture of Vitamin B_{12} and its analogues when cultured in a cobalt adequate medium'. Only two forms have been recognised as physiologically active in ruminant nutrition. These are adenosylcobalamin and methylcobalamin (Schneider and Stronskii, 1987).

1.3.1 Adenosylcobalamin

An important form of Vitamin B_{12} in ruminant nutrition is adenosylcobalamin which functions as a co-factor of an enzyme, methylmalonyl Co enzyme-A mutase (E.C. 5.4.99.2). This enzyme governs the conversion of methylmalonyl CoA to succinyl CoA during propionate metabolism in hepatocytes as shown in Figure 1.

McDonald *et al* (1981) stress the vital nature of propionate (and other volatile fatty acids) in ruminants as a major energy source. Thus a deficiency of Vitamin B_{12} will result in a dearth of the enzyme involved in methylmalonyl conversion and thus affect the Kreb's Tricarboxylic Acid Cycle.

This was first reported by Marston *et al* (1961). The net effect of deficiency is a build up of methylmalonyl CoA in the first instance. Excess methylmalonyl CoA was found to be excreted in urine and to result in elevated levels in the blood (Marston *et al* 1961). An excess of methylmalonyl CoA could lead to a failure to clear and a build-up of propionate in deficient animals.

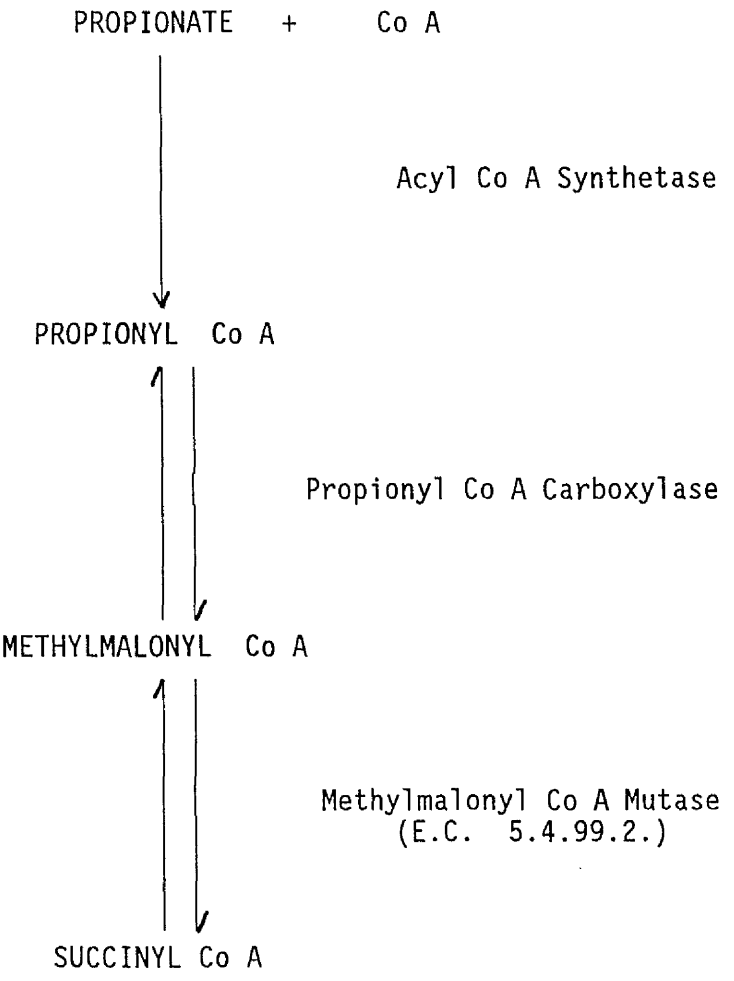


FIGURE 1: The Conversion of Propionate to Succinyl Co-enzyme A. (After MacDonald *et al*, 1981).

The same authors subsequently reported an inverse relationship between voluntary feed intake and half-life of propionate clearance.

Somers (1969) reported a similar failure to clear acetate during its metabolism in the deficient animal which Underwood (1981) suggested was a function of the impairment of propionate clearance.

Similar impairment in methylmalonyl CoA isomerase activity was reported by Gurnai *et al* (1961) and by Smith and Monty (1959) in rats. Gawthorne (1968) was able to demonstrate that urinary excretion of excess methylmalonyl CoA was stopped by Vitamin B_{12} injections.

1.3.2 Methylcobalamin

The second physiologically active form of Vitamin B_{12}, methylcobalamin, has been shown to affect methionine synthesis (Underwood 1977) and thus folate metabolism.

The function of methylcobalamin in mammalian tissues is as a co-factor for the enzyme, 5-methyltetrahydrafolate : homocysteine methyl transferase (E.C. 2.1.1.13) whose common name is methionine synthetase, and which catalyses the reformation of methionine from homocysteine. This process is shown diagramatically in Figure 2.

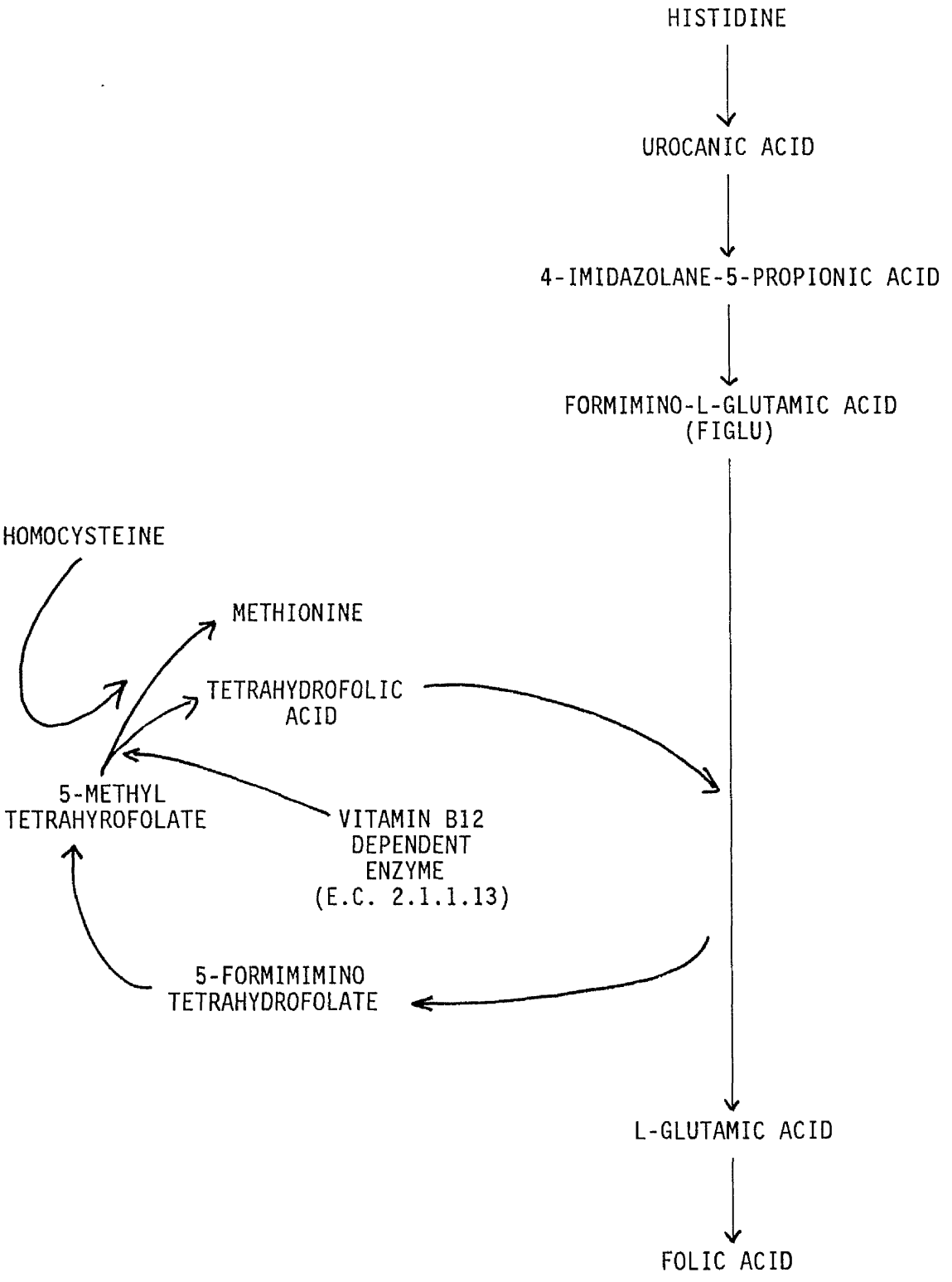


FIGURE 2: The Relationship between Vitamin B12 and FIGLU in the Methionine Synthetase Pathway (After Givens, 1978).

Vitamin B_{12} regulates the availability of tetra-hydrofolic acid, a pre-cursor of folic acid (Givens, 1978). Folic acid production initially involes the breakdown of the amino acid histidine to formimino-L-glutamic acid (FIGLU) which requires tetra-hydrofolic acid for further reaction.

It has been suggested that when Vitamin B_{12} levels are low tetra-hydrofolic acid levels in the liver fall and prevent further FIGLU metabolism excesses of which are then removed by the liver. Smith and Osborne-White (1973) reported very low levels of tetra-hydrofolate and folacin, (the collective name for a group of compounds derived from folic acid) in the livers of Vitamin B_{12} depleted sheep. Smith *et al* (1974) restored liver folate levels by treatment with either Vitamin B_{12} or L-methionine thus suggesting that the effect of Vitamin B_{12} operates via methionine. Vitamin B_{12} deficiency was shown by Gawthorne and Smith (1974) to cause a secondary deficiency in methionine due to this influence.

Therefore when Vitamin B_{12} status is low, re-cycling of tetra-hydrofolic acid is reduced, preventing L-glutamic acid production, a pre-cursor of folic acid. Deficiency in folacin is characterised by nutritional anaemia and poor growth (MacDonald *et al*, 1981).

Underwood (1981) suggested that secondary methionine deficiency may impair nitrogen retention in the deficient animal which may be important in wool and overall body growth.

The site of absorption of Vitamin B_{12} was stated by Smith and Marston (1970a,b) to be the small intestine. The Vitamin is associated with the fibrous part of the digesta and following release in the acid conditions of the abomasum passes into the small intestine relatively intact.

Those authors stated that while the efficiency of absorption is low and the majority is lost through faecal excretion once absorbed Vitamin B_{12} is preferentially stored in the liver.

1.4 CLINICAL SIGNS OF COBALT DEFICIENCY

Exposure of ruminant animals to prolonged deprivation of cobalt will result in a clinical deficiency syndrome but it is important to remember that deficiency is not static. Although clinical deficiency is more easily detected and treated, sub-clinical deficiency by its very nature will probably have the greater economic impact since animals will achieve less than optimal productivity (Andrews, 1965).

The appearance of a severely clinically deficient animal is one of extreme emaciation and listlessness (Underwood, 1981) and is difficult to distinguish from starvation (Underwood, 1977). He described a gradual loss of appetite in the sub-clinically deficient animal resulting in a failure to grow and perhaps leading to a frank weight loss. McDonald *et al* (1981) suggested that prolonged high levels of propionate in blood could affect nervous mechanisms mediated by metabolite concentration and which control appetite thus leading to a reduction in voluntary feed intake.

Muscular wasting or marasmus resulted from the failure in appetite and anaemia, resulting in pale mucous membranes in sheep was reported by Smith *et al* (1950). Gawthorne *et al* (1966) reported low packed cell volume (PCV) and haemoglobin concentration in deficient animals. Pale and fragile skin together with photo-sensitive coat change and lachrymal exucation have also been reported (Underwood, 1981). Chronically deficient animals develop a depraved appetite referred to as "Pica" and severe anaemia culminating in death.

1.4.1 Pathological Changes

Internal tissue and metabolite changes are also associated with cobalt deficiency in sheep and cattle. MacPherson and Moon (1974) reported lower plasma glucose levels in deficient steers compared with cobalt adequate control cattle. Filmer (1933) recorded under-development of bone marrow erythropoetic tissue, fatty deposition around the liver and distinct discolouration of the spleen (associated with erythrocyte destruction) during post mortem examination of deficient animals. He further noted that red cell numbers and haemoglobin levels were always sub-normal and sometimes extremely low in deficient animals. The anaemia associated with deficiency was initially classified as normocytic and hypochromic (Filmer, 1933), however Gawthorne *et al* (1966) later characterised the anaemia in lambs as normocytic and normochromic.

Underwood (1977) indicated that inappetance not anaemia was responsible for the major symptoms of deficiency. He further

stated that inappetance and marasmus preceded any considerable, presumably fatal, anaemia.

1.4.2 Biochemistry Associated with Cobalt Deficiency

MacPherson *et al* (1976) reported the following biochemical changes in severely cobalt deficient sheep; abnormally low plasma glucose levels (as found in steers by the same authors), abnormally low plasma alkaline phsophatase activity, abnormally high glutamate oxaloacetic transaminase (GOT) and pyruvate levels together with lower plasma ascorbic acid and thiamine levels. Underwood (1977) stated that the high GOT and low ascorbic acid levels found by these authors in palsma may have been a function of the liver damage associated with cobalt deficiency and additionally that high pyruvate levels may have influenced thiamine deficiency.

MacPherson *et al* (1973) reported a suspected link between Cerebrocortical Necrosis (CCN) and cobalt deficiency in sheep. The same authors found in 1976 that cobalt supplementation reduced the occurrence of CCN and suggested that low plasma ascorbic acid levels found in deficient sheep may be an important factor in increased susceptibility to respiratory and enteric infection among cobalt deficient sheep. Confirmation of this phenomenon has been reported by Garton *et al* (1981) who described cerebral cortex degeneration in cobalt deficient sheep (ewes and lambs) and Fell *et al* (1985) who found similar cellular atrophy. Both groups concluded however that changes may be associated with hepatic dysfunction similar to hepatic encephalopathy as described next.

The development of a pathological syndrome associated with clinical cobalt deficiency was descibed by Cordes and Gardener (1971) as White Liver Disease (WLD). This is a condition of some economic importance (Sutherland *et al*, 1979) which was found initially in New Zealand and subsequently in Australia (Richards and Harrison, 1981). Similar conditions have been reported in Holland (Wensvoort and Herweige, 1975), Norway (Ulvund and Overas, 1979), Northern Ireland (McLoughlin *et al*, 1984 and McLoughlin *et al*, 1986) and in experimental animals in Scotland (Rowett Research Institute Annual Report, 1980).

While the exact aetiology is unclear, cobalt and Vitamin B_{12} deficiency are commonly associated with a condition which is characterised by fatty change in hepatocytes and both bile duct and lymphocyte proliferation.

1.5 COBALT DEFICIENCY AND IMMUNITY

1.5.1 General Considerations

Gershwin *et al* (1985) stated that because Vitamin B_{12} is essential for protein and nucleic acid biosynthesis, deficiency will produce a depression in immune competence. However the immune system of sheep or any other animal is extremely complex and involves both independent and conjoint functioning of cellular and humoral factors throughout the body.

Andrews *et al* (1970) suggested that the current state of knowledge on the interaction between cobalt deficiency and immune function in relation to common pathogens was extremely limited.

1.5.2 Non-Specific Immunity and Cobalt Deficiency

Following the work of Boyne and Arthur (1979) who reported impaired neutrophil killing ability in selenium deficient calves, Wright *et al* (1982) reported a similar reduction of neutrophil killing ability in cobalt-deficient calves while subsequently Fisher and MacPherson (1986) were the first to report similar impairment in neutrophil killing ability in cobalt deficient ewes.

MacPherson *et al* (1976) also reported suspected impairment of immunocompetence in sheep. In an experiment these authors found 9 out of 12 cobalt deficient sheep suffering either pneumonic or enteric infection. This compared unfavourably with 1 from 12 cobalt adequate sheep kept under identical housing conditions.

1.5.3 Cobalt Deficiency and Neonatal Effects

Cobalt deficiency has also been shown to affect new-born lambs. Duncan *et al* (1981) reported higher perinatal mortality in lambs born to Vitamin B_{12} deficient ewes (ie deficient both at mating and during pregnancy). Five deficient ewes gave birth to 9 lambs of which 3 were still-born, 2 died at birth and the remainder were weak and unable to suckle, whereas three control ewes produced normal lambs.

Fisher and MacPherson (1986) reported poorer 'viability' in lambs born to deficient ewes. In two experiments examining the effect of cobalt deficiency in the pregnant ewe they reported that lambs born to deficient ewes took significantly longer to

stand, find the udder and suckle post-partum than sufficient controls.

Although both experiments examined the effect of cobalt deficiency and supplementation on the pregnant ewe, the effect of **half-supplementation** (ie full supplementation with cobalt in either the first or the latter half of gestation) demonstrated that cobalt deficiency in either resulted in similar poor viability (as measured by time to stand and suckle post-partum) in those lambs.

Both experiments demonstrated that increased neonatal mortality was also found in lambs from cobalt deficient ewes (Fisher and MacPherson, 1986). Those authors suggested that while the causes of decreased viability were complex, some depression in transfer of passive immunity from ewe to lamb was found in deficient ewes as measured by radial immunodiffusion of IgG in serum and zinc sulphate turbidity (ZST) in serum.

1.5.4 Cobalt Deficiency and Parasitic Infection

Parasitic infection among cobalt deficient animals has been studied with varying degrees of success and agreement for the past 30 years. Although many shepherds have formed opinions concerning possible increased susceptibility of pining sheep to parasitic infection, until recently relatively few scientific studies existed characterising this phenomenon.

Corner (1941) suggested that while cobalt deficiency was not directly connected with infestation, "pining sheep usually carry

considerable infection", although he stated that it had been their experience that after supplementation, "the question of worms need not specifically be considered".

Richard *et al* (1954) quoted previous work of Weir *et al* (1948), Badr (1949) and their own work which found that feeding trace mineralised salts and bone meal enabled lambs to withstand the stomach worm *Haemonchus contortus*.

Richard *et al* (1956) attempted to characterise which minerals, if any, were involved in this apparent enhancement of immuno-competence. In two experiments which investigated firstly cobalt versus manganese and secondly cobalt versus bone-meal, it was found that double supplementation with bone-meal and cobalt resulted in the least pathogenic effect of the parasite.

Threlkeld *et al* (1956) designed a project to determine if a relationship existed between cobalt deficiency and parasitism in ruminants. These authors quote Shumard and Herrick (1954) who demonstrated that culturing faeces of lambs receiving trace element supplements resulted in variable larval development dependent upon the nutritional status of the donor lamb used. Threlkeld *et al* (1956) used 3 month old lambs (weaned at one week), fed a cobalt deficient basal ration in which cobalt deficiency was recognised by a failure to gain weight, very low packed cell volume (PCV) and haemoglobin levels.

Thereafter lambs were allocated to groups in a 2 x 2 factorial design experiment (ie ± infection, ± cobalt) where the infective

challenge of 70000 larvae (L3) consisted of 90% *Haemonchus contortus*, 5% *Ostertagia circumcincta* and 5% *Trichostrongylus axei*. These authors found that supplementation with cobalt led to greater parasite establishment, a higher percentage of gravid females and greater average female worm length.

Downey (1965) attempted to examine the relationship between trichostrongylid infestation and host-cobalt status further by supplementing 31 week old Greyface lambs in an experiment similar to the 2 x 2 factorial described above. Infection consisted of 70000 *H. contortus* larvae. He recorded weight loss due to infection in both supplemented and non-supplemented groups. A more rapid rise in worm egg count was found during early patency among deficient lambs. By 30 days post-infection, supplemented animals were found to be excreting more than ten times the number of eggs found from deficient lambs (1745 versus 164). This was coupled to a more severe anaemia in supplemented sheep.

Downey (1965) stated that higher worm egg counts in supplemented animals may be attributable either to increasing number of larvae establishing or to greater fecundity of egg laying females. He quotes Weir *et al* (1948) in support of his findings that supplementation of infected animals resulted in relatively greater parasitic burden.

The findings of Threlkeld *et al* (1956) that the establishment and fecundity of *H. contortus* was determined by host cobalt

status and his own work led Downey to believe that the parasite had an apparent requirement for either cobalt or Vitamin B_{12}.

Further studies by Downey (1966a) using a *H. contortus* - infection suggested that initially, greater pathogenicity occurred in cobalt supplemented, helminth infected sheep. The same author subsequently developed the concept of Weir *et al* (1948) that parasitic infection impaired micronutrient (Vitamins A and C) utilisation. To test this theory he attempted to assess the effect of parasitism on serum Vitamin B_{12} levels and the relationship between cobalt status and parasitic infection in lambs.

This was achieved by superimposing an infection of 17000 *Ostertagia circumcincta* larvae on a long standing *T. axei* infestation in 6-7 month old lambs. He demonstrated significantly lower serum Vitamin B_{12} values in infected animals compared to non-infected controls.

In a second experiment Downey (1966b) assessed the relationship between sub-clinical cobalt deficiency and *O. circumcincta* infection. In contradiction of his earlier work using *H. contortus* he observed that marginal cobalt deficiency resulted in higher worm egg counts, lower haemoglobin levels and poorer weight gains compared with similarly infected lambs receiving adequate cobalt. He therefore concluded that marginal cobalt deficiency and mild *O. circumcincta* infection were additive in their effects.

More recently Wright *et al* (1982) reported similar findings in cattle. Calves biochemically defined as deficient showed no ill-effects due to low cobalt status and these authors felt that an additional 'stress' was necessary to cause any detrimental effects.

The additional 'stress' was provided by a single dose of 10000 *O. ostertagi*. Infected animals thereafter showed reduced live-weight gains compared with uninfected control animals.

Those authors concluded from their observations that cobalt deficiency led to an indirect increase in vulnerability to disease.

1.6 SUPPLEMENTATION OF COBALT AND VITAMIN B_{12}

1.6.1 Early Strategies to Correct Cobalt Deficiency

Filmer (1933) while investigating the nature of cobalt deficiency found that supplementation by feeding with liver was "highly curative". Filmer and Underwood (1934) suggested that liver contained a factor dependent on cobalt which in turn had curative action.

Underwood (1977) however cited unsuccessful attempts to correct cobalt deficiency by liver supplementation (Kercher and Smith, 1955a,b and 1956 and Marston and Lee, 1952).

Oral administration of Vitamin B_{12} was shown to be much less effective than injection since ruminants were found to absorb Vitamin B_{12} poorly. (Smith and Marston, 1970a,b).

1.6.2 Current Approaches

There are numerous methods commonly employed to correct cobalt deficiency. These include:

1. treatment of pasture with cobalt salts or fertiliser;
2. injection with Vitamin B_{12};
3. incorporation of cobalt salts into licks or feeds;
4. oral dosing of animals with cobalt salts;
5. metered dosing through drinking water;
6. intra-ruminal slow release devices;
7. supplementation via cobalt containing anthelmintics.

Top dressing of pasture:

Underwood (1981) reported that cobalt deficiency could be cured or prevented through soil or pasture treatment with specialised cobalt containing fertilisers.

The most economical method of correcting deficiency was stated by Underwood (1977) to be top dressing of pasture with fertilisers containing cobalt salts. Application rates depended largely on husbandry methods employed. Rossiter *et al* (1948) reported that annual or biannual application of 100-150 grams per hectare of cobalt sulphate, maintained adequate cobalt levels in pasture otherwise deficient.

Andrews and Anderson (1954) reported satisfactory pasture cobalt levels in deficient areas of New Zealand for 3-6 years after a single application of 500-600 grams of cobalt sulphate per hectare. However, MacPherson (1981b) reported 'availability' problems associated with certain soil conditions and types when using pasture dressing as corrective therapy.

Vitamin B_{12} injection:

Koch and Smith (1951) reported correction of cobalt deficiency in lambs by Vitamin B_{12} injection (as hydroxycobalamin) at the rate of 100-150 ug per week. Injections of cobalt to cure deficiency may be inappropriate since insufficient cobalt would reach the rumen where it is required for microbial conversion to Vitamin B_{12} (Underwood, 1981).

Incorporation of cobalt salts into licks and feed:

Dunlop (1947) reported the successful treatment of cobalt deficiency in Scotland with mineralised block 'licks' and by incorporation of a cobalt salt into the diet of sheep. Underwood (1977) suggested that this inclusion, which he described as widespread, often takes place indiscriminately where no evidence of clinical deficiency has previously been found.

Both Underwood (1977) and MacPherson (1981b) discussed possible problems with salt-licks or dietary inclusion where variable consumption by animals did not ensure adequate intake by any individual animal.

Oral dosing with cobalt salts:

Lee (1950) reported that the dosing of sheep twice weekly with 2 mg or weekly with 7 mg cobalt (as the sulphate) was completely adequate in preventing deficiency even under conditions usually resulting in acute cobalt deficiency. The same author, while investigating the possibility of increasing dose rate to reduce the required frequency of administration, reported that 35 mg of cobalt administered one in 5 weeks only delayed the onset of symptoms. Filmer (1941) increased the dose rate to 140 mg per month and found that ewes and lambs did not suffer clinical disease but he described an "Ill-thrift" in these animals which was absent in sheep dosed more frequently.

Dunlop (1947) described a similar failure to thrive, presumably due to sub-clinical deficiency, in sheep. He found however that monthly dosing with 100 mg cobalt "worked well on affected farms".

A larger dose of 300 mg cobalt (as oxide or sulphate) administered to lambs grazing deficient pasture was compared to untreated lambs and those given weekly 7 mg doses by Andrews *et al* (1966). They found that both supplementation rates and forms of cobalt worked equally well but in contrast to Dunlop (1947) both failed to stop deficiency totally. Cobalt carbonate and chloride have proved equally satisfactory as sources of cobalt (Cuhna *et al*, 1964, after Underwood 1977).

Underwood (1977) stated that the requirement of ruminants for frequent, regular dosing lay in the inability of cobalt to be

stored in the liver or elsewhere in the animal. Phillipson and Mitchell (1952) found that direct abomasal and duodenal infusion via cannulae was less effective in alleviating deficiency than conventional oral drenching.

Metered dosing of cobalt through drinking water:

Satisfactory prevention of cobalt deficiency was reported by MacPherson (1981a) by incorporation of cobalt salts into drinking water of animals through a metering device.

Intra-ruminal slow-release devices:

Dewey *et al* (1958) first reported the use of an orally administered heavy cobalt oxide iron pellet (subsequently called a 'bullet') which lodged in the reticulum/rumen and released cobalt into the rumen liquor. Further success with this method of supplementation has been reported by other Australian (Lee and Marston, 1969), New Zealand (Andrews *et al*, 1959 and Andrews and Hart, 1962) and American (Skerman *et al*, 1959) workers.

Millar and Andrews (1964) reported that one-third of animals regurgitated radioactive cobalt oxide pellets shortly after administration and that a number of pellets had reduced release rates attributable to calcium phosphate coating in the rumen. Similar problems were found by Poole and Connolly (1967) in Ireland. Inclusion of an abrasive 'grub-screw' to prevent calcium phosphate coating has shown inconsistent results (Millar and Andrews, 1964, Poole and Connolly, 1967 and Lee and Marston, 1969).

Millar and Albyt (1984) reported a comprehensive comparison between oral cobalt sulphate, cobaltous-EDTA, Vitamin B_{12} thrice weekly injection (as hydroxycobalamin) and a ruminal pellet in the prevention of cobalt deficiency in marginally deficient sheep as measured by serum Vitamin B_{12} and liver cobalt levels.

They found no difference between the sulphate and EDTA forms of cobalt. After 5 injections of Vitamin B_{12} significantly raised liver B12 levels occurred when compared to all treatments (except cobalt sulphate). Bullets significantly increased serum Vitamin B_{12} levels initially compared with untreated controls until 4 weeks post-treatment after which levels diminished.

Supplementation via cobalt-containing anthelmintics:

Dunlop (1947) first reported the usefulness of dosing susceptible animals with cobalt during normal 'gathers' for worming at which time a mineral supplement could also be administered.

Similarly oral dosing of sheep and lambs with micronutrients during normal husbandry gathering has been described by Underwood (1981). However the production of trace element supplemented anthelmintics have combined normal husbandry practices with the requirement for supplementation whereby animals are treated with anthelmintic and supplement in one dose. No evidence was available describing the efficacy of this method of cobalt supplementation at the outset of this project but more recent studies have been published which will be examined in the discussion section of Chapter 5.

1.7 SELENIUM AND VITAMIN E IN ANIMAL NUTRITION

1.7.1 The Discovery of Selenium

Elemental selenium was discovered in 1817 by Berzelius and Gahn in Sweden and named after the Greek God of the moon, "Selene". Interest in biological and nutritional aspects of selenium was not aroused until more than 100 years later when selenium was originally examined in relation to its toxic effects in livestock by Moxon (1937). He found acute and chronic selenium poisoning (selenosis) to be the causes of 'blind staggers' and 'alkali disease' respectively in cattle.

The first essential physiological role for selenium was discovered in 1957 by Schwarz and Foltz who found that selenium supplementation prevented dietary induced liver necrosis in rats. They suggested a molecule responsible for the prevention of the disease and named it 'Factor 3'. In the same year Patterson *et al* (1957) independently reported prevention of exudative diathesis in chicks after selenium supplementation.

Scott *et al* (1959) reported increased Factor 3 activity in Vitamin E supplemented rats fed a selenium deficient diet and this probably represented the first documentation of the complex inter-relationship which was later found to exist between selenium and Vitamin E.

Underwood (1977) stated that within two years of the discovery of a nutritional role for selenium, it was found in both New Zealand and North America that selenium supplementation of

calves and lambs relieved the symptoms of naturally occurring muscular dystrophy. He further stated that growth and fertility responses found in these deficient animals after supplementation with selenium were "greater than was achieved by tocopherol (Vitamin E)".

The first reports of an independent role for selenium whereby it did not merely function as a substitute for Vitamin E were published by McCoy and Weswig (1969) in experiments where Vitamin E supplementation reduced mortality and weight loss in rats less effectively than selenium.

Thompson and Scott (1969) furnished additional evidence that selenium was a dietary essential for growth in chicks, regardless of Vitamin E supplementation. Chicks consuming low selenium diets showed poor growth and higher mortality even when supplemented with 200 ppm d-alpha-tocopherol. Additionally, they found that very high levels of tocopherol (1000 ppm) prevented mortality but failed to match the growth effects found with selenium-only supplementation.

The precise role of selenium in animal nutrition was first reported by Rotruck *et al* (1973), who described it in the structure of the enzyme, glutathione peroxidase (GSH-Px, E.C. 1.11.1.9). This finding was confirmed by Flohe *et al* (1973) who showed that selenium was present in GSH-Px in stoichiometric amounts.

Ganther *et al* (1976) described the distribution of GSH-Px in tissue and cells which varied with species, tissue and selenium status of the animal. Underwood (1977) reported the highest GSH-Px activity in liver, moderate activity in erythrocytes, heart muscle, lung and kidneys and a small amount of activity in the intestinal tract and skeletal muscle of sheep.

Underwood (1981) cited other studies which reported on the connection between selenium intake and GSH-Px activity in other species including further investigation of chicks (Scott, 1973 and Omaye and Tappel, 1974) and rats (Chow and Tappel, 1974 and Hafemann *et al*, 1974).

The GSH-Px activities in tissues of lambs from ewes either depleted of or supplemented with selenium were described by Godwin *et al* (1975) who recorded depressed GSH-Px values in erythrocytes, muscle and plasma of 2-3 week old lambs from deficient ewes. These showed further marked decreases in concentration by 10 weeks of age.

1.7.2 The Discovery of Vitamin E

Mattill and Conklin (1920), using a milk-based diet to induce sterility in rats were the first to speculate that deficiency of the substance now known as Vitamin E may produce disease in animals. Evans and Bishop (1922) discovered a substance present in natural feedstuffs required for normal fertility in rats whose existence was confirmed shortly thereafter (Sure, 1924 and Mattill *et al*, 1924) and subsequently named Vitamin E. It was

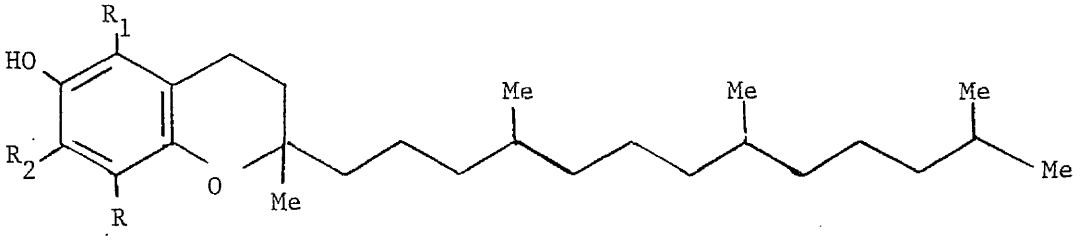
detected in large quantity in wheat germ oil and isolated by saponification (Evans *et al*, 1938).

In a series of experiments Evans *et al* (1938) reported that the anti-sterility activity was due to the presence of a lipid-soluble alcohol named alpha-tocopherol. Additional tocopherols, beta, gamma and delta were also isolated (Evans *et al*, 1936 and Emerson *et al*, 1937, Green *et al*, 1960). All have considerably less "activity" than the alpha- form (MacDonald *et al*, 1981).

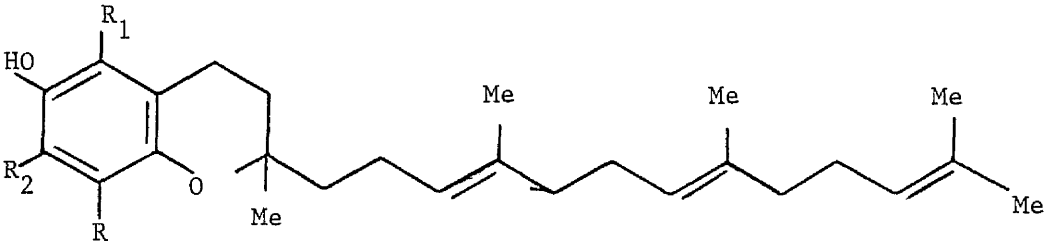
Activity is determined by the degree of methylation on the chroman ring, ie the tocopherol with the most methyl groups on the ring, the alpha- form with three, has most antioxidant activity but as the methyl groups are replaced activity decreases.

Some differences in activity between tocopherols have been found. Ames (1956) reported a ratio of biological activity of 100:33:1:1 for alpha-, beta-, gamma- and delta- forms of tocopherol respectively but Griffith (1959) found the ratios 100:34:18:1 for the same compounds. However more recent studies (Leth and Sondergaarde, 1977), using the foetal rat absorption assay ascribed relative biopotencies of 100, 56, 16 and 0.5% to the alpha-, beta-, gamma- and delta- forms of tocopherol. These findings are summarised in Figure 3.

Compounds related to tocopherols have also been shown to exhibit Vitamin E activity, namely tocotrienols which have largely the same molecular structure except that the degree of unsaturation



Generic Structure of a Tocopherol



Generic Structure of a Tocotrienol

FORM	R	R_1	R_2	% ACTIVITY	
α-Tocopherol	Me	Me	Me	100	a
β-Tocopherol	Me	H	Me	56	a
γ-Tocopherol	H	Me	Me	16	a
δ-Tocopherol	H	H	Me	0.5	a
α-Tocotrienol	Me	Me	Me	13	b
β-Tocotrienol	Me	H	Me	13	b
γ-Tocotrienol	H	Me	Me	4	b
δ-Tocotrienol	H	H	Me	<0.4	b

a = After Leth & Sondergaarde, 1977

b = After Al Tekrity, 1986

Figure 3: Structures and Activities of Tocopherols and Tocotrienols

found in the side-chain of the trienol derivative is greater than is found in tocopherol compounds.

As with tocopherols, within tocotrienols, decreasing Vitamin E activity was found dependent upon the degree of methylation. The most 'active' tocotrienol, the alpha- form, has been reported as having only 25% of the activity of alpha-tocopherol (MacDonald *et al*, 1981).

However, Al Tekrity (1986) cited activities of tocotrienols in the ratio 13:13:4:0.4 for the alpha-, beta-, gamma- and delta-forms using dL-alpha tocopherol as the 100% reference standard. These compounds are also illustrated in Figure 3.

While the different relative biopotencies of tocopherol and tocotrienol compounds are important, most experiments examining the role of Vitamin E metabolism and associated disease in ruminants usually refer only to alpha-tocopherol activity.

1.8 SELENIUM METABOLISM

1.8.1 General Considerations

Underwood (1977, 1981) stated "that selenium is necessary for growth and fertility in animals and for the prevention of a variety of disease conditions which show a variable response to Vitamin E supplementation". However mechanisms operating in various disease states can be better understood by considering the function and metabolism of selenium in detail.

Selenium occurs in animals bound to protein by means as yet not completely understood (Underwood, 1981). One suspected mode of binding in protein molecules involves disulphide linkage (R-S-Se-S-R) (Jenkins 1968). Ganther, (1971) suggested that per-sulphide (S-SeH) linkage would provide suitable cleavage to release selenium for biological activity.

Inclusion of selenium in sulphydryl compounds was reported by Stadelmann (1974) especially when selenium concentrations were high. This may not be surprising due to the proximity of selenium and sulphur in the Periodic Table and in related chemical activity. Further evidence of similar chemical characteristics of selenium and sulphur was cited by Underwood (1981) from Broderius *et al* (1973) and Sprinkler *et al* (1971).

Whanger *et al* (1973) described a suspected selenoprotein found in muscle of selenium supplemented lambs and absent in deficient counterparts but which was found to have a relatively low molecular weight (10000) and spectroscopic absorptive peaks broadly similar to those of Cytochrome C.

1.8.2 Glutathione Peroxidase

Selenium however is most commonly regarded as a component of the enzyme glutathione peroxidase, (GSH-Px, E.C. 1.11.1.9) identified by Mills (1957) as a heat labile, non-haemoglobin protein. Studies by Mills (1957) indicated that GSH-Px catalysed the reduction of hydrogen peroxide by reduced glutathione according to the reaction:

$$2GSH + H_2O_2 \xrightarrow{\text{Glutathione peroxidase}} GSSG + 2H_2O$$

The findings of Rotruck *et al* (1973) and Hoekstra (1973) that selenium was a stoichiometric constituent of GSH-Px in sheep blood provided primary evidence of a functional requirement for selenium.

Good correlations have since been found between the activity of GSH-Px and selenium concentration in blood of different species: Chicks (Noguchi *et al*, 1973), rats (Hafemann *et al*, 1974), sheep (Oh *et al*, 1976) cattle (Anderson *et al*, 1978) and pigs (Thompson *et al* 1976). Allen *et al* (1975) also reported increased GSH-Px activity in muscles after selenium supplementation in sheep.

The importance of GSH-Px lies in its ability to remove various types of toxic radicals produced during normal cellular metabolic activity. The reduction of hydrogen peroxide has already been indicated but other hydroperoxides are reduced according to the general reaction:

$$ROOH + 2GSH \longrightarrow R\text{-}OH + H_2O + GSSG$$

Underwood (1981) reported that GSH-Px exerted a broad role in protecting tissues against oxidative damage. Further he cited the hypothesis of Noguchi *et al* (1973) that Vitamin E and selenium supplementation of selenium deficient animals corrected exudative diathesis in chicks where Vitamin E acted as a specific lipid-soluble antioxidant in cellular membranes and

selenium functioned as a component of cytosolic GSH-Px, reducing peroxides.

One hypothesis suggested that GSH-Px was important in primary destruction of peroxides before damage of cellular membranes, while Vitamin E acted within the membrane itself, preventing chain reactive auto-oxidation (McKay and King, 1980).

GSH-Px occurs in many body tissues and fluids (Underwood, 1981). Scholtz *et al* (1981) recorded highest GSH-Px activity in whole blood after supplementation of calves with selenium. Thereafter GSH-Px activity in tissue occurred in decreasing order in testes, spleen, heart, thyroid, kidneys, lung, liver and finally adipose tissue. Not surprisingly it has been found that distribution is related to dietary selenium intake (Scholtz *et al*, 1981 and Ganther *et al*, 1976). Interestingly though, a non-selenium dependent form of GSH-Px has been detected in the testes of rats (Underwood, 1981).

1.8.3 Other Roles for Selenium

Other selenoenzymes have been reported but are of limited interest in ruminant nutrition. Formate dehydrogenase (FDH), involved in the oxidation of formic acid to carbon dioxide and water was revealed as a selenoenzyme during experiments measuring uptake of radio-isotope ^{75}Se by *Escherichia coli* (Vokal-Borek, 1979).

Similar studies have revealed selenium-dependent glycine reductase activity in *Clostridium stricklandii* (Vokal-Borek, 1979)

while a further selenoprotein was isolated by Calvin (1978) from the tails of rat sperm.

Underwood (1981) reported an additional role for selenium as a complexing agent in protecting against heavy metal toxicity, namely cadmium and mercury.

1.8.4 Selenium Absorption

Physiological studies using ^{75}Se indicated that the main site of selenium absorption in ruminants was the duodenum and that absorption did not take place in either the rumen or abomasum of sheep or the stomach of pigs (Wright and Bell, 1966). The same authors however found that selenium as selenomethionine was assimilated better than selenium as sodium selenite. Lopez *et al* (1969) found in radio-isotope studies that ^{75}Se selenium migrated to tissues such as blood, muscle, hair, wool, bone and organs in treated lambs as GSH-Px.

1.9 THE FUNCTION OF VITAMIN E

Vitamin E has been recognised as a biological antioxidant for many years (Tappel, 1962). Hickman (1949) proposed that Vitamin E may act as a protective agent against oxidative damage to membrane structural components at the cellular level, eg to prevent damage from toxic radicals produced in the respiratory burst associated with phagocytosis.

Although eight forms of Vitamin E occur, their relative antioxidant activities discussed earlier mean that alpha-toco-

pherol is the most important at the cellular level. McMurray and Rice (1982) found that irrespective of relative antioxidant activity and concentration fed in the diet, only alpha-tocopherol was found in any significant concentration in the blood or tissues of pigs and cattle.

Tappel (1953) proposed that one function of alpha-tocopherol was to inhibit peroxidation of fatty acids *in vivo*. The first evidence of possible intracellular antioxidant activity in biological membranes was furnished by Diplock (1974b) who suggested that Vitamin E acted as a stabiliser in microsomal membranes.

McCay and King (1980) reported that there was no doubt that tocopherols had antioxidant properties and as such functioned as free-radical scavengers at the intracellular level in membranes of organelles. This activity was particularly evident when large amounts of unsaturated fats were present in the diet. Taylor *et al* (1976) recorded the highest levels of alpha-tocopherol in the highly unsaturated sub-cellular fraction of the cell membrane in animals.

Diplock (1983) hypothesised that fatty acids in cellular membranes permitted the lipophylic carbon chain found in tocopherols to form an integral part of the membrane. He proposed that d-alpha-tocopherol reacted with the arachidonyl chain in membrane phospholipids and allowed methyl groups found at the C-4 and C-8 position of alpha-tocopherol to fit into pockets created by cis-double bonds of the fatty acid protecting the membrane from oxidative damage and thus maintained stability. Lucy (1972) suggested that

membranes lacking in Vitamin E would be unstable and have increased permeability to radicals which could additionally cause damage.

The intra-cellular role of Vitamin E was confirmed by Burton and Ingold (1984) who demonstrated loss of *in vivo* antioxidant function in alpha-tocopherol upon removal of the phytyl chain. *In vitro* antioxidant activity was not impaired by chain removal.

It is likely that Vitamin E acts both as an integral part of membrane structure conferring stability and as a free-radical scavenger inhibiting *in vivo* tissue fatty acid perxodation. The hydroxy group is the most important structural feature of Vitamin E and the one which confers biological antioxidant activity (McMurray and Rice, 1982). Tocopherol is able to donate a phenolic hydrogen to lipid-free radicals formed during peroxidation and stop damage but the hydrogen's role appears to be catalytic since tocopherol is reformed by the action of ascorbic acid (Willison, 1983).

A possible connection between antioxidant activity of selenium and Vitamin E against polyunsaturated lipids was proposed by McMurray (1980) and is illustrated in Figure 4.

1.10 SYMPTOMS OF VITAMIN E/SELENIUM DEFICIENCY (VESD SYNDROME) IN RUMINANTS

1.10.1 General Considerations

Vitamin E and selenium deficiency continue to cause losses and problems in livestock production from a number of specific

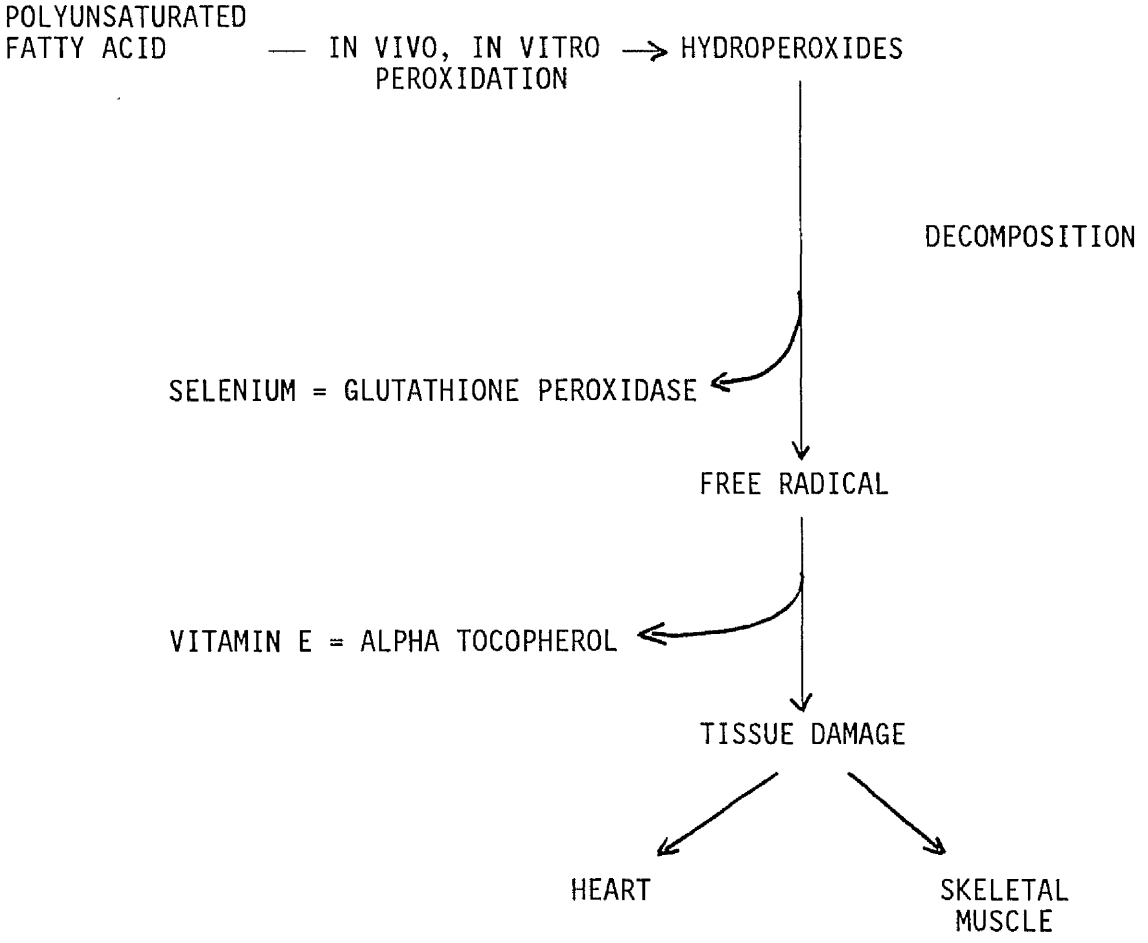


FIGURE 4: A Possible Connection Between Selenium and Vitamin E During Peroxidation of Polyunsaturated Fatty Acids, (after McMurray, 1980).

disease states which occur despite the recently acquired knowledge regarding requirements and interactions (McMurray, 1980). Since the discovery that selenium prevented dietary induced liver necrosis in rats fed a Vitamin E deficient diet, the beneficial effects of selenium and Vitamin E in the treatment or prevention of a number of diseases in laboratory and farm animals has been recorded.

Examples include the prevention of nutritional muscular dystrophy, cardiac muscle degeneration and unthriftiness in sheep and cattle and additionally reduction in depressed fertility and embryonic mortality in ewes, and depressed fertility and placental retention in cattle (Vokal-Borek, 1979).

The aetiology of these diseases is complex and probably involves besides Vitamin E and or selenium deficiency, the effects of antioxidants, sulphydryl containing amino-acids and unsaturated fatty acids. Nonetheless the requirement for selenium or Vitamin E or both is essential in the therapy of these diseases.

The close inter-relationship between selenium and Vitamin E makes it difficult to examine only selenium deficiency or only Vitamin E deficiency in disease. Thus, deficiency is examined below from the disease viewpoint.

1.10.2 Exudative Diathesis

Thompson and Scott (1970) reported prevention of exudative diathesis in chicks by administration of both selenium and Vitamin E. Noguchi *et al* (1973) proposed differing roles for selenium

and Vitamin E in preventing exudative diathesis; selenium acted at the extra-cellular level and Vitamin E intracellularly.

Earlier Gries and Scott (1972) reported another condition of selenium deficient chicks, pancreatic atrophy (or pancreatic fibrosis) which was prevented by selenium supplementation but which did not respond to Vitamin E supplementation.

1.10.3 Mulberry Heart Disease (MHD)

Grant (1961) detailed selenium and Vitamin E responsiveness in Mulberry Heart Disease (MHD, also known as dietetic micro-angiopathy), a condition affecting heart musculature in pigs and this was confirmed by Simeson *et al* (1979). Prevention of MHD in piglets by treatment of pregnant sows with selenium and Vitamin E was reported by Van Fleet *et al* (1973).

1.10.4 Effect of VESD on Liver Function

Two diseases of rodents associated with the feeding of low selenium and Vitamin E diets have been reported by Schwarz (1958); dietary liver necrosis in rats and multiple necrotic degeneration in mice.

A potentially fatal condition associated with selenium deficiency is Hepatosis Dietetica (HD) affecting pigs up to 3 months of age. Massive liver necrosis was found post-mortem although no clinical signs were evident (Underwood, 1981).

Obel (1953) demonstrated that HD could be prevented by Vitamin E supplementation, however Mahan *et al* (1971) have since reported failure to prevent HD even after high levels of Vitamin E supplementation. Eggert *et al* (1957) found HD responsive to selenium supplementation.

Underwood (1981) cited Moir and Masters (1979) who reported that HD could be found either singly or in any combination with MHD or nutritional muscular dystrophy - all of which are diseases of pigs associated with either selenium or Vitamin E deficiency.

1.10.5 Nutritional Muscular Dystrophy

The most common selenium deficiency disease in livestock is nutritional muscular dystrophy. The disease is known by other names; nutritional (degenerative) myopathy, white muscle disease, "Weisses Fleisch" and still lamb disease (Vokal-Borek, 1979) and has been recognised for at least 100 years (Rice and McMurray, 1986). Clinical names for the disease also exist including paralytic myoglobinuria, enzootic muscular dystrophy, enzootic paraplegia and enzootic myositis. All references in this section will hereafter refer only to nutritional muscular dystrophy when describing the syndrome.

The condition was first described by Olcott (1938) in rats and was thought to be due to Vitamin E deficiency. It has since been observed in other livestock species of economic importance; sheep (Hartley and Dodd, 1957), adult cattle (Blaxter and Sharman, 1958) and pigs (Scott *et al*, 1967). A similar disease

was also found in yearling cattle in Europe (Allen *et al*, 1975, Anderson *et al*, 1976 and McMurray and McEldowney, 1977).

McMurray and McEldowney (1977) demonstrated that nutritional muscular dystrophy was prevented by parenteral treatment with either selenium or Vitamin E before turnout.

Rice and McMurray (1986) stated that the aetiology of nutritional muscular dystrophy in cattle remained unclear and proposed that Vitamin E and or selenium deficiency was a pre-disposing factor to the disease rather than a direct initiator. This proposition was previously and indirectly supported by the work of Arthur (1981) and Siddons and Mills (1982) who were unable to induce nutritional muscular dystrophy in yearling cattle despite feeding a semi-synthetic selenium and Vitamin E deficient diet for extended periods.

Rice and McMurray (1986) suggested that possible initiators of nutritional muscular dystrophy included inclement weather and or unaccustomed exercise. They cited other authors (including themselves) views that high levels of dietary polyunsaturated fatty acids may also act as an initiator of nutritional muscular dystrophy.

Pathology of nutritional muscular dystrophy:

Underwood (1977) stated that white muscle disease was most common in 3-6 week old lambs. He defined a primary condition called congenital muscular dystrophy where affected lambs usually died within a few days of birth, if untreated. Muscles

overlying cervical vertebrae were particularly affected with characteristic white striations.

He did however state that lambs could be affected at any time up to the age of twelve months and lambs affected later in life with 'delayed muscular dystrophy' showed a stiff stilted gait, occasionally with an arched back, were reluctant to move about, lost condition, became prostrate and died if left untreated (Underwood 1981). Godwin (1968) described abnormal cardiographs from affected lambs.

Bradley and Fell (1981) demonstrated that muscle tissue affected by nutritional muscular dystrophy was usually phagocytosed *in situ*. Muscular regeneration only took place after supplementation with Vitamin E or selenium and when the animal was not in a deficient state.

Biochemistry of nutritional muscular dystrophy:

Low GSH-Px activity has been associated with nutritional muscular dystrophy but attempts to correlate probability of the disease with enzyme levels were unsuccessful (Oh *et al*, 1976, Whanger *et al*, 1977b).

Both groups have reported the occurrence of nutritional muscular dystrophy where blood selenium and GSH-Px activities were similar to those found in ewes and lambs exhibiting no evidence of the condition.

Blincoe and Dye (1958) reported serum glutamic oxaloacetic transaminase (SGOT) levels 5-10 times higher in nutritional muscular dystrophy affected animals and that elevation was approximately proportional to the amount of muscle damage found. Whanger *et al* (1969) reported elevated SGOT and lactate dehydrogenease (LDH) activity in affected lambs.

However, Oksanen (1967) reported that "the SGOT value may be only moderately increased in animals with marked clinical symptoms" and conversely "a degeneration of the myocardium, even an acute one, may also produce only a moderate increase in the SGOT value, although this often ends in death".

Anderson *et al* (1976) cited Boyd *et al* (1964) and Hidiroglou *et al* (1967) who found aspartate aminotransferase (AAT) (also known as Glutamic Oxaloacetic Transaminase, GOT), useful in diagnosing muscle disease in cattle but Anderson *et al* (1976) preferred creatine phosphokinase (CPK) as a potentially more sensitive and selective indicator of muscle damage. Godwin *et al* (1974) in a study of "some biochemical features of white muscle disease in lambs" reported significantly lower urinary excretion of calcium and magnesium in deficient lambs than in non-supplemented lambs fed a diet deficient in selenium.

1.10.6 Ill-Thrift

A poorly defined condition in sheep and cattle known as ill-thrift was extensively described in New Zealand (Andrews *et al*, 1968, Hartley, 1967) and in Australia (Jones and Godwin, 1963)

which occurred in lambs and shearling sheep at pasture and in beef and dairy cattle of all ages (Underwood, 1977).

The syndrome varied from sub-clinical growth deficit to clinical unthriftiness with associated anaemia and diarrhoea. Upon the onset of clinical disease weight loss was rapid and mortality occasionally occurred (Underwood, 1981). No characteristic microscopic lesions were apparent and ill-thrift might or might not be associated with nutritional muscular dystrophy and infertility syndromes (Underwood, 1981).

Vokal-Borek (1979) stated that ill-thrift could be confused with other trace mineral deficiencies (eg copper or cobalt deficiency) and/or parasitic infection. McLean *et al* (1959 and 1963) reported a selenium responsiveness in lactating ewes to a condition characterised by rapid weight loss and scouring of the sort normally associated with heavy parasitic infection.

Prevention of ill-thrift by selenium treatment with subsidiary effects on growth and wool yield have been reported (Andrews *et al*, 1968, Drake *et al*, 1960, Hartley and Grant, 1961 and Jones and Godwin, 1963).

Increased wool yield resulting from supplementation of selenium adequate sheep has been reported in Canada (Slen *et al*, 1961), Scotland (Quarterman *et al*, 1966) and the USA (Oldfield *et al*, 1963). Production improvement after supplementation in lambs was reported by Blaxter (1963).

Very significant improvement in ill-thriven lambs in New Zealand, was manifested as striking increases in growth and wool yield in some cases (Andrews *et al*, 1968). Mortality associated with ill-thrift and found under normal husbandry conditions was reduced and significant weight gains were reported after supplementation (Underwood, 1977 and 1981).

Hartley (1967) however reported that Vitamin E supplementation alone did not relieve the symptoms of unthriftiness. Similar responses to selenium supplementation in unthrifty young Merino sheep were reported by McDonald (1975) in Australia.

1.10.7 VESD and Reproduction

Selenium and Vitamin E have been implicated in reproductive disorders. Underwood (1977) stated that "in all species studied, selenium deficiency has resulted in impaired reproductive performance in both males and females". An early report of this phenomenon by Hartley and Grant (1961) linked nutritional muscular dystrophy to a higher incidence of barren ewes. Underwood (1977) cited studies reporting higher seasonal incidence of infertility in ewes in parts of New Zealand and association with both nutritional muscular dystrophy and unthriftiness.

Although no increased fertility in ewes was found on an individual animal basis, Hartley *et al* (1960) did report relatively increased lambing percentages achieved through reduction in the number of barren ewes after supplementation with 5 mg selenium as sodium selenite. Hartley (1963) added that in

such barren animals high embryonic mortality occurred between 3 and 4 weeks after conception around the time of implantation. Again oral dosing with sodium selenite cured that problem but Hartley (1963) reported however that neither Vitamin E nor an antioxidant were effective in reducing barrenness.

Buchanan-Smith *et al* (1969) reported that a combination of selenium and Vitamin E were required to ensure reproductive capability in ewes fed selenium deficient diets. Mudd and Mackie (1973) demonstrated that selenium and Vitamin E injections one month prior to mating were adequate to improve ewe prolificacy in selenium deficient areas of Scotland.

Peter (1980) reported that selenium supplementation of ewes during pregnancy resulted in decreased lamb mortality and greater birth weight. Percentage weaning rates of lambs from supplemented ewes were higher than those of untreated animals but Kott *et al* (1983) failed to find significant improvement in reproductive performance in selenium adequate ewes supplemented with selenium only, Vitamin E only or selenium and Vitamin E.

Reproductive disorders associated with deficiency have also been reported in hens (Cantor and Scott, 1974), quail (Jensen, 1968) and pigs (Mahan *et al*, 1974 Edwards et al, 1971 and Nielson *et al*, 1979). Mace *et al* (1963) reported reduction in losses from premature, weak or dead calves in cows supplemented with a sodium selenite/Vitamin E mixture injected one month before calving.

Reduction in the incidence of retained placentae in cattle was reported by Trinder *et al* (1969) following selenium supplementation. Subsequent investigation showed lower blood selenium in cattle suffering retained placentae than in unaffected animals. Julien *et al* (1976) confirmed this finding in the USA in mature dairy cows where retained placentae was prevented after a single injection containing sodium selenite and Vitamin E, while Segerson *et al* (1977) suggested that selenium supplementation improved uterine function and thus aided placental expulsion.

1.11 SELENIUM AND IMMUNITY IN ANIMALS

The immune system is a complex and highly specific defence mechanism which recognises and eliminates cellular and non-cellular entities deemed "self" or "non-self", through the action of its non-specific, humoral and cell-mediated components. Considerable evidence exists, detailed below, that selenium status significantly affects the function of all components of the immune system. However in light of the lack of studies investigating the effect of selenium in immunity in relation to ruminant nutrition, the review below will also examine the effect of selenium nutrition in the immune response of laboratory animals.

1.11.1 Non-Specific Immunity and Selenium

Immunity is non-specific where it has developed in the absence of antigenic stimulus, ie the indigenous resistance used as the first line of defence against pathogenic infection.

Selenium deficient rats (McCallister *et al*, 1980) or goats (Aziz *et al*, 1984) produced neutrophils which showed severely depressed migratory ability to the chemotactic agent, Leukotriene B4 (LTB4), when compared with supplemented animals.

Supplementation in goats resulted in enhanced neutrophil response both *in vivo* and *in vitro* (Aziz and Klesius, 1986). However McCallister *et al* (1980) reported that selenium deficiency had no effect on random migration of rat neutrophils. This contrasted with the findings of Aziz *et al* (1984) that random migration was inhibited in selenium-deficient goats but that the effect was reversible by selenium supplementation. Aziz and Klesius (1986) consequently concluded that selenium deficiency decreased LTB4 production in caprine neutrophils and thus LTB4-mediated neutrophil chemotaxis.

Serfass and Ganther (1975) demonstrated impaired killing ability in neutrophils of selenium deficient rats. An analogous situation demonstrated enhanced neutrophil bactericidal activity against *Staphylococcus aureus* after supplementation with selenium (Gyang *et al*, 1984).

Selenium deficiency was shown by Boyne and Arthur (1979 and 1981) to impair significantly the ability of calf neutrophils to kill ingested *Candida albicans in vitro*. Boyne and Arthur (1979) reported lower superoxide (O_2^-) production and peroxidase activity in neutrophils from selenium deficient cattle when compared to supplemented controls. The oxidative respiratory burst associated with phagocytosis as measured by chemilumine-

scence was found to be impaired in selenium deficient caprine neutrophils phagocytosing a yeast-based product, Zymosan (Aziz *et al*, 1984).

A significantly lower macrophage content in peritoneal exudates of selenium-deficient mice was reported by Serfass and Ganther (1976) than in selenium supplemented control animals. Boyne and Arthur (1985a) using peritoneal macrophages harvested from rats described significantly impaired candidacidal activity.

1.11.2 Humoral Immunity and Selenium

Immunity to specific challenge is expressed by a humoral response via immunoglobulins leading to antigen destruction. Studies exist which show that slelenium supplementation resulted in enhanced humoral antibody response to antigenic stimulus. Higher immunoglobulin G (IgG) titres to sheep red blood cell antigen (SRBC) have been reported by Spallholz *et al* (1973) in selenium supplemented mice. Similar enhanced antibody responses, cited by Finch *et al* (1986) were found in challenged rabbits (Berenshtein, 1972), swine (Peplowski *et al*, 1981) and cattle (Norman and Johnston, 1976) after selenium supplementation.

However, Boyne *et al* (1985) have shown that selenium deficient rats were more or alternatively less susceptible to bacterial infection than selenium supplemented animals depending on the infective organism *(Staphylococcus aureus* or *Salmonella typhimurium* respectively).

The inconsistent effect of selenium supplementation and deficiency on immune function was also emphasised by the findings of Serfass *et al* (1974) that selenium deficient rats infected with *Salmonella enteritidis* demonstrated higher mortality rates than selenium adequate controls.

To add further emphasis on the inconsistent nature of selenium studies by Finch and Turner (1986) have shown that marginally selenium deficient lambs elicited a strong immune response and that selenium supplementation of these animals resulted at best in only marginal improvement in antibody response.

Increased incidence of calf pneumonia was claimed to be attributable to selenium deficiency by Cowie and Dear (1977), but studies by Wooliams *et al* (1986) showed no reduction in incidence of pneumonic lesions in selenium supplemented lambs.

Similar failure to enhance antibody response to *Salmonella dublin* in both sheep and cattle supplemented with selenium (as sodium selenite) when compared to deficient control was reported by Finch *et al* (1986). The same authors in additional experiments found that selenium supplementation as barium selenate only marginally increased antibody titres to the same challenge when compared to sodium selenite supplementation.

Reduced nitrogen responses to phytohaemagglutinin (PHA) were reported by Turner *et al* (1986) in four unsupplemented lambs (of sixteen) fed a selenium deficient diet. Titres were among one-tenth of those found in the healthy unsupplemented lambs.

However, these authors stated that lambs which showed signs of muscle damage (as white muscle disease) and lymphocyte failure were not necessarily the least thrifty nor those which exhibited the lowest GSH-Px activity.

Those studies examined above illustrate the very inconsistent nature and effects of selenium deficiency or supplementation on humoral immune response to specific pathogens or to mitogenic stimulation.

1.11.3 Cell-Mediated Immunity and Selenium

Cell-mediated immunity provides specific immunity to a number of pathogens via three stages shown below:

1. Recognisition of antigen.
2. Proliferation and differentiation of T-lymphocytes.
3. Expression of immune response.

These mechanisms are involved in many aspects of immunity including autoimmunity, homograft rejection, and response to intracellular bacterial, viral and parasitic infections. Selenium supplementation has been shown to affect *in vivo* cell-mediated immune response.

Supplementation with selenium was shown by Martin and Spallholz (1977) to increase delayed hypersensitive immune response in guinea pigs while Koller *et al* (1986) also reported increased natural killer cell (a T-cell proliferate) mediated cytotoxic effects on tumours in rats supplemented with selenium.

Turner *et al*, (1985) found increased nitrogen response to stimulation with PHA and Poke Weed Mitogen (PWM) in selenium supplemented lambs. Conversely however, Parnham *et al* (1983) described decreased mitogen stimulation to Concanavilin A (Con A) and lipopolysaccharide (LPS) in selenium deficient mice. Similar decreased mitogenic activity in selenium deficient rats was reported by Eskew *et al* (1985).

Meeker *et al* (1985) found decreased natural killer mediated cytotoxicity in selenium deficient mice but no effect on antibody mediated lymphocyte cytotoxicity to chicken red blood cells (CRBC) antigen. However, Eskew *et al* (1985) found increased antibody mediated lympocyte cytotoxicity to SRBC in the rat.

Only limited studies have taken place on cell mediated immune responses in selenium deficient ruminants. Aziz and Klesius (1986) recorded no effect of selenium deficiency in caprine mitogen response to stimulation with Con.A but found decreased leukocyte migration inhibitory factor production. Interleukin-2 (IL-2), the lymphokine which initiates and maintains T cell proliferation during immune response was reported by those authors to be unaffected in selenium deficient goats.

Eight unsupplemented lambs on a low selenium/Vitamin E diet were reported by Finch *et al*, (1986) to show a decline and eventual failure in T-cell reactivity. Three of these animals examined post-mortem showed extensive cardiac and skeletal muscle lesions usually associated with deficiency.

Those authors found however that lymphocyte reactivity was restored in the remaining five lambs after supplementation with both selenium and Vitamin E. Ewes deficient in both selenium and Vitamin E were found to retain lymphocyte reactivity in the absence of any supplementation (Finch *et al*, 1986).

1.12 VITAMIN E AND IMMUNITY

According to Gershwin *et al* (1985) the role of alpha-tocopherol in immunity has not been intensively investigated because the metabolic function and basis for its "essentiality" as a nutrient still remain to be clearly established. As with selenium a greater volume of studies exist on the potential beneficial effects of Vitamin E supplementation than on the possible detrimental effects of deficiency. Furthermore studies examining Vitamin E and immunity in ruminant animals are limited.

Sheffy and Williams (1980) stated that since the function of both selenium and Vitamin E in maintenance of erythrocyte membrane integrity is recognised, the fact that erythrocytes and lymphoid cells originate from the same pluripotential stem cells could mean that deficiency (of either selenium or Vitamin E) may affect immune response mechanisms.

1.12.1 Non-Specific Immunity and Vitamin E

In studies examining the role of Vitamin E in non-specific immune responses of animals, Heinzerling *et al* (1974b) found a four-fold increase in phagocytosis of *Diplococcus pneumoniae* in Vitamin E supplemented mice and Gershwin *et al* (1985) reported

increased polymorphonuclear leukocyte (PMN) function over a two-three week period in humans supplemented with Vitamin E.

Decreased mortality due to *E. coli* infection and increased weight gains in chicks fed 150 or 300 iu Vitamin E/kg diet was reported by Heinzerling *et al* (1974a).

Studies examining supplementation of 100, 150 and 300 iu Vitamin E/kg diet was reported to decrease mortality and increase resistance in chicks and turkeys to *E. coli* infection (Nockels, 1979, Tengerdy and Nockels, 1975).

Decreased mortality rates in *Eimeria tenella* infected Vitamin E supplemented chicks was reported by Colgano *et al* (1984) when compared to non-supplemented controls. Additionally those authors found increased weight gain in Vitamin E supplemented chicks but it remained unclear whether this resulted from true productivity improvement or from simple removal of coccidial infection.

1.12.2 Humoral Immunity and Vitamin E

Nockels (1979) reported improved humoral immune response to SRBC and tetanus toxoid in mice receiving supplements of 60 and 120 iu Vitamin E per kg per day as measured by plague forming cell (PFC) and haemagglutinin titres. Reduced PFC activity in mice associated with a Vitamin E deficiency was partially corrected by N,N-diphenyl-p-phenylenediamine (an antioxidant) but not as effectively as by Vitamin E itself (Nockels, 1979). Significantly decreased spleen weight, PFC activity and haema-

gglutinin titres was reported by Tengerdy, (1980) in Vitamin E deficient mice.

Gershwin *et al* (1985) cited undisclosed studies in which a 20-fold increase in Vitamin E supplementation above normal resulted in no differences in resistance to *Listeria monocytogenes* infection in mice.

Nockels (1979) found that an initial boost of 1000 iu Vitamin E followed by dietary supplementation of 300 iu/kg diet fed resulted in significantly increased resistance in sheep to a non-specified Chlamydial infection. Also using Chlamydial infection (administered intra-tracheally) Stephens *et al* (1979) reported enhanced recovery in lambs supplemented with Vitamin E.

Additional studies (Reddy *et al*, 1985) have demonstrated that Vitamin E supplementation at the rate of 1400 iu/week (injection) of 2800 iu/week (orally) for 12 weeks inhibited viral replication in calves infected with infectious bovine rhinotracheitis (IBR) virus.

Significantly decreased lymphocyte stimulation indices (LSI) to PHA were reported by Cipriano *et al* (1982) in Vitamin E deficient calves. PHA induced mitogenesis was reported by these authors to be significantly greater after either oral or intramuscular Vitamin E supplementation.

Additionally they found Vitamin E supplementation of calves resulted in enhanced humoral immune response to bovine herpes

virus-type I (HBV-I) when compared to unsupplemented controls. Calves supplemented with either 125 or 500 iu Vitamin E/day over a 24 week period showed significantly increased mitogen responses to LPS (a B-lymphocyte stimulater) when compared to unsupplemented controls (Reddy *et al*, 1986). However those authors stated that lymphocyte proliferative response to Vitamin E supplementation was not linear.

No difference in vaccination titres was reported by Anderson *et al* (1986) in Vitamin E deficient calves infected with IBR, tetanus toxoid or Johne's Disease *(Mycobacterium paratuberculi)* when compared to controls.

Tengerdy (1980) suggested that Vitamin E may have an indirect role in immune response and cited the findings of Machlin that prostaglandin levels were higher in Vitamin E deficient rats. Vitamin E supplementation has since been shown to inhibit oxidation of L-arachidonic acid to prostaglandins (Tengerdy, 1986).

Prostaglandins are known to be suppressors of immunological cellular activity such as mast cell histamine release and neutrophil chemotaxis. Dogs fed low selenium/Vitamin E diets showed depressed lymphoproliferative responses to mitogens, which were completely restored by Vitamin E supplementation (Sheffy and Schultz, 1979). Vitamin E was also found to reduce prostaglandin levels in chicken organs which resulted in enhanced antibody production (Tengerdy and Brown, 1977).

1.12.3 Cell Mediated Immunity and Vitamin E

Increased delayed-type hypersensitive response was reported by Tengerdy (1980) in tuberculin challenged guinea pigs supplemented with 60 mg Vitamin E per kg diet. Similar experiments by the same author using both selenium (1 ppm) and Vitamin E (120 mg/kg diet) supplementation in mice found similar enhanced reactions to tuberculin challenge.

However Tengerdy (1980) reported that Vitamin E supplemented mice were not protected against challenge by virulent *Mycobacterium tuberculosis*, (an infection known to elicit a cell-mediated immune response). Experiments with a number of species of laboratory animals (mice, rats and guinea pigs) have shown that T- and B- lymphocytes responses to mitogenic stimulation (Bendich *et al*, 1984), mixed lymphocyte responses (Corwin and Gordon, 1982) and PFC level were severely depressed in Vitamin E deficient animals (Corwin and Schless, 1980, Gebremichael *et al*, 1984).

Gershwin *et al* (1985) reported selective action of Vitamin E in mitogenesis when it was supplied at ten times the normal rate. It stimulated a response from murine spleen cells at low CoA levels when supplied at the rate mentioned above but such supplementation had not effect at CoA levels likely to stimulate automitogenesis. Normal Vitamin E supplementation did not result in additional mitogenesis, and these authors stated that the effect of Vitamin E on T-cell mitogenesis was modified by the dietary polyunsaturated fatty acids (PUFA) level, ie greater mitogenic effect at low PUFA levels.

Significant humoral antibody enhancement in mice supplemented with 10 times normal levels of Vitamin E were reported by Tanaka *et al* (1979) to be due to stimulation of T-helper cell activity. Additionally, depression in T-cell activity to mitogen (Con A) in Vitamin E deficient dogs has been reported by Sheffy and Schultz (1978). Interestingly depressed B-cell mitogenic response was also found by the same authors.

However, Sheffy and Williams (1979) stated that the suppressive activity found in serum from Vitamin E deficient dogs was able to inhibit *in vitro* T-cell responses in serum from nutritionally adequate animals. Similar proliferation inhibition was found when 'deficient' canine serum was added to the serum of nutritionally adequate pigs, cats, cows and humans and thus demonstrated that this was a general phenomenon and not restricted to canine cells.

In experiments described previously Reddy *et al* (1986) found a significantly increased lymphocyte stimulation index, (LSI) with the T-cell mitogen Con.A in calves fed 125 and 500 iu Vitamin E per day when compared to non-supplemented controls. This led those authors to state that these results indicated that Vitamin E played a role in the regulation of both cell-mediated and humoral immune responses.

Tengerdy (1986) went one stage further when he described an adjuvant effect of Vitamin E in a *Brucella ovis* vaccine against epididymitis in both immature and mature rams. He cited Afzal *et al* (1986) who found that cell mediated immunity played an

even greater role than that of humoral immune response mechanisms.

1.13 SELENIUM AND VITAMIN E SUPPLEMENTATION

A number of different methods have been used for the prevention and treatment of selenium and Vitamin E deficiency, which can be conveniently divided into 'direct' or 'indirect' methods as listed below.

Indirect:

1. Pasture treatment/soil amendment*
2. Inclusion in specialist fertiliser*

Direct:

1. Addition to ration mineral supplement
2. Inclusion in trace mineralised 'lick'
3. Feed blending
4. Parenteral injection
5. Oral dosing
6. Ruminal 'heavy' pellet
7. Glass bolus*
8. Anthelmintic supplement

*denotes supplementation via this method is for selenium only at this time.

1.13.1 Indirect Methods of Supplementation

Soil amendment and foliar application:

Treatment of pasture with selenium compounds has been extensively practiced in New Zealand (Allaway *et al*, 1967 and Hartley, 1967). However Underwood (1981) stated that potentially toxic levels of selenium could occur immediately after application. Grant (1965) and Allaway *et al* (1966) all found that the addition of 2 lbs (or 900g) selenium per acre (as sodium selenite) increased selenium content of alfalfa from a very low level to a level which protected susceptible lambs from white muscle disease. A single application was shown to maintain those levels for three years. However Underwood (1981) cautioned that this procedure had not been widely used because added selenium is poorly absorbed by most plants and especially those growing in acid soils (Cary *et al*, 1967).

Possible toxicity problems have been disregarded by Watkinson and Davies (1967) who stated that "with proper precautions to minimise pasture contamination, 1 oz per acre or possibly 2 oz per acre, as sodium selenite, (14-28g per acre) should present no hazard, at least for a few years". Underwood (1977) however suggested that pasture or foliar applied selenium would undoubtedly remain on foliage and thus cause problems.

Specialist fertiliser:

Specialist fertiliser containing selenium as sodium selenite was found by Nielsen *et al* (1979) to increase barley selenium con-

tent from 0.01 to 0.05 ppm through application of 100 grams selenium per acre. Halpin *et al* (1985) claimed that there was an advantage in using specially prepared superphosphate fertiliser containing added selenium as this was able to moderate selenium deficiency indirectly by ensuring uniform distribution in the soil and thus uptake by plants.

1.13.2 Direct Methods of Supplementation

Mineral supplements:

Andrews *et al* (1968) used selenium-containing minerals (available commercially) for incorporation into pig and poultry diets providing 0.15 ppm selenium (DM). Similar work by Frost (1965) demonstrated that selenium-supplemented feedstuffs prevented exudative diathesis and nutritional myopathy in chickens. The use of selenium only, Vitamin E only or fully mineralised trace salt licks has been quoted by Underwood (1977) as effective in curing this deficiency.

Trace mineralised "lick":

Trace mineral fortified salt with 26 mg selenium/kg when offered to ewes and lambs, decreased nutritional muscular dystrophy in lambs without increasing tissue selenium concentrations greatly above that recorded in unsupplemented lambs grazing different, non-selenium deficient areas (Paulson *et al*, 1968, Jenkins *et al*, 1974).

Provision of a salt lick containing selenium to ewes and lambs was also cited by Underwood (1981) to have been successful in preventing diseases associated with a low selenium diet (approximately 0.003 ppm) and maintained adequate selenium levels as measured by whole blood GSH-Px activity. This method was found to be as effective as any of the other direct supplementation methods.

Feed blending:

Incorporation of selenium and/or Vitamin E into manufacturers concentrated feedstuffs has been successful in the past in preventing deficiency and is still widely used. The use of grains and alfalfa grown in seleniferous areas of the United States has been quoted by Underwood (1977) as being used in feed blending. In this process sufficient grains are fed to animals to offset deficiencies usually associated with locally grown feedstuffs.

The importance of gauging accurately the selenium content of grains from seleniferous areas was emphasised by Allaway *et al* (1967) since the variable nature of selenium content might lead to problems of toxicity.

Underwood (1981) also stressed the importance of being aware of appropriate conditions of husbandry and the need to introduce minimum levels of selenium into the environment. While particular attention should be paid to indirect methods of supplementation, recent legislation in Finland requiring compulsory application of selenium fertilisers emphasised the

belief that with proper application and monitoring, the potential benefits of such supplementation outweigh adverse effects.

Parenteral injection:

Direct administration of either Vitamin E or selenium can and does take place on an individual animal basis. Parenteral administration of Vitamin E and selenium to calves, lambs and mature sheep as a means of preventing and/or curing deficiency has been used in the past with success (Muth, 1963, Kuttler *et al*, 1961, Mudd and Mackie, 1973, Jenkins *et al*, 1974, and Kott *et al*, 1983).

Jenkins and Hiridoglou (1972) reported that experimentally, and in the field situation, sodium selenite (in sterile aqueous solution) was best administered via the intramuscular route but Kuttler *et al* (1961) suggested selenium presentations in oil (as barium selenite or zinc selenite) proved longer acting in preventing deficiency.

Sub-cutaneous injections (10-30 mg) of sodium selenite for cattle and 1-5 mg for sheep at 3-monthly intervals were described by Underwood (1977) as being effective in preventing selenium-responsive diseases.

Nutritional muscular dystrophy in calves and lambs could be prevented by the addition of 2700 iu Vitamin E/kg mineral supplement as alpha-tocopherol acetate (Jenkins *et al*, 1974). Hartley (1967) reported that the dosing of new-born lambs with

0.18 mg selenium and with 1.12 mg seven days later was effective in preventing nutritional muscular dystrophy.

Similarly parenteral injection of cattle with Vitamin E (as alpha-tocopheryl acetate) before spring turn-out has been reported as preventing deficiency diseases commonly associated with that procedure (Underwood, 1981).

Injections of 10 and 30 mg selenium to calves and adult cattle respectively administered 3-monthly were reported by Andrews *et al* (1968) to cure deficiency and that 5 mg selenium at mating and a further 5 mg during gestation prevented reproductive problems and protected ewes and lambs from selenium deficiency. Mudd and Mackie (1973) reported that the supplementation of ewes with 272 iu of Vitamin E and 6 mg selenium prior to mating produced improved lambing percentages compared with non-treated control sheep and claimed a significant economic advantage in selenium deficient areas. Similar prevention of reproductive diseases associated with deficiency have been reported in sheep by Kott *et al* (1983) after injection with Vitamin E.

Oral dosing:

Success with oral-dosing of selenium-containing compounds has been reported in control of unthriftiness in sheep in Australia using 0.1 mg selenium per kg bodyweight (as sodium selenite) administered at marking and again four months later (McDonald, 1975).

Direct administration of oral selenium or injectable preparations was cited by Underwood (1977) from Cousins and Cairney (1961), Dornenbal (1975) Kuttler and Marble (1961) and Ostradius and Alberg (1961) as being adequate in preventing deficiency without toxic accumulation of selenium in tissue. Oral dosing with 1-5 mg selenium (as sodium selenite) was suggested by Underwood (1977) to be the most commonly used means of preventing selenium-responsive diseases in sheep.

Ruminal heavy pellet:

The disadvantage of frequent handling of individual animals to administer supplement led Kuchel and Buckley (1969) to further develop 'heavy' pellets originally proposed by Cousins and Cairney in 1961 and further improved by Paulson *et al* (1968). The pellet used by Kuchel and Buckley was composed of iron oxide and selenium (as either calcium selenate, barium selenate or elemental selenium) in the ratio 9:1, which lodged in the reticulo-rumen after administration.

The pellet released sufficient selenium to maintain significantly enhanced blood and tissue selenium levels for twelve months after administration when compared to sheep grazing selenium-adequate pasture. Various ratios of elemental selenium to finely divided metallic iron (ranging from 1.25-10% selenium) were all discovered to increase blood selenium levels significantly, within one week of administration when compared to untreated control animals.

Underwood (1977), ever concerned with potential toxicity associated with selenium, quoted the findings of Paulson *et al* (1968) and Cousins and Cairney (1961) using the original pellets (which contained more than the 10% selenium in the pellet of Kuchel and Buckley) that even by 6-12 months after pellet-administration, tissue selenium levels were not greater than those found in untreated non-deficient sheep.

Maximum GSH-Px activity was described by Paynter (1979) as occurring in sheep 20 weeks after being treated with a similar heavy pellet with an iron:selenium ratio of 19:1 (w/w). A benefit of this type of supplementation is the continuous supply of selenium to animals which may be grazing deficient areas without the need for frequent handling of stock (Handreck and Godwin, 1970).

These authors using ^{75}Se found that heavy pellets released 0.5-1.3 mg selenium per day of which 30% was excreted in urine and resulted in blood selenium levels stabilising around 0.18-0.34 ug per ml without toxic effects.

A selenium containing pellet (as sodium selenite) was prepared by Hiridoglou *et al* (1971) for subcutaneous implant. The device was adequate in preventing white muscle disease in lambs born to treated ewes and also in preventing white muscle disease when administered to newborn calves. These authors however reported that high tissue selenium levels around the site of implantation was one possible disadvantage.

Soluble glass bolus:

A recent development in supplementation has extended the heavy pellet approach further using soluble glass boluses containing a number of micronutrients including selenium which are released slowly over an extended period from the reticulo-rumen following oral dosing.

Experimentation has shown that boluses correct selenium deficiency (as measured by GSH-Px activity) rapidly in ewes, lambs and cattle grazing selenium-deficient sites (Telfer *et al*, 1983). Glutathione peroxidase has been maintained at normal levels for at least one year in cattle and sheep without adverse toxic effects (Carlos *et al*, 1985, Driver *et al*, 1985, 1986).

Anthelmintic supplementation:

Oral dosing of sheep and lambs with micronutrients during normal husbandry gathering has been described by Underwood (1981). However the production of trace element supplemented anthelmintics has combined normal husbandry practices with the requirement for supplementation whereby animals are treated with anthelmintic and supplement in a single dose. No evidence was available describing the efficacy of this method of selenium supplementation at the outset of this project but subsequent studies are examined in the discussion section of Chapter 5.

1.14 *OSTERTAGIA CIRCUMCINCTA* INFECTION IN SHEEP

1.14.1 General Considerations

Sheep flocks are invariably infected with helminth parasites to some degree. In many tropical and sub-tropical regions, where more favourable conditions exist for the survival of infective stages, gastro-intestinal parasites are recognised as a major cause of death (Wilson, 1982). Even in temperate regions the sub-clinical disease state where sheep appear normal but perform consistently below potential remains difficult to detect.

Significant losses due to inferior weight gain and wool growth, carcass condemnation and costs associated with prophylaxis and treatment of sub-clinically infected animals can occur. Reid and Armour (1978) evaluated the economic losses associated with parasitic infection and reported a weight deficit of between 2.23 and 2.95 kg per lamb in untreated grazing animals compared to anthelmintic treated controls resulting in £2.20-£3.00 lost profit per lamb (1976 prices).

1.14.2 History and Life Cycle of *Ostertagia circumcincta*

Ostertagia circumcincta, speciated by Stadelmann in 1894, is a slender reddish brown worm which occurs in the abomasum of sheep and goats and is strongly adapted to the class *nematoda* of the phylum *Nemathelminthes* in the bursate superfamily *Trichostrongylus*.

This parasite has a direct life cycle, where no intermediate host is involved in parasite development before infection. Eggs measuring 80-100 microns (u) x 40-50 microns (Soulsby, 1981) are deposited by adult female *O. circumcincta* in the abomasum of the host. These pass out in the faeces and hatch to first stage larvae which feed on faecal bacteria (Smyth, 1976). Within the faeces larvae undergo two moults to become infective third-stages during a period known as the pre-parasitic phase. Rose (1961) working with the equivalent cattle parasite, *Ostertagia ostertagi*, showed that the amount of time spent in the first three phases was influenced by temperature, moisture and aeration.

During their second ecdysis, the cuticle from the second stage larva is retained (Taylor, 1930) and thus the third stage larvae are extremely resistant to drought and temperature extremes. First and second stage *O. ostertagi* larvae were found by Rose (1961) to be extremely susceptible to desiccation.

When moist conditions prevail, third stage larvae migrate up and into herbage from whence following consumption by grazing sheep they pass into the rumen and exsheath. Migration to the abomasum follows and larvae penetrate into the lumen of the gastric glands where further moutling produces the fourth larval stage (Reid and Murray, 1980). Once ensconced in the lumen, parasites grow rapidly and begin emerging to the luminal surface some 7 to 10 days after ingestion (Smith *et al*, 1985b) having caused much damage to the mucosa. Figure 5 illustrates the activity at the mucosal level after infection with the parasite.

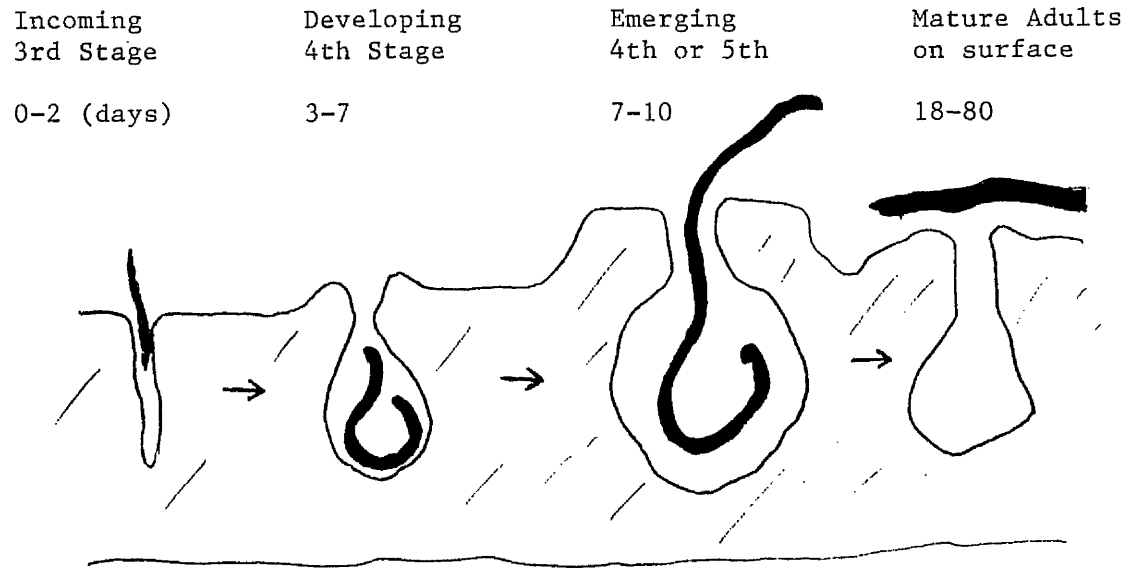


Figure 5: The Parasitic Phase of O. circumcincta is confined to the mucosa of the abomasum.

(After Smith et al 1985b).

After further growth and moulting to produce the fifth larval stage, (or immature adult) parasites emerge fully from the lumen and mature on the mucosal surface. Full maturation is reached some 17-18 days after ingestion of the third stage larvae and consequently within three weeks of infection eggs are usually detectable in the faeces (Reid and Murray, 1980). Figure 6 shows the complete life cycle of *O. circumcincta*.

Adult male worms measure 7.5-8.5 mm in length and females 9.8-12.2 mm (Soulsby, 1981). Spicules (accessory sex-organs) in the male are slender, 0.28-0.32 mm in length with two dissimilar terminal processes. The vulva in the female is usually covered by a flap and opens in the last fifth of the body. Additionally a thickened band consisting of 4-5 transverse striations is found in the female (Soulsby, 1981).

Hypobiosis:

The only interruption to the life cycle of the parasite is a diapausal-like phenomenon called hypobiosis possibly arising from external trigger factor(s), discussed below. Hypobiosis results in arrestment of larval development and may be a manifestation of acquired immunity (Urquhart *et al*, 1962). Gordon (1970) suggested that this phenomenon may be partly due to the environmental experience of the parasite. Hypobiosis was catalogued in cattle infected with *O. ostertagi* initially by Martin *et al* (1957) and definitively in Scotland by Anderson *et al* (1985) and has subsequently been described in many other countries (Armour, 1980). Primary evidence of hypobiosis in sheep in Great Britain was furnished by Reid and Armour (1972).

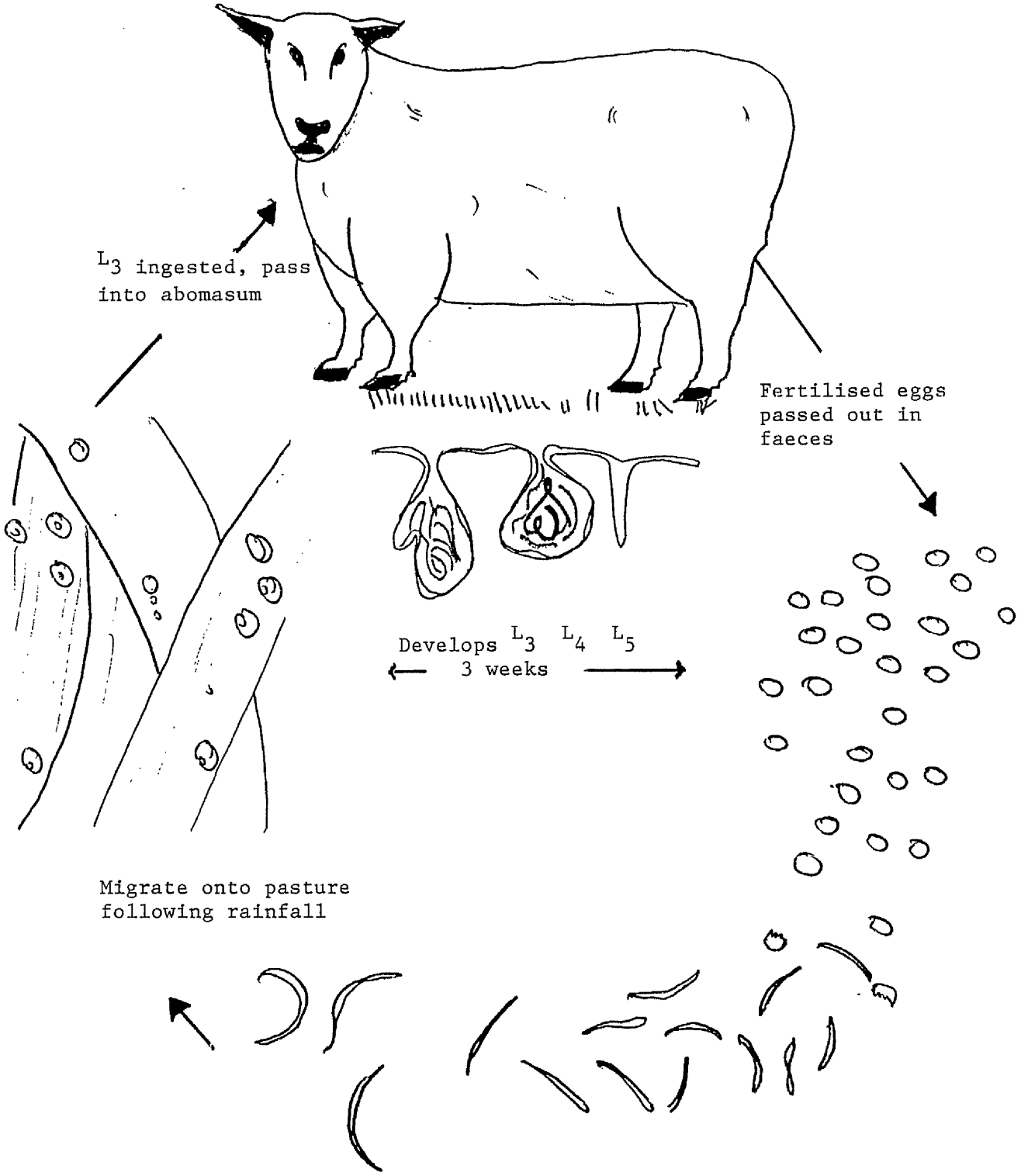


Figure 6: The Life Cycle of Ostertagia circumcincta

The mechanism which induces hypobiosis as yet remains unclear although Armour (1980) cited polarisation of opinion on possible aetiology. He discounted the first suggestion that endocrine and immunological factors produced the effect.

Evidence that hypobiosis may be due to an environmental effect on the parasite was first presented by Anderson *et al* (1965) and later confirmed by Armour, Jennings and Urquhart (1969a). The latter authors, using tracer calves found a ten-fold increase in the number of fourth-stage (inhibited) larvae in calves grazing in late autumn when compared with summer grazed controls. Seventy per cent of the total worm burdens of autumn grazed animals consisted of L4 larvae and those authors showed that percentage inhibition rate was independent of the number of days grazed or the level of burden established and was in fact dependent on the season. Additionally, similar proportions of inhibited larvae were found in animals slaughtered at various intervals after removal from grazing.

The exact trigger for hypobiosis as yet remains obscure but it has been proposed that animal age and previous experience of infection may result in different inhibition rates (Michel, Lancaster and Hong, 1979). Armour (1980) therefore concluded from these facts and additional studies (Armour and Bruce, 1974, Michel *et al*, 1974) that a direct effect on the metabolism of the free-living stages was the mechanism responsible.

Previous studies (Armour, Jennings and Urquhart, 1969b) demonstrated however a tendency to hypobiosis (as measured by rela-

tive proportion of L4) in calves grazing pasture contaminated with a natural field infection when compared to calves grazing pasture 'seeded' with a laboratory-raised strain of the parasite.

Little attention has been paid to the trigger factors causing synchronous redevelopment of inhibited larvae despite its obvious epidemiological role. Such studies as exist suggested that arrestment usually occurs at a time when normal development would possibly result in poor survival rates due to harsh environmental conditions (Reid and Armour, 1972, 1973). Armour (1980) theorised therefore that a primary function of hypobiosis was in fact to ensure the survival of the next generation of the parasite, regardless of the clinical disease caused (Reid and Armour, 1973).

This manifestation of ostertagiasis was initially known as the 'winter' form but has more recently been known as Type II Ostertagiasis. The epidemiological implications of this phenomenon are discussed in the next sections.

1.14.3 Epidemiology of *O. circumcincta*

Epidemiology in animal disease was founded on studies carried out in the late 19th Century on the causes and control of rinderpest cited by Halpin, 1976. Armour (1980) however cited Gordon (1949) who stated that while epidemiology was usually associated with the occurrence of disease, in the case of "parasitic infection, in which the borderline between safe and dangerous burdens is indefinite and where manifestations of

disease are often sub-clinical and insidious in onset, it might be more desirable to think of epidemiology in terms of population dynamics."

Host and environmental factors contribute to the propagation of *O. circumcincta* and development of clinical ostertagiasis and have been defined as occurring in four general categories by Armour (1980) namely:

1. increase in the infective mass;
2. alteration in the susceptibility of existing stock;
3. introduction of susceptible stock into an infected area;
4. introduction of infected stock into a non-endemic area.

Increase in infective mass:

A seasonal increase in the infective mass of *O. circumcincta* was quoted by Urquhart *et al* (1986) as occurring in Europe from mid-summer onwards due to large numbers of third larval stage *O. circumcincta* in herbage. Armour (1980), had previously suggested a number of factors which could influence contamination levels.

Firstly biotic potential, the capacity of an organism for biological success via fecundity, was recongised as an important factor in measurement of prolificacy. *H. contortus*, and *Ascaris suum* were cited by Armour (1980) as producing thousands of eggs compared to the relatively few produced by *Trichostrongylus* spp. A similar proposal was made for *O. circumcincta* by Coop *et al*, (1977) who found similar worm egg counts in sheep infected with different numbers of parasites and appears to support the

suggestion of Michel (1969) using the equivalent cattle parasite *O. ostertagi* that total egg output of the population was restricted to an upper limit which remained constant regardless of infective challenge.

Secondly ostertagiasis is particularly common where stocking rates are high (Cameron and Gibbs, 1966). Also the provision of 'Clean Grazing' (where infected animals are treated with anthelmintic and moved to new, 'clean' pasture for the rest of the current and the whole of the next season) was stated by Mitchell *et al* (1984) to provide further evidence of the influence of stocking rate and grazing management on levels of pasture contamination and therefore infective mass.

Armour (1980) opined that stock management was particularly important in disease outbreaks where no mutliplication of the parasite takes place outside the final host. The date of turnout to grass in spring has also been shown to influence subsequent helminth pasture contamination in housed cattle in temperate regions (Borgsteede, 1977).

Environmental factors:

Translation, the survival, dissemination and availability of free living stages of the parasite has been examined previously. Environmental factors such as drought have obvious effects on larval survival but indirect effects similar to that found in fascioliasis, where the intermediate host *Lymnea truncatula* is additionally affected by environmental factors, must also be considered (Armour, 1980).

Seasonal effects have been shown to affect development times of free living stages. Crofton (1963) demonstrated a reduction in development time for free living stages directly related to increasing mean day/night temperatures greater than 10°C.

Anderson *et al* (1965, 1969) and Michel (1967, 1969) reported that normal seasonal progression, ie temperature rise in summer and fall in winter, resulted in a concertina effect on *O. ostertagi*'s life cycle, where shorter development time was evident in summer and greater development time in colder months eventually resulting in cessation of eggs being shed in the faeces. This usually happens in September in a typical season. Subsequent studies have found similar results in sheep infected with *O. circumcincta* (Gibson and Everett, 1971, Thomas and Boag, 1972 and Reid and Armour, 1973).

Additionally, the ability of infective stages to survive or over-winter is also important as mild winters have enabled sheep trichostrongyle larvae to survive well in South Africa (Muller, 1968), England (Gibson and Everett, 1971) and Australia (Donald, 1968, Anderson, 1972).

1.14.4 Altered Stock Susceptibility

Spring rise, post-parturient rise (PPR) and peri-parturient relaxation in immunity (PPRI):

Although the influence of stocking density will be of paramount importance in fully susceptible animals, the immune status of the host also contributes to determination of the infective

mass. A rise in nematode faecal egg counts in spring in ewes was first described by Zawadowsky and Zvjagvintzew in Russia in 1933 (cited by Armour, 1980), but Taylor (1934a,b) and later Morgan and Sloan (1947) in Scotland were responsible for the global recognition of a phenomenon subsequently called 'Spring-rise'.

Crofton (1958) in demonstrating that similar increased egg counts were found in autumn-lambing flocks suggested that the event was associated with parturition and lactation and proposed the term 'post-parturient rise'. Characterisation and comparison between worm egg count and prolactin activity led Salisbury and Arundel (1970) to name this event 'peri-parturient rise' (PPR). The fact that lactation involved the hormone prolactin was implicated as a possible modulator of the rise (Connan, 1972 and Kelly and Dineen, 1973).

Armour (1980) referred to the more recent and preferred name of 'peri-parturient relaxation in immunity' (PPRI). Relaxation in immunity was also thought to be associated with the synchronous redevelopment of arrested larvae (L4's), female worm fecundity, maturity of infective challenge and failure to eliminate existing infections (Armour, 1980).

Age related effects:

Alteration in susceptibility of existing stock has been described by Armour, (1980) in adolescent or mature sheep already harbouring a sub-clinical parasitic infection. Michel (1976) had previously suggested that a dynamic situation existed in the

host-parasite relationship whereby worm populations were maintained by a balance between loss of existing worm burden and establishment of incoming infection. The influence of age related immunity is examined in Section 1.17.2.

The influence of diet:

A change of diet at housing was suggested by Brunsdon (1964) as being the cause of a marked increase in worm egg output in New Zealand sheep infected with *O. circumcincta* possibly related to a reduction in feed intake. Urquhart *et al* (1986) cited that this phenomenon was also found in *H. contortus* infected sheep in the USA (Whitlock and Georgi, 1976) and in Nigeria (Akerejola, 1977).

Metabolic studies have shown that *O. circumcincta* infection in sheep can result in impaired feed conversion as measured by negative nitrogen balance (Parkins *et al*, 1973) and production impairment (Sykes and Coop, 1977) as discussed in Section 1.15.

Mineral and trace element deficiencies have also been involved both as a consequence of and interacting with parasitic infection. Decreases in plasma inorganic phosphate in *O. circumcincta* infected calves (Waymack and Torbert, 1969).

Hypophosphataemia was reported at three weeks post-infection (ie coincident with mucosal changes associated with infection) in sheep (Coop *et al*, 1976) and ultimately a severe osteoporosis in lambs associated with double deficiency of both calcium and phosphorus (Field *et al*, 1975) indicative of impaired calcium

and phosphorus absorpotion. Additional studies have confirmed these findings (Wilson, 1982).

The converse situation where trace element (cobalt) deficiency affected pathogeneisis of infection has already been discussed in this review.

Breed, species and sex differences:

Breed differences may be important in influencing the degree of infection (Armour, 1980). He cited his own unpublished observations (1957) and reports of greater burdens found in Angora goats grazing land with yearling Merino sheep (LeJambre and Royal, 1976) as evidence. The latter authors further suggested that variability in resistance to challenge may well exist both between and within breeds of the same species. Scrivener (1964) demonstrated that Targhee and Panama breeds of sheep were more resistant to *Ostertagia* spp. infection than Suffolk, Hampshire and Rambouillet breeds. In addition to breed differences, host sex was shown by Vengor *et al* (1971) to be important in the pathogenesis of infection since ram-lambs appeared more susceptible to natural infection with *Ostertagia* spp. and *Strongyloides* spp. than ewe lambs.

Cross-species contamination:

Coop *et al*, (1985b) reported a variable reponse to infection in lambs given 3500-4000 *O. ostertagi* L3 daily for 6 weeks. Weight arrestation, biochemical and pathological features normally associated with *O. circumcincta* infestation were also present in

this situation. Despite obvious implications for cross-infection and thus epidemiology those authors concluded that this could be a possible method of immunisation of susceptible individuals.

Inter-current infection:

Enhanced pathogenecity due to inter-current infection with both *Nematodirus battus* and *Eimeria* spp. has been reported in lambs (Reid, 1976). Similar findings in cattle and pigs, have been cited by Armour (1980) who reported that it was difficult to apportion enhanced pathogenicity to either infection of immunosuppressive effects. Urquhart *et al* (1973) however found impaired immune expulsion of *Nippostrongylus brasiliensis* in *Trypanosoma brucei* infected rats. More recently Coop *et al* (1986) reported reduced liveweight gain in sheep concurrently infected with both *O. circumcincta* and *Trichostrongylus vitrinus* compared to animals receiving single infections.

The effect of anthelmintic treatment:

Anthelmintic treatment according to Armour, (1980) may affect immunity and thus susceptibility where treatment has expelled an immunising infection and therefore allowed re-infection. This hypothesis was formulated on the basis of studies using *H. contortus* infection in sheep (Benitez-Usher *et al*, 1977).

Earlier studies by Dineen and Wagland (1967) found that frequent (fortnightly) anthelmintic treatment resulted in increased susceptibility of sheep to infection compared to those treated less

frequently (4-8 weekly). Thomas and Boag (1973) reported similar findings where anthelmintic treated sheep had higher eventual burdens than untreated sheep grazing infected pasture continously.

Hormonal effects:

Armour (1980) cited Armour (1967) who recorded increased nematode faecal egg counts in *O. ostertagi* infected calves treated with cortisone. Although the exact mechanism causing this effect remained unclear, the epidemiological implications via increased infective mass on pasture are evident.

Hypersensitivity:

Barger *et al* (1973) and Barger and Southcott (1975) recorded that *O. circumcincta* and *T. coloubriformis* resistant sheep ceased to grow wool upon infection and implicated a hypersensitive response in the gut (Anderson, 1972, 1973) as the cause.

Leakage of plasma pepsinogen (and other macromolecules), usually indicative of extensive parasite associated mucosal damage (Murray *et al*, 1970), may be functionally dependent on the same hypersensitive response. Worm burdens in sheep which developed a hypersensitive response were low and Armour (1980) suggested that wool growth effects may be attributable to similar leakage of amino-acids essential for wool growth into the gastrointestinal tract.

Clean grazing:

Mitchell and Fitzsimons (1983) demonstrated significant improvement in lamb performance and reduced contamination levels in ewes and lambs managed under 'clean' grazing conditions both as a novel system and under a system managed for eight years and compared with traditional set stocking on permanent pasture. A comparison was also made with pasture managed under 'clean' grazing conditions for 8 years and tracer lambs compared in all three situations indicated that extended use of clean grazing resulted in lower infection rates in grazing lambs and lowered levels of overwintered larvae on pasture. Mitchell and Fitzsimons (1983) cited Whitelaw (1978) who previously demonstrated that a lack of immunity to infection could be associated with 'clean' grazing and similar findings were recorded by the latter authors. The consequences of the phenomenon are discussed in the next section.

1.14.5 Introduction of Susceptible Stock into an Infected Area

Clinical outbreaks of disease can often be associated with novel infection particularly in naive juveniles and including those recently weaned, which had been moved onto infected grazing, eg movement of parasite naive sheep from non-endemic areas to infected pasture (Armour, 1980).

Eggs of cattle *Ostertagia* spp. and *Cooperia* spp. deposited in late autumn have been reported to have caused clinical parasitism in calves grazing that area during the following June in Canada (Smith and Archibald, 1969 and Slocombe, 1974) and

similar phenomena have been reported in Northern Ireland (Taylor *et al*, 1973) and Norway (Tharaldson, 1970). Recently Connan, (1986) reported ostertagiasis in May in spring lambs grazing pasture where *O. circumcincta* larvae had presumably over-wintered in large numbers.

1.14.6 Introduction of infection into Non-Endemic Areas

The introduction of infected stock has an obvious effect on the number of parasites available for infection and therefore causes an increase in the infective mass available as previously discussed. Moreover helminth infection may also be introduced additionally via application of manure products such as slurry to a previously non-infected area (Moore and Downey, 1978).

1.15 PATHOPHYSIOLOGY OF *O. CIRCUMCINCTA* INFECTION IN SHEEP

Pathophysiological effects of experimental infection with single or multiple doses of *O. circumcincta* have been reported within 4 days of administration at the time when larvae first become established in the gastric mucosa (McLeay *et al*, 1973, Anderson *et al*, 1976). Gross lesions in the abomasum are usually associated with the growth and development of larvae within glands and their subsequent emergence to the mucosal surface (Armour *et al*, 1966, Soulsby, 1981).

During this time elevation of plasma pepsinogen occurs and reduction in feed intake and/or utilisation if found (Anderson *et al*, 1965, 1966, Holmes and McLean, 1971, and Sykes and Coop, 1977). Coop *et al* (1977) calculated that abomasal parasitism caused a

22% reduction in feed intake. Using pair-fed animals, the same authors demonstrated that a reduction in the efficiency of utilisation occurred since infected sheep gained weight at half the rate of pair-fed uninfected controls. This was thought to be of considerable impact in the field situation by Wilson (1982) who felt that intermittent anthelmintic treatment may not remove a potential appetite suppressing factor in situations of continuous larval intake.

Holmes and McLean (1971) using a single *O. circumcincta* infection of sheep, postulated that the rise in plasma pepsinogen found in infected animals may be related to a transient permeability to macromolecules and not as Jennings *et al* (1967) suggested to a large increase in the permeability of the mucosa of infected animals allowing pepsinogen diffusion into the bloodstream and permitting plasma protein leakage into the gastro-intestinal tract. Studies using radio assay techniques have also shown elevation in levels of plasma gastrin in the infected animal (McLeay *et al*, 1973 and Anderson *et al*, 1976, 1981). Anderson *et al* (1981) suggested that elevation of the pH of gastric digesta associated with parasitic infection could be an important factor contributing to increased release of gastrin in affected sheep but did not record hypergastrinaemia in later experiments (Anderson *et al*, 1985) but suggested that this may be due to the release of initial parasitic stimulus.

Increased plasma pepsinogen and other effects have been investigated by a number of authors. Cellular replacement in the abomasal mucosa resulted in separation of the inter-cellular

junctional complexes sometimes called the 'leak lesion', resulting in mucosae with enhanced permeability to macromolecules (Murray *et al*, 1970). Epithelial hyperplasia and inflammation resulted in a thickening of almost all of the parasitised mucosal surface. Coop *et al* (1977) described hypertrophy of the fundic mucosa and the presence of lesions specifically associated with penetration of worm larvae as the main feature in *O. circumcincta* infected sheep.

Loss of differentiated chief and parietal cells and replacement with non-secretory undifferentiated equivalents results in failure to secrete hydrochloric acid for digestion (Anderson *et al*, 1965, Armour *et al*, 1966) and a consequent rise in pH of abomasal contents. Increased hydrochloric acid secretion at a higher concentration in non-parasitised abomasal pouches, (separated from the rest of the abomasum) was reported by McLeay *et al* (1973) in *O. circumcincta* infected sheep. Microscopic and ultrastructural examination of these pouches suggested that they had been subjected to considerable secretory stimuli. The same authors reported elevation of abomasal sodium concentration in infected sheep with 4 days of infection and additional elevation after the infection-associated characteristic rise in pH.

A rise in serum plasma pepsinogen in addition to the characteristic rise associated with rise in abomasal pH was reported recently by Anderson *et al* (1985) in *O. circimcincta* infected sheep. McKellar *et al* (1984) explained a similar effect found in *O. ostertagi* infected cattle by stating that direct stimulation of abomasal lumen by diffusible parasite exudate was the cause.

In experiments using various dosing rates of *O. circumcincta* and *O. trifurcata*, Todd *et al* (1951) demonstrated a marked fall in circulatory red blood cells in infected sheep which developed macrocytic hyperchromic anaemia.

Marked reductions in packed red cell volumes (PCV), haemoglobin concentration and circulating red blood cell volume were reported by Horak and Clark (1964) in *Ostertagia circumcincta* infected sheep and those authors also reported decreased plasma albumin, gamma globulin and total circulating protein in the same animals.

Holmes and McLean (1971) using radio-labelled proteins demonstrated marked protein loss and plasma pepsinogen elevation between days 5 and 9 after infection which was associated with a greater albumin turnover rate. Albumin hypercatabolism and plasma protein leakage however were found by the same authors to decline between days 14 and 21 after infection although plasma pepsinogen levels remained elevated. Serum albumin concentration was not significantly affected.

Recent studies (Yakoob *et al*, 1983) indicated that ewes previously grazed on contaminated pasture demonstrated pathophysiological changes similar to a secondary experimental infection, as measured by effects on pepsinogen, albumin and plasma protein levels when compared to animals experiencing primary infection of mixed trichostrongyle larvae (including 90% *O. circumcincta*). Final burdens in animals receiving secondary infection however remained low.

Similar severe pathophysiological effects in sheep were reported by Anderson *et al* (1985) after direct transference via abomasal cannulae of larva and adult *O. circumcincta* into previously parasite free sheep. While the greatest effect was experienced in animals receiving adult/larval mixtures, larval stages alone were found to elicit an immediate marked and measurable physiological response.

1.16 CLINICAL ASPECTS OF OSTERTAGIASIS

Type I ostertagiasis has almost certainly been recognised since the observations of Hogg (1807) which have been discussed in Section 1.1. The disease was stated by Reid and Murray (1980) to be the primary cause of parasitic gastro-enteritis in sheep. Although a disease associated primarily with lambs, occurring in the summer months (Taylor, 1934a), the condition was most frequently associated with lambs under intensive grazing conditions (Reid and Murray, 1980). Examination of previous investigations allows the following observations:

The condition is sudden in onset and results in animals displaying clinical signs thus; profuse, watery diarrhoea resulting in soiling of fleece with faecal material around the tail and perineum; affected animals become dull, wool lacks lustre and weight gain is arrested. As diarrhoea persists weight loss occurs and without treatment animals become dehydrated resulting in marked weakness and a subsequent staggering, uncoordinated gait followed by recumbency and death (Reid and Murray, 1980).

Type II (winter) ostertagiasis, the aetiology of which was described earlier, affects hoggs, gimmers and young ewes in the late winter to early spring (January-April) in both grazing and housed situations (Taylor, 1934b and Reid and Armour, 1973) and has been extensively studies.

The main findings may be summarised thus:
Affected animals become progressively thinner, demonstrate a loss of wool and develop either a profuse watery diarrhoea or marked softening of the faeces. However, faecal change may be intermittent in character. Performance is generally affected and lambs born to infected ewes tend to be of low birthweight. The ovine condition is essentially similar to bovine Type II ostertagiasis and results from the use of permanent grass paddocks in autumn and early winter (Reid and Murray, 1980).

1.17 IMMUNITY TO *O. CIRCUMCINCTA* INFECTION IN SHEEP

"The immune system has not been conspicuously successful in producing absolute resistance to helminth infections in mammals. In a sense it has been detrimental since the IgE-mediated reactions appear to have evolved largely for the control of these parasites" (Tizard, 1986).

The same author declared that the inefficiency noted in immune control was unsurprising since the organism had an obligatory parasitic existence and as such "was able to survive and procreate dependent on reaching a form of accommodation within the host either by confronting, overcoming or evading the host's immune

system" (Tizard, 1986). Resistance to parasitic disease according to Urquhart *et al* (1986) could be grouped under two broad categories:

1. **Resistance not related to immunity:**
   a) species resistance
   b) age resistance
   c) breed resistance

2. **Acquired immunity:**
   a) the effect of the host immune response
   b) evasion of the host immune response

1.17.1 Species Resistance

The effectiveness of management procedures such as 'clean' grazing systems in parasite control to limit infection will be dependent on the degree of specificity shown by the parasite (Coop *et al*, 1985a). Evidence that *O. ostertagi* does not transmit well to sheep has been reported and any development which occurs in sheep is limited (Porter, 1953, Herlich and Stewart, 1954, Herlich, 1958, 1974, Smith and Archibald, 1977, Borgsteede, 1981, After Coop *et al*, 1985a).

Thus, larvae of *O. ostertagi* can undergo development in sheep, but few reach the adult stage (Urquhart *et al*, 1986). Bisset (1980) noted that a few *O. ostertagi* developed to maturity in sheep but in general were smaller than those recovered from comparable *O. ostertagi* infected calves.

Considerable variation in response in fifteen 2.5-3 month old and 4-4.5 month old worm free sheep infected with 3500-4000 *O. ostertagi* was reported by Coop *et al* (1985b) but characteristic symptoms of and effects similar to ovine ostertagiasis were found. Weight loss in the youngest lambs was noted but no specific effect was demonstrated in older animals. Worm burdens in all animals consisted mostly of early fourth stage larvae (EL4's) and developing worms. Loss of differentiation in abomasal mucosa was noted post mortem. Those authors concluded that distribution was certainly not as uniform as in lambs exposed to continuous *O. circumcincta* infection (Coop *et al*, 1977, 1982) and further emphasised the limited cross species infectivity associated with *Ostertagie* spp. parasites.

1.17.2 Age Resistance Related to Age Immunity

The fact that lambs are more susceptible than adult sheep to experimental infection with gastro-intestinal nematodes is well known (Smith *et al*, 1985a). Early studies in sheep immunised with irradiated or normal *H. contortus* larvae demonstrated that lambs aged six months or less were not resistant to *H. contortus* larvae and that older sheep treated similarly displayed a high degree of resistance (Manton *et al*, 1962, Urquhart *et al*, 1966).

Similar findings in sheep infected with *T. colubriformis* were reported by Dineen *et al* (1978) who explained inconsistent results with the phenomenon of 'responders' and 'non-responders'. Smith *et al* (1985b) proposed that the reason for age-related unresponsiveness was not known since Soulsby (1981) described previous findings where even foetal lambs were able to

mount protective immune responses against bacterial and viral infections.

In two separate but identical experiments, Smith *et al* (1983b, 1984, 1985a) compared the local immune responses in 4.5 and 10 month old parasite-naive and immune lambs challenged with 50000 *O. circumcincta*. When compared to controls, previously infected 4.5 month old lambs were partially resistant to infection as expressed as larval stunting, arrestment of development, secondary local immune response and increased lymphoblast and IgA cell output 3-5 days post-challenge.

Similar comparisons with 10 month lambs from previous experiments demonstrated that younger lambs were measurably less resistant to challenge and displayed singificantly depressed local immune responses. Depsite similar timing in response in the two age groups, Smith *et al* (1985a) suggested that poorly developed ability to mount secondary mucosal immune response may have explained the relative failure of younger animals to resist gastro-intestinal nematodes compared to 10 month old lambs.

1.17.3 Breed Resistance to *O. circumcincta*

Variation in resistance of certain breeds of domestic ruminants to parasitic infection was cited by Urquhart *et al* (1986) who reported trypanosome tolerance displayed by West African N'dama humpless cattle and not demonstrated by other breeds when under heavy trypanosome challenge and that mechanisms controlling apparent tolerance were unknown.

A higher degree of resistance to *H. contortus* infection in East African Red Masai sheep was cited by Urquhart *et al* (1986) than in other breeds and as previously mentioned Scrivener (1964) reported differing resistance between breeds of sheep to *O. circumcincta* infection.

Further analysis has demonstrated inter-breed differences in resistance to *H. contortus*, possibly related to higher gene frequency of the haemoglobin A (HbA) gene such as is found in Merino, Scottish Blackface, and Finn Dorset sheep the consequences of which are higher haematological values, lower worm egg counts and smaller burdens compared to animals with haemoglobin type B (HbB). Urquhart *et al* (1986) also stated that similar findings have been reported in the field in Australia (Evans and Whitlock, 1964), the United States (Jilek and Bradley, 1969) and Kenya (Allonby and Urquhart, 1976).

Attempts to verify these findings experimentally produced unconvincing results (Armour, 1980) although Altaif and Dargie (1975) suggested that high affinity of HbA for oxygen and/or its easy change to HbC may explain reported differences in degree of anaemia resistance between experimental and field cases of *H. contortus* infection.

Intra-breed differences in response to *T. colubriformis* infection was reported by Dineen *et al* (1978) who classified individual lambs as 'responders' or 'non-responders' dependent on their ability to limit pathogenic disease as discussed above.

1.17.4 Acquired Immunity to Helminths

Immune responses to helminths are complex actions possibly dependent upon antigenic stimuli by secretory/excretory and other parasitic products released during development from L3 to adult (Urquhart *et al*, 1986). Despite this, Urquhart *et al* (1986) reported that once immunity has developed, adult sheep may ingest up to 50000 L3 *O. circumcincta* daily without any clinical signs of parasitic gastritis.

Host resistance can be sub-divided thus:

a) The development of immunity.

b) Attempts to limit re-infection.

1.17.5 Development of Immunity

The immune response in *O. circumcincta* infected sheep has been extensively studied by Sinski (1975) who cited the finding of Soulsby (1966) that precipitates formed around *Ascaris suum* larvae placed in antiserum demonstrated the importance of antibodies in resistance to gastro-interstinal worms (Sinski, 1975). Sinski however suggested that immune-based resistance to parasites involved humoral factors on one hand and cellular activity on the other.

In experiments using haemagglutination and detection of precipitating antibodies in sheep infected with *O. circumcincta*, Sinski (1975) found that average alpha-globulin levels were always higher in single and multiple infection animals when compared to

uninfected controls and that levels of gamma-globulin increased gradually but demonstrated considerable variation.

Sharp rises in gamma-globulin were noticed immediately after infection but no appreciable change in gamma-globulin was found in uninfected controls. Serum mucoid levels also increased associated with hypoalbuminaemia during infection however Sinski (1975) stated that the rises in alpha-globulin and mucoid levels may be specifically associated with inflammatory processes.

Increased gamma-globulin levels in the above experiment in *O. circumcincta* infected sheep agreed with the findings of Horak and Clark (1964) in similarly infected sheep and Ross and Todd (1965) in *O. ostertagi* infected calves. Sinski (1975) was initially unable to determine whether antibodies in mucosal fluid were homocytotropic as reported by Hogarth-Scott (1969) and Curtain and Anderson (1972) but in a later study (Sinski *et al*, 1978b) confirmed the previous workers' findings. Three month old Merino sheep infected with 100000 *O. circumcincta* demonstrated haemagglutinating titres against third larval *O. circumcincta* antigen from three weeks post-infection and from five weeks post-infection titres to adult *O. circumcincta* antigen. Precipitating antibodies to *O. circumcincta* first appeared between 7 and 9 weeks post-infection.

Active antibodies (reacting to both L3 and adult *O. circumcincta*) were demonstrated in gamma-globulin prepared from abomasal mucosa. Additionally the IgG1 sub-class homocytotropic antibody (which elicited anaphylactic type hypersensitivity

reaction) was found in infected animals in the same experiment. Basophilic degranulation in sensitised cells *in vitro* was also noted in this experiment.

Further studies using similar experimental designs (Sinski and Bezubik, 1980) revealed increased alpha-globulins, beta-lipoproteins and and alpha-macroglobulin levels accompanied by decreases in alpha- and beta-globulins in *O. circumcincta* infected sheep. Immunoassay in this experiment demonstrated increases in IgG1, IgG2, IgM and IgA quantified as a doubling of IgG1 and tripling of mucosal IgA by 8 weeks post-infection. Haemagglutinating antibodies to L3s and adult antigens were also found.

Sinski and Bezubik (1980) however concluded that resistance may not be related to serum antibody titres in a number of gastro-intestinal parasitic diseases, particularly where infection occurred via mucosal surfaces such as the gastro-intestinal tract.

Other studies exist examining local immune response to *O. circumcincta* infection. Lambs given small daily doses of *O. circumcincta* gradually became resistant to infection where resistance was expressed as a decline in worm egg output by around 8 weeks post-infection (Coop, Sykes and Angus, 1977, 1981; Gibson and Everett, 1978) and was accompanied by reduced worm burdens, a high rate of larval inhibition and increased resistance to challenge (Gibson and Everett, 1978).

Smith *et al* (1981) stated that the nature of the mechanism of acquired resistance remained unknown but that it was likely that immune mechanisms operating in the abomasal mucosa were involved. The same authors also reported changes in gastric lymph secretions of sheep when total secretions doubled upon infection. Lymph IgA concentration became elevated in immune sheep compared to animals infected with a primary challenge and secretions in both primary and secondary infected animals reached levels at least five times greater than that found in uninfected controls. However IgM and IgG concentrations remained unaffected. Anti-larval IgA and IgG was found on lymph secretions of all infected animals and both infected groups demonstrated antigen bound IgM.

In studies examining the local immune response to primary *O. circumcincta* infection, Smith *et al* (1983c) reported a significant increase in lymphoblast frequency, indicative of an immunological response, in infected sheep by 8 days post-infection. No significant difference in either lymph IgA or IgA anti-worm antibody was detected.

Additional studies (Smith *et al*, 1983b) demonstrated immunity to secondary *O. circumcincta* infection manifested by a reduction in the number and degree of development of infective larvae compared to parasite-naive controls. Secondary local immune response was detected in gastric lymph after challenge and consisted of increased lymph cell output (of lymphoblasts and IgA-containing cells) which peaked 4 or 5 days post-infection and was followed by a ten-fold increase in IgA-immunoglobulin. IgA

anti-worm antibody peaked at 7 or 8 days after challenge and from these findings the authors concluded that cellular immunologic events rather than IgA secretion were the putative mechanism causing arrested larval development.

Early studies by Stewart (1955) reported that continuous larval intake resulted in a hypersensitive immune response in sheep exposed to both *H. contortus* and *T. colubriformis* and that further infection was limited by a phenomenon called 'self-cure'. A transient rise in blood histamine levels at 4 days post-infection was noted, accompanied by a dermal sensitivity response and oedema associated with infection.

In experiments using anaesthetised immune and parasite naive sheep he observed (via laparotomy) oedema, increased peristalsis and segmentation in the abomasum within 10 minutes of introduction of exsheathed larvae in hypersensitive sheep. Stewart (1955) then suggested that 'self-cure' was not limited to *H. contortus* but was also evident when *O. circumcincta* or *T. axei* infections were present.

Urquhart *et al* (1986) summarised primary *O. circumcincta* and *Trichostrongylus* spp. infections by stating that while immunity to luminal parasites was still not fully understood despite extensive research, it was generally agreed that infection produced a state of gut hypersensitivity associated with increased numbers of mucosal mast cells in the lamina propria. Worm specific IgE was produced and became attached to mast cells which in the presence of antigen (primary or secondary infec-

tion) released vasoactive amines which caused increased capillary and epithelial permeability and hyperproduction of mucus.

Consequently those authors stated that the normal course of parasitism consisted of "a build-up of infestation of adult worms followed by their expulsion and subsequent immunity (in sheep); in later life only small short-lived adult infections are established and eventually the infective larvae are expelled without any development at all".

1.17.6 Limiting Re-Infection

Urquhart *et al* (1986) described attempts to limit re-infection which consisted of the ability to prevent larval migration or to arrest larval development. Adults which did develop were stunted in size, possibly with reduced fecundity. The studies cited below have examined these suggestions more fully.

In attempting to quantify resistance to infection at a local level, Smith *et al* (1984) found that 1000 L3 *O. circumcincta* used to infect previously challenged and parasite-naive sheep were insufficient to elicit a marked local immune response. However, 50000 larvae used to infect equivalent animals did cause a marked local response. Previously infected sheep were resistant to both infection levels when compared to parasite-naive animals where resistance was measured indirectly as arrested development and the roduction of stunted adults. The above authors described limited resistance in sheep infected with 1000 L3 *O. circumcincta* but marked resistance in animals

infected with 50000 L3 when proportionally fewer worms per infective challenge were recovered from the latter group, the majority of which were arrested EL4's.

Additionally, those authors proposed that a temporary rise in pepsinogen activity in lymph of sheep infected with 50000 L3 *O. circimcincta* indicated a hypersensitive reaction related to the presence of large numbers of mucosal mast cells occurring between 24 and 48 hours after challenge which was followed by a marked cellular and IgA response which peaked 3 and 6 days respectively post-infection.

Further studies produced positive evidence of a functional role of the cellular response to *O. circumcincta* challenge, in which partial immunity was adoptively transferred to naive sheep using washed immune lymphocytes collected from hyperimmune sheep during the blast response (Smith *et al*, 1985). In addition to furnishing apparent protective immunity, as measured by loss of worms or stunting, transfer of mastocytosis was reported following mast cell enumeration (Smith *et al*, 1986).

Experiments examining age effects on secondary local immune response (Smith *et al*, 1985) demonstrated that younger lambs produced a much poorer secondary immune response to *O. circumcincta* than mature sheep. Those authors concluded that this was a possible explanation for the phenomenon of immune unresponsiveness to gastro-intestinal nematodes of young ruminants.

Urquhart *et al* (1986) quoted unpublished studies showing that both lambs and calves "failed to develop any useful degree of immunity to re-infection with *Ostertagia* spp. until they had been exposed to constant re-infection for an entire grazing season."

1.17.7 Evasion of Host Immune Response

Complementing Tizard's (1986) view of 'obligatory' parasitism, Urquhart *et al* (1986) opined that despite evidence that animals developed vigorous immune responses to helminths, it remained clear that parasites have captalised on defects in the immune system's armoury and cited three examples of parasitic evasion of host immune response.

The first of these was cited earlier in this review and reported as neonatal immune unresponsiveness in lambs and calves to *Ostertagia* spp. infection despite foetal immune response to viral or bacterial pathogens (Soulsby, 1981). Urquhart *et al* (1986) suggested that the parasite may contribute to this phenomenon and cited unpublished studies where no effective immunity developed during this period of immune unresponsiveness. The period of immune unresponsiveness was considerably lengthened in *H. contortus* infected Merino sheep which were reared from birth in an endemic area and kept there for the rest of their lives.

Concomitant immunity whereby host immune response against incoming larvae has little or no effect on established adults was similarly categorised by Urquhart *et al* (1986) as an example of evasion of host immune response. Those authors cited

schistosome infection as the best example of this and suggested that failure to recognise surface cytoplasm as antigen may be the cause but cautioned that the chitinous cuticle found in helminth infections, including *O. circumcincta*, may not respond in the same way.

A third and final evasion of host immuneresponse was stated to be polyclonal stimulation of immunoglobulin.

In this situation helminth antigens stimulated large scale non-specific IgE production (in addition to normal IgE anti-worm antibody) helping the parasite initially by coating mast cells with non-specific IgE and resulting in lowered attraction to the parasite with ultimate limitation of mast-cell degranulation capability. Additionally when large quantities of non-specific IgE are produced, there is a lowered likelihood of adequate quantities of specific IgE being made.

CHAPTER 2

ANALYTICAL TECHNIQUES

2.1 EXPERIMENTAL PROCEDURES

2.1.1 Animals

Lambs used in the experiments described in Chapters 3-5 were from Scottish Blackface and Scottish Blackface-Swaledale cross ewes which had been treated with progesterone sponges (Veramix, Upjohn Ltd., Crawley, W. Sussex) to ensure synchronous oestrus before mating with Blueface Leicester rams. Micronutrient status of ewes which provided lambs for experimentation was normal except for those in Experiment 1, (Chapter 3) details of which are recorded there.

All experiments were carried out in a housed situation where hay and water were fed to animals *ad libitum* and concentrate allocation given in two feeds per day. Normal husbandry practices were applied to experimental animals including 'worming' of ewes at housing (Valbazen, Smith-Kline Animal Health, Stevenage), 'creep' feeding of lambs before weaning at 16 weeks and compulsory summer dipping. Pregnant ewes were vaccinated with a multi-component clostridial vaccine (Heptavac-P, Hoechst Animal Health, Milton Kenyes) 2-4 weeks before lambing and lambs were immunised with 2 ml Ovivac-P (Hoechst Animal Health) at 8 and 13 weeks of age.

2.1.2 Experimental Design

Experiments 1 and 2, detailed in Chapters 3 and 4 respectively, were essentially of a 2 x 2 factorial design examining the effects of the presence or absence of *Ostertagia circumcincta* infection in lambs either deficient or supplemented/sufficient in

cobalt or selenium/Vitamin E. Precise experimental designs are listed in the relevant chapters. In Chapter 5, the effect of treatment with micronutrient supplemented anthelmintic (Panacur SC, Hoechst Animal Health) of surviving animals was examined in terms of parasitological, biochemical and production effects.

2.1.3 Ration Formulation

The diets in Experiments 1 and 2 were formulated to produce specific micronutrient deficiencies. Precise details of each diet including its micronutrient content are contained in the relevant chapters. Diets were formulated to meet the protein, energy and mineral requirement of lambs gaining 0.1 kg per head per day (ARC, 1980).

Feedstuff samples were submitted to the West of Scotland College's Analytical Services Unit for copper and cobalt determination, protein and energy evaluations and feeding rates were adjusted when necessary. Selenium and Vitamin E content was determined by the North of Scotland Agricultural College's Analytical Laboratory in Aberdeen.

2.1.4 Infection

Ostertagia circumcincta larvae, supplied by the Veterinary Parasitology Department of the Unviersity of Glasgow, consisted of a laboratory strain established from field origin. The number of larvae in 20, 25 ul samples of homogenous larval suspension were counted under a 10 x 5 objective light microscope (Vickers Scientific Instruments, London) to determine the concentration of

larvae per ml and calculate the volume which contained 2000 L3 *O. circumcincta* for administration of infection to lambs.

2.1.5 Blood Sampling

Blood samples were taken from ewes and lambs in the pre-experimental phase on an intermittent basis to assess micro-nutrient status. During the whole experimental period however, blood samples were taken for biochemical analysis weekly. Blood sampling in the recovery phases of Experiments 1 and 2 took place on the day of treatment and 7, 14 and 28 days post treatment.

Biochemical determinations such as Vitamin B_{12}, Vitamin E and methylmalonic acid concentrations, and assessment of antibody levels were carried out in blood collected by jugular venepuncture into uncoated siliconised evacuated 10 ml blood tubes (Vacutainer, Becton-Dickinson, New York). Serum was collected by placing the tubes in a 30°C water bath for 20 minutes and after clot retraction centrifugation was carried out at 1000 G (Magnum Centrifuge, MSE, London) for a further 20 minutes and serum transferred to 2 ml flat bottom tubes (Tek-Lab Medical Supplies, Durham) and stored frozen (-20°C).

Whole blood glutathione peroxidase (GSH-Px) was determined on blood collected by jugular venepuncture into lithium heparin coated 10 ml vacutainers (Becton-Dickinson) containing 143 USP units of heparin. Packed cell volume (PCV) determination was carried out on these samples and blood lysates for GSH-Px analysis prepared. The remainder of the blood was centrifuged, plasma was removed and frozen for later determination of biochemical

parameters including pepsinogen, albumin and total protein content.

Blood samples for white cell function tests were collected by jugular venepuncture in either of two tubes, the choice of which varied depending on which test was performed. The neutrophil function test (NFT, described later in this Chapter) used blood samples which were collected from four lambs from each group in weeks 1, 3, 5, etc into uncoated 10 ml vacutainer tubes containing 1 ml of 1.5% EDTA (sodium salt) in 0.8% phosphate buffered saline (PBS). Samples for Nitroblue tetrazolium reduction assessment (NBT - also described later in this chapter) were taken from 4 further lambs from each group during even numbered experimental weeks (2, 4, 6, etc) into 10 ml uncoated vacutainers containing 200 USP units of heparin (lithium form) in PBS. Haematological examination (as described later) was carried out on blood samples used for white cell function tests.

2.1.6 Statistical Analysis

Statistical analysis in Experiments 1 and 2 was carried out using the Genstat IV statistical package (Lawes Agricultural Trust, 1984) and Minitab Data Analysis Software (Release 7.1, Pennsylvania State University). Analysis of variance and covariance analysis were used unless otherwise stated and data was log-transformed where necessary.

2.2 BIOCHEMISTRY

2.2.1 Serum Vitamin B_{12} Determination

Test serum samples were diluted 1 to 9 in an acetate buffer, pH 4.85 containing 20 mg/l potassium cyanide. After steaming at 106°C for 30 minutes, the serum proteins were precipated and the Vitamin B_{12} present converted to stable cyanocobalamin. 2 ml of protein free supernatant was diluted with 8 ml of assay broth and sterilised at 121°C for 30 minutes before inoculation with a suspension of *Lactobacillus leichmanii*. After incubation for 18-24 hours, growth of *L. leichmanii* was determined spectrophotometrically and compared to growth in samples with known Vitamin B_{12} content and with growth found in aqueous Vitamin B_{12} standards. Precise details of Vitamin B_{12} analyses in this experiment were according to the method cited by Fisher (1988).

2.2.2 Serum Methylmalonic Acid (MMA) Determination

Standards were prepared from a 10 µmol/ml MMA (Sigma Chemical Co., Poole, Dorset) stock standard in acetone (BDH, Poole Dorset) which was then diluted to a 0.5 µmol/ml working standard in acetone. 100 µl of working standard was added to a quick-fit tube and made up to 500 µl with distilled water, 500 µl of serum was placed in similar tubes and 100 µl of a 0.1 µmol/ml ethylmalonic acid (EMA, Aldrich Chemical Company, Poole, Dorset) was then added to each tube as internal standard. After addition of 500 µl acetone while vortex-mixing each tube, 2 ml of a saturated sodium chloride (BDH) solution (300 g/l) in 0.5M sulphuric acid (Analar Grade, BDH) (Sodium chloride:sulphuric acid ratio =

1:9) was added to each tube. 1 ml ethyl acetate (BDH) was dispensed into each tube which was shaken vigorously for 20 seconds and centrifuged at 1000 G for 10 minutes. The ethyl acetate layer was transferred to a 1 ml microtube and then evaporated to dryness at 60°C on a Dri-Block heater under oxygen free nitrogen (BOC Gases). Ethyl acetate extraction and evaporation was repeated once more. 100 ul of a 1:10 mixture of acetylchloride (BDH)/butanol (BDH) was added to each microtube which were then incubated at 70°C in a water bath for 20 minutes. After cooling, 300 ul hexane (BDH) and 600 ul distilled water was added and each tube shaken vigorously for a few seconds before the hexane layer was transferred to a small gas chromatogram autosampler vial for determination of MMA concentration.

Separation was carried out in a Hewlett Packard 439 gas chromatogram where 2.5 ul of sample was injected in hexane using a 1:10 split ratio onto a 25m, 0.22 mm Chrompack CP-SIL 5B capillary column using helium as a carrier gas. Oven temperature profile for separation was:

120°C x 2 minutes

5°C rise per minute up to 150°C

40°C rise per minute up to 230°C

Hold at 230°C x 5 minutes.

Results from a flame ionisation detector held at 280°C were collected and processed using a Shimadzu CR3A Integrator. These were determined by calculation of mean ratio of EMA:MMA peak areas in standards to determine the response factor (RF). MMA concentration in samples was calculated by expressing MMA area as

a fraction of EMA internal standard and multiplication by the product of original MMA standard concentration and the RF.

2.2.3 Whole Blood GSH-Px Activity Determination

GSH-Px activity was determined according to the method of Paglia and Valentine (1967) as modified by Anderson, Berrett and Patterson (1978). Briefly, this method used GSH-Px present in the whole blood sample lysate, activated by the presence of reduced glutathione, glutathione reducatase and NADPH to convert cumene hydoperoxide substrate to free radicals in a kinetic assay at 30°C. The rate of NADPH oxidation was measured, related to red cell fraction in the blood and thus GSH-Px activity noted.

2.2.4 Serum Vitamin E Determination

Vitamin E was determined in serum using a simple hexane extraction technique. 500 µl of serum, placed in a 5 ml screw capped tube had 500 µl ethanol (Standard Grade, BDH) added during vortex-mixing and was allowed to stand for a few minutes. 500 µl samples of a working standard of 2.15 µg/ml alpha-tocopherol in ethanol (= 2.495 µmol/l) was prepared by dilution of a 0.215 mg/ml stock standard of d-alpha-tocopherol (Kodak Chemical Co., Liverpool) and processed using the experimental technique described below. Comparison of processed and non-processed alpha-tocopherol standards revealed > 90% recovery.

2.5 ml hexane (Analar Grade, BDH) was added to all tubes which were then shaken vigorously for 40 seconds. Centrifugation at 500 G for 5 minutes followed and exactly 2.0 ml of the hexane

layer was transferred to clean microtubes and evaporated to dryness at 50°C on a Dry-Block heater under oxygen free nitrogen (BOC). The residue was dissolved in 200 µl methanol (HPLC Grade, BDH) before being transferred to autosampler vials for Vitamin E determination by comparison with standards of known Vitamin E concentration. 94 µl samples were injected (Varian 9090 Autosampler) onto an HPLC system consisting of a Spectra-Physics SP8700 pump, a 15 cm x 4 cm Superspher (BDH) 4 µm RP-18 column and a Kratos Analytical Spectorflow 980 Fluorimeter. Column flow rate was 2 ml/minute of a 96:4 degassed methanol:water, (HPLC Grade, BDH) isocratic mixture. Fluorimetric detection used a 210 nm excitation wavelength and a 370 nm emission cut-off filter which produced results collected and integrated by a Spectra-Physics 4270 Integrator.

2.2.5 Serum Creatine Kinase (CK) Determination

Creatine Kinase (E.C. 2.7.3.2.) was determined by a Diagnostic Kit (Boehringer-Mannheim, Lewes Sussex, Number 126322) using the CK NAC-Activated UV-system. Essentially the method used CK present in test serum to convert creatine phosphate into creatine and ATP, the latter being used to generate NADPH, the production of which was measured at 340 nm in kinetic assay to 30°C.

2.3 CLINICAL METHODS

2.3.1 Biochemistry of Clinically Affected Lambs

Blood samples for determination of Blood Urea Nitrogen (BUN, Kit No. 620235, Boehringer-Mannheim) Serum Glutamic Oxaloacetic

Transaminase (SGOT, Kit No. 124362, Boehringer-Mannheim) and Non-Esterified Fatty Acids (NEFA, Kit No. 9947540, Alpha Laboratories, East Leigh, Hampshire) were removed from clincally affected animals which were then euthanased *in extremis* by intravenous overdosage with pentobarbitone-sodium (Euthatal, RMB Animal Health, Essex).

2.3.2 Post Mortem Examination Procedures - Casualty Lambs

In Experiments 1 and 2 a number of deaths occurred and these lambs were examined post mortem thus:

Casualty lambs were weighed, placed dorsally on an examination trolley and the thoracic and abdominal cavities opened and examined. Gross findings were recorded and samples of any affected organs or tissue were removed and fixed in a 10% formol saline solution for later histopathological examination. The central nervous system (brain, spinal cord and eyes) were removed from a number of casualty lambs in Experiment 1 and fixed in 10% buffered formol saline for later neuropathological examination. Liver samples were removed to allow the determination of copper and cobalt content by atomic absorption spectroscopy. The gastrointestinal tract was examined to assess abomasal and rumen pH and to assess parasitic damage. Any affected tissue was removed and fixed for later histopathological examination. Fresh faeces were taken from the rectum for worm egg count (WEC) determination.

2.3.3 Histopathological Techniques

Tissue for histopathological examination was prepared using standard methods as described by Campbell (1951) and examined under 20 x 40 light objective microscope.

2.3.4 Clinical Cough Score

A semi-objective cough index was determined in lambs in Experiment 2 whereby they were graded for symptoms associated with pneumonia immediately before and four days after treatment with antibiotics (Terramycin LA, Pfizer Animal Health, Sandwich).

Coughing was scored with 0-3 points representing absence of cough (0), occasional cough (1), repeated coughing (2) or paroxysmal coughing (3). Nasal discharge was scored for its presence of absence by 1 or no points. Similar scoring was adopted for dyspnoea (difficulty in breathing), tachypnoea (increased respiratory rate) or hyperpnoea (increased respiratory effort). Scoring for temperature, 0-5, was based on a sliding scale where $< 39.1^{\circ}C = 0$, $39.1 - 39.5^{\circ}C = 1$, $39.6 - 40.0^{\circ} C = 2$, $40.1 - 40.5^{\circ}C = 3$. $40.6 - 41.0^{\circ}C = 4$ and $> 41.0^{\circ}C = 5$. Other non-specific features associated with pneumonia such as dullness, inappetance or low condition were awarded arbitrary single points. Scores for each animal were counted and pre- and post-treatment values assessed for significant statistical differences.

2.4 PRODUCTION

2.4.1 Weighing

Lambs in Experiments 1 and 2 were weighed on a fortnightly basis for the duration of each experiment using a conventional weigh crush (Ritchie's Farm Implements, Forfar). In the recovery phases of Experiments 1 and 2 (Chapter 5), lambs were weighed on the day of treatment then 7, 14 and 28 days post treatment.

2.5 PARASITOLOGY

2.5.1 Plasma Pepsinogen Determination

Plasma pepsinogen was determined by measuring the serum conversion of an acidified albumin substrate to soluble peptides and a tyrosine terminal residue which was then detected using colourimetry via alkaline Folin-Ciocalteau reagent and comparison with tryosine standards. Precise details of the techniques were described by Edwards *et al* (1960) and later modified by Porter (1977).

2.5.2 Worm Egg Count (WEC)

Worm egg counts were determined in fresh faecal samples taken weekly from lambs and processed according to the modified McMaster technique of Gordon and Whitlock (1939) as listed in the ADAS Manual of Veterinary Investigation Laboratory Techniques - Part 7, Parasitology (1978). This essentially involved disruption and filtration of faecal material in water; centrifugation of the filtrate and salt flotation of nematode eggs released from

the deposit left for geometric counting in a glass McMaster chamber and the result calculated as eggs per gram (epg).

Prior to patency and when WEC's were negative, a modification of this technique known as the Clayton-Lane method was adopted to determine egg count. The 20 ml universal containers which had been used in the normal WEC determination were smeared with sealing gel around the rim, and over-filled with saturated sodium chloride solution until a positive meniscus had been created. The container was then sealed with a polypropylene disc before centrifugation at 400 rpm (Superminor Centrifuge, MSE) for 5 minutes. Eggs, if present, floated to the disc which was examined and any eggs found represented one egg per gram.

2.5.3 Total Worm Count Determination

Gastrointestinal tracts of infected and sacrificed lambs were examined for quantitative assessment of total worm count (TWC). Abomasa were removed (or tied in the case of slaughtered lambs) before being opened along the greater curvature and the contents collected in a plastic vessel. The mucosa was washed and massaged between thumb and forefinger under a stream of water and this fluid collected in the same vessel. Around 500 ml of 10% formol saline was added and the total volume made up accurately to 2 litres with water and a representative sample collected.

The mucosal material from the abomasum was scraped from the serosal surface using the edge of a scalpel blade and suspended in a pepsin (BDH)/hydrochloric acid (BDH) solution (made as 10g pepsin in 30 ml of concentrated hydrochloric acid and made up to

1 litre with distilled water). The solution was incubated at 37°C for two hours or until it was assessed that mucosal integrity had been lost and larvae had been released. This solution was formalised, made up accurately to 2 litres with distilled water and a representative sample retained.

A small intestine (SI) wash was made by removing the intestine and running a volume of water into one end. Water trapped in the intestine was passed between the thumb and forefinger along its entire length and collected, formalised, diluted and sampled as above.

The number of larvae and adults in each of the abomasal wash, the abomasal digest and the small intestine wash was calculated by counting the total number of each in 10 x 4 ml replicate representative samples in a disposable culture dish under a 10 x 5 light microscope (Vickers Scientific Instruments) and calculating the total number present in 2 litres. This represented the total burden from the infected lamb. The ratio of male:female adult worms from each lamb was also determined in the ten samples. Additionally, 50 each of male and female adult worms were collected and measured.

2.5.4 Larval Culture

At the end of Experiment 2, 3 groups of lambs consisting of 4 each from the selenium deficient, the Vitamin E deficient and adequate groups selected for slaughter, were placed in restraining cages to facilitate faecal collection. Male lambs were fitted with harnesses and faecal collection bags but in female

lambs, failure to separate urine from faeces in similar bags would have prevented faecal culture for the purpose of harvesting parasites. Their faecal matter was therefore collected on a tray below the cage and urine allowed to pass through. Faeces were then washed to remove urinary contamination and cultured in the same way as faecal material collected from male lambs. 200 ml polystyrene tubs (Fibracan SC20, Food Service Disposal, Glasgow) were half filled with faecal material from an individual lamb (its WEC and weight of faecal output having been determined), numbered, dated and then incubated at 20-25°C until eggs had matured to third larval stages (usually 2-3 weeks). Thereafter, each tub was filled with luke-warm water and left for three hours before semi-quantitative recovery. Liquid from the faeces was filtered through two standard milk filters, (Chambers Bros., Ayr) in Buchner apparatus under reduced water pressure. The residue was covered with a third filter and Baermannised in a funnel of warm water where larvae migrated to the closed end of the funnel as the water cooled during a 6 hour period. Thereafter, the tap closing the funnel was opened and larvae collected and counted using the method detailed previously.

2.6 HAEMATOLOGY

2.6.1 Packed Cell Volume (PCV) Determination

PCV was determined from fresh homogenous blood samples. A small glass tube was filled with blood (via capillary action) and the tube closed with a plasticine bung. Tubes were centrifuged in a Haematocrit (MSE, London) centrifuge at setting 8, and percentage PCV determined using a Haematocrit reader.

2.6.2 Differential White Cell Counts

One drop of fresh homogenous blood was placed on a standard glass slide and smeared using the narrow edge of another slide to make a film. The film was fixed by immersion in Wright's Giemsa stain (Sigma) for 15 seconds followed by immersion in distilled water for 2 minutes and then thoroughly rinsed. 100 white cells were counted on a random basis under 20 x 40 light objective microscope (Kyowa Opticals, Japan) and the percentage of each type recorded.

2.7 IMMUNITY

2.7.1 White Cell (Neutrophil) Isolation Technique

Isolation of white cells in this experiment was similar to the method described by Carlson and Kaneko (1973). Non-coagulated blood (anti-coagulant being EDTA for Neutrophil Function Tests, (NFT) and Heparin for Nitroblue Tetrazolium Reudction, (NBT)) was centrifuged (within two hours of collection) at 1000 G for 20 minutes and the plasma portion and the uppermost part of the red cell column (mono fraction) aspiration off and discarded. The remaining sample was resuspended in 4 ml of isotonic (0.8%) PBS and transferred to 50 ml conical based centrigute tubes. 20 ml of distilled water was added to each sample and left for 1 minute to allow lysis. Isotonicity was restored by the addition of 10 ml 2.7% sodium chloride in PBS and tubes centrifuged at 500 G for 10 minutes before aspirating and discarding the supernatant to leave a white cell 'button'. The button was resuspended in 25 ml PBS and gently mixed by inversion before recentrifugation and

removal of the supernatant. Washing procedure was repeated twice more before the button was finally suspended in 1.0 ml PBS ready for use. Previous determinations of total and differential white cell counts revealed a typical concentration of 10 million neutrophils/ml in the button.

2.7.2 Neutrophil Function Test

This test involved the incubation of neutrophils isolated as above, with a suspension of *Candida albicans* for 75 minutes. After staining to determine the proportion of dead ingested yeast cells, the killing ability or index (KI) of neutrophils was recorded. Precise experimental details are identical to those reported by Fisher (1988).

2.7.3 Nitroblue Tetrazolium Reduction (NBT)

This test measured possible defects in respiratory burst during phagocytsis in neutrophils from sheep under different experimental regimes. Measurement of superoxide produced during the respiratory burst was made using superoxide to donate electrons for the reduction of yellow Nitroblue Tetrazolium to blue coloured formosan which was detected spectrophotometrically. Residual, resting, superoxide activity was also determined to remove individual variation and results were expressed as a change in optical density (between resting and stimulated neutrophils), designated dOD.

Neutrophils for activity assessment in this experiment were isolated via the method listed above and processed using a modi-

fication of the experimental technique reported by Nagahata *et al*, (1986). A fresh culture of *Candida albicans* was grown in Soubraud's broth 2-3 days before use as the neutrophil stimulant in this experiment. Concentration of yeast cells was determined using a Coulter Counter (Model 2) and the cells washed four times in PBS before dilution with PBS to give a final concentration of 10 million yeast cells/ml. A 0.1% solution of Nitroblue Tetrazolium (Grade III, Sigma) was suspended in Earles solution (Flow Laboratories, Rickmansworth). Fresh pooled serum, collected on the day of test, was added to the reaction mixtures to enable opsinisation of the yeast cells. Test mixtures were prepared in glass culture tubes thus:

Stimulated assay:

0.2 ml 0.1% NBT in Earles solution

0.1 ml neutrophil suspension

0.4 ml *C. albicans* solution

0.1 ml Earles solution

0.3 ml fresh pooled serum

Non-stimulated assay:

0.2 ml 0.1% NBT in Earles solution

0.1 ml neutrophil suspension

0.5 ml Earles solution

0.3 ml fresh pooled serum

Tubes were then incubated in a 37°C water bath for 60 minutes and the reaction stopped by the addition of 3 ml 0.5 N hydrochloric acid. All tubes were centrifuged at 1000 G for 10 minutes and the supernatant aspirated off and discarded. The hydrochloric

acid washing step was repeated twice more before the pellet was suspended in 3 ml dimethyl formamide (DMF, Sigma) and formosan extracted by placing the tubes in a boiling water bath for 30 minutes. After being allowed to cool, 2 ml of 10 N potassium hydroxide was added to enhance colouration and tubes were thoroughly mixed prior to centrifugation. The upper DMF layer was transferred into silica cuvettes and optical density determined at 710 nm. dOD was calculated by subtracting the optical density of the resting unstimulated neutrophils from the optical density determined for stimulated counterparts.

2.7.4 Determination of Antibody Response to *O. circumcincta* in ELISA

A third larval stage *Ostertagia circumcincta* antigen was manufactured by grinding larvae with a teflon pestle in a ground glass homogeniser. Protein concentration was determined (Micro BCA Protein Assay Kit, Pierce, Oud-Beijerland, Holland) and the homogenate diluted to 5 µg protein/ml with bicarbonate coating buffer, pH 9.6 (3.7g sodium bicarbonate (BDH) + 6.0g disodium carbonate (BDH) made up to 1 litre with distilled water). 150 µl of antigen solution was added to Dynatech M129B Microelisa plates which were then incubated for 24 hours at room temperature. After incubation, plates were washed five times with PBS containing 0.05% Tween 20 (Sigma) (PBST), before control and sample sera were added in duplicate at 1:200 dilution (optimal dilution having been previously determined by serial dilution), and the plate incubated overnight at room temperature.

Equine anti sheep IgG was obtained from Moredun Research Institute, Edinburgh (W.D. Smith) and biotinylated thus:

Immunoglobulin solution was dialysed using standard visking tubing against 0.1M sodium bicarbonate, (pH 8.2) overnight at 4°C, set at 1 mg/ml protein concentration. Biotin-N-hydroxy succinimide ester (Sigma) was dissolved at 1 mg/ml in dimethyl-sulphoxide immediately prior to use. 120 µl of biotin ester/ dimethyl sulphoxide (DMSO, Sigma) was added per ml of immuno-globulin solution, mixed and then left at room temperature for 4 hours. The solution was then dialysed against PBS/Azide over-night at 4°C and the optimum dilution determined.

150 µl biotinylated equine anti sheep IgG conjugate (1:100 dilu-tion in PBST containing 10% horse serum) was added to each well and the plate incubated for 1 hour at room temperature. After washing five times with PBST, 150 µl of a 1:300 dilution of avidin peroxidase (Sigma) in PBST was added to each well and plates incubated at room temperature for one hour before washing as before.

A substrate mixture was made thus:
24.3 ml of 19.2 g/l citric acid (BDH) was added to 25.7 ml of 28.4 g/l sodium dihydrogen phosphate (BDH) : 50 ml de-ionised water and shortly before use, 40 mg of ortho-phenyline diamine (O.P.D., Sigma) and 40 µl hydrogen peroxide were added. 150 µl of mixture was added to each well and colour allowed to develop for 30 minutes at room temperature. The reaction was stopped by adding 50 µl of 2.5 M sulphuric acid (Analar Grade, BDH). Optical density was measured at 492 nm using a Titertek Multiscan spectrophotometer (Flow Laboratories) and results expressed as

percentages of a pooled reference serum from a hyperimmune lamb which had been assayed in all plates.

2.7.5 Determination of Antibody Response to *Clostridium tetani* in ELISA

The ELISA method listed below assessed the vaccine response to the *C. tetani* component of the multivalent vaccine used in lambs. This technique was used in preference to the conventional toxin neutralisation test in mice as listed in British Pharmacopea (Veterinary), 1989. Dynatech M129B micro-ELISA plates were coated with a 1/1500 dilution of *C. tetani* toxin (Calbiochem) in 0.01M phosphate-buffered saline (PBS) pH 7.2 with a final protein content of 5.9 µg/ml (Micro BCA Protein Assay Kit, Pierce). Coating was carried out at 37°C for 3 hours before use. Plates were then emptied and washed three times with 0.25 ml PBST per well. 250 µl 0.1% bovine serum albumin (Sigma) in PBS was then added to each well and the plates incubated at 37°C for 1 hour before three washes with PBST.

Standard and test sheep sera were then added at appropriate dilutions in PBST (ranging from 1:200-1:5000 in test serum samples). The standard serum, obtained from a pool of ten sheep standardised using a toxin neutralisation test in mice and found to have 5.5 iu activity per ml was diluted between 1/1000 and 1/16000 before application to the micro-ELISA plate.

The plates were incubated at 37°C for 1 hour and washed three times with PBST before addition of a 1/2000 dilution of donkey anti-sheep IgG-horseradish peroxidase conjugate (Sigma) in PBST before further incubation at 37°C for 1 hour. After washing three

times with PBST a solution of 0.042 mM tetramethyl benzidine (ICN Biomedicals) (TMB) in 0.1M sodium acetate-citric acid buffer, pH 6.0 (= 1:100 dilution), containing 8.8 mM hydrogen peroxide (= 1:1000 dilution) was added and the plated incubated at room temperature for 15 minutes. The reaction was stopped by the addition of 50 µl 1M sulphuric acid (BDH, Analar) to each well and the absorbance measured at 450 nm using a Titertek Multiscan spectrophotometer (Flow Laboratories).

All volumes used were 200 µl unless otherwise stated and the plates were sealed during each incubation period. All standards and test samples were added to the plate in duplicate and mean values calculated. The tetanus antitoxin concentration of test samples was determined by comparison of absorbance with those of the standard curve.

CHAPTER 3

THE INTER-RELATIONSHIP BETWEEN COBALT DEFICIENCY, *Ostertagia circumcincta* INFECTION AND IMMUNE FUNCTION IN SHEEP

3.1 INTRODUCTION

Enhanced susceptibility to infection in cobalt deficient sheep has been previously reported (MacPherson *et al*, 1976). Impaired neutrophil function which was later demonstrated in cobalt deficient animals was suggested as a possible cause of an apparent increased vulnerability to disease (Wright *et al*, 1982, Fisher and MacPherson, 1986, MacPherson *et al*, 1987).

Wright *et al* (1982) described greater susceptibility to infection with *Ostertagia ostertagi* in cobalt deficient cattle. Additionally, Fisher and MacPherson (1986) reported that low cobalt status in pregnant ewes resulted in poor viability of lambs born to such ewes.

Downey (1965) in studies examining *Ostertagia circumcincta* in sheep infected with *Trichostrongylus* spp., initially reported apparent enhanced pathogenicity in animals receiving a cobalt supplement which suggested an obligatory cobalt requirement by the parasite. In further studies (Downey, 1966a), however, described the reverse effect in sheep where cobalt deficiency resulted in enhanced pathogenicity of *Ostertagia circumcincta* infection.

The experiment detailed below was designed to examine the effect of cobalt deficiency (maternal or otherwise) on:

a) the pathogenesis of *Ostertagia circumcincta* infection; and
b) immune function in lambs including their response to vaccination.

3.2 MATERIALS AND METHODS

3.2.1 Experimental Animals

Ewes:

Ewes of varying cobalt status, as designated in Table 1 which produced lambs of varying cobalt status as detailed below were used in the experiment. The first group (1) of 20 cobalt deficient ewes produced 17 cobalt deficient lambs, whereas groups 2 and 3 (40 cobalt adequate ewes) provided 43 lambs for the main part of the experiment.

TABLE 1: Ewes Used to Provide Lambs for Experiment 1

GROUP NUMBER	NUMBER OF EWES	STATUS	DESCRIPTION
1	20	Deficient	No cobalt given at mating or during gestation
2	20	Half Supplemented	No cobalt given at mating but supplemented from mid-pregnancy until parturition
3	20	Fully Supplemented	Full cobalt supplement at mating, during pregnancy and lactation

Lambs:

Sixty lambs consisting of 17 from deficient and 43 from sufficient ewes were assigned to the three broad cobalt status groups defined below and in Table 2 (which were arranged in a 3 x 2 factorial design). The first group, termed **Deficient**, were

produced by ewes kept on a cobalt deficient ration prior to mating and throughout pregnancy. These lambs had little or no cobalt reserves, received no cobalt supplement and were therefore entirely dependent on dietary intake of cobalt for essential physiological functions.

TABLE 2: Experimental Design Used in Experiment 1

Group 1 (9 animals)	-	From cobalt deficient ewes No cobalt supplement Infected with *O. circumcincta*	**DEFICIENT**
Group 2 (8 animals)	-	From cobalt deficient ewes No cobalt supplement Non-infected	
Group 3 (10 animals)	-	From cobalt adequate ewes No cobalt supplement Infected with *O. circumcincta*	**DEPLETED**
Group 4 (11 animals)	-	From cobalt adequate ewes No cobalt supplement Non-infected	
Group 5 (11 animals)	-	From cobalt adequate ewes Cobalt supplemented Infected with *O. circumcincta*	**SUFFICIENT**
Group 6 (11 animals)	-	From cobalt adequate ewes Cobalt supplemented Non-infected	

All lambs were fed a diet deficient in cobalt.

Lambs in the second status group also received a cobalt deficient ration and received no cobalt supplement during the course of the experiment. This group was termed **Depleted**. This procedure allowed the exhaustion of cobalt reserves derived from the ewe

and permitted examination of the pathogenesis of *Ostertagia circumcincta* infection in lambs of diminishing cobalt status.

The third main status group, **Sufficient**, came from cobalt sufficient ewes and received a weekly oral dose of 7 mg cobalt (as $CoSO_4$) throughout the experiment which allowed this group to act as a control for the depleted and deficient lambs.

The three main cobalt status groups were then sub-divided into **Infected** and **Non-infected** sub-groups, to allow direct comparison between status and infection in related groups and to allow suitable analysis.

Additionally, within the original constraints of maternal cobalt status the lambs were stratified according to age, sex and weight and randomly allocated to the six experimental groups shown in Table 2. Normal animal husbandry procedures were employed at all times during this experiment including vaccination against *Clostridial* infection as detailed in Chapter 2.

3.2.2 Diet and Ration Formulation

All lambs were fed a ration deficient in cobalt (< 0.05 mg cobalt per kg (DM) which consisted of Timothy hay (0.07 ± 0.006 mg cobalt per kg (DM)), micronised (flaked) maize (0.01 ± 0.001 mg cobalt per kg (DM) and prairie meal (0.06 ± 0.0015 mg cobalt per kg (DM)).

Hay and water were offered *ad libitum* to all animals during the experiment. At the start of the experiment the lambs remained

unweaned but were gradually introduced to the cobalt deficient 'creep' ration described above. Rations were formulated using ARC (1980) guidelines to ensure suitable protein and energy content to achieve a daily liveweight gain of 0.1 kg. Adequate minerals were also added to the diet where appropriate consisting of salt, calcium and phosphate at appropriate rates.

Regular representative samples of dietary constituents were submitted to The West of Scotland College's Analytical Services Unit for trace mineral, protein and energy evaluations (Table I, Appendix I). Rations were adjusted as necessary to ensure adequate protein and energy provision for maintenance and production.

3.2.3 Experimental Parameters

Lambs were 9-12 weeks old at the start of the experiment. Infection consisted of 6000 L3 *Ostertagia circumcincta* per week administered at 3 x 2000 L3 oral doses given on alternate days to simulate field conditions. This dosage was calculated on the basis of a 15 kg lamb consuming 3.5 kg herbage per day (wet weight) containing approximately 300 *Ostertagia circumcincta* L3 per kg grass (wet weight) (Armour, personal communication).

The experiment continued for 16 weeks after initial infection after which time a representative sample of lambs from appropriate groups were sacrificed for total worm counts. These details are summarised in Table 3.

TABLE 3: Summary of Experimental Parameters

Date of First Infection	:	24 June 1987 (Day 0)
Age of Lambs	:	9-12 weeks
Duration of Experiment	:	16 weeks
Infective Dose	:	3 x 2000 L3 *O. circumcincta* per week
Date of Sacrifice	:	12 October 1987
Cobalt Supplement	:	7 mg Co (as $CoSO_4$) per week

3.2.4 Monitoring Procedures

Lambs in this experiment were penned for sample collection and administration of larvae three times per week. Liveweight was measured fortnightly and fresh faecal samples taken from the rectum weekly for the assessment of worm egg counts (WEC's). Blood samples were taken on a weekly basis and used for monitoring procedures such as Vitamin B_{12}, serum MMA and other determinations described in Table 4.

Neutrophil function tests (NFT) and Nitroblue Tetrazolium Reduction (NBT) assay were determined alternatively on a weekly basis using blood samples taken outwith the main blood sampling dates. Haematological differential white cell blood films and PCV determinations were carried out on samples taken for white cell function tests mentioned above. Table 4 summarises the monitoring procedures carried out during this experiment.

TABLE 4: Experimental Parameters in Experiment 1 (Sampling Frequency)

Biochemistry	:	Vitamin B_{12} (weekly)
		Serum Methylmalonic Acid (6 times during experiment)
		Vitamin E (periodically)
		Glutathione Peroxidase (periodically)
		Serum Protein and Albumin (weekly)
Clinical Pathology	:	Post mortem examination Histopathology Neuropathology SGOT, BUN, NEFA determination Spectroscopic Liver Analysis
Production	:	Liveweight (fortnightly)
Parasitology	:	Plasma Pepsinogen (weekly)
		Worm Egg Count (weekly)
		Total Worm Count (at date of sacrifice)
Haematology	:	Differential White Cell Count (weekly)
		Packed Cell Volume Determination (weekly)
Immunity	:	White Cell Function Tests - Neutrophil Function Test (alternate weeks) - NBT Reduction (alternate weeks)
		ELISA - *O. circumcincta* Antigen (fortnightly) - *C. tetani* Antitoxin (2 weeks post-vaccination)

3.3 RESULTS

3.3.1 Biochemistry

Vitamin B_{12}:

Accepted criteria defining cobalt status and indicating deficiency levels are listed in Table 5.

TABLE 5: Assessment of Cobalt Status in Sheep

STATUS	SERUM VITAMIN B_{12} (ng/l)	LIVER COBALT (mg/kg) <3 mths	LIVER COBALT (mg/kg) >3 mths	LIVER VITAMIN B_{12} (µg/g) (wet weight)	
Deficient	<200	0.04	0.06	very low	<0.07
Marginally Deficient	200-400			low	0.07-0.10
Adequate	>400	>0.08	>0.10	border	0.11-0.19
				normal	>0.19

(After Paterson, 1988)

In this experiment Vitamin B_{12} was used as the main criterion indicating cobalt status in lambs. Only on two isolated occasions, 98 and 119 days post infection, did infected sub-groups have significantly lower serum Vitamin B_{12} concentrations than their non-infected equivalents in depleted and sufficient groups ($P < 0.05$) and consequently mean Vitamin B_{12} levels incorporating both infected and non-infected sub-groups were used to determine Vitamin B_{12} levels in each of the three cobalt status groups.

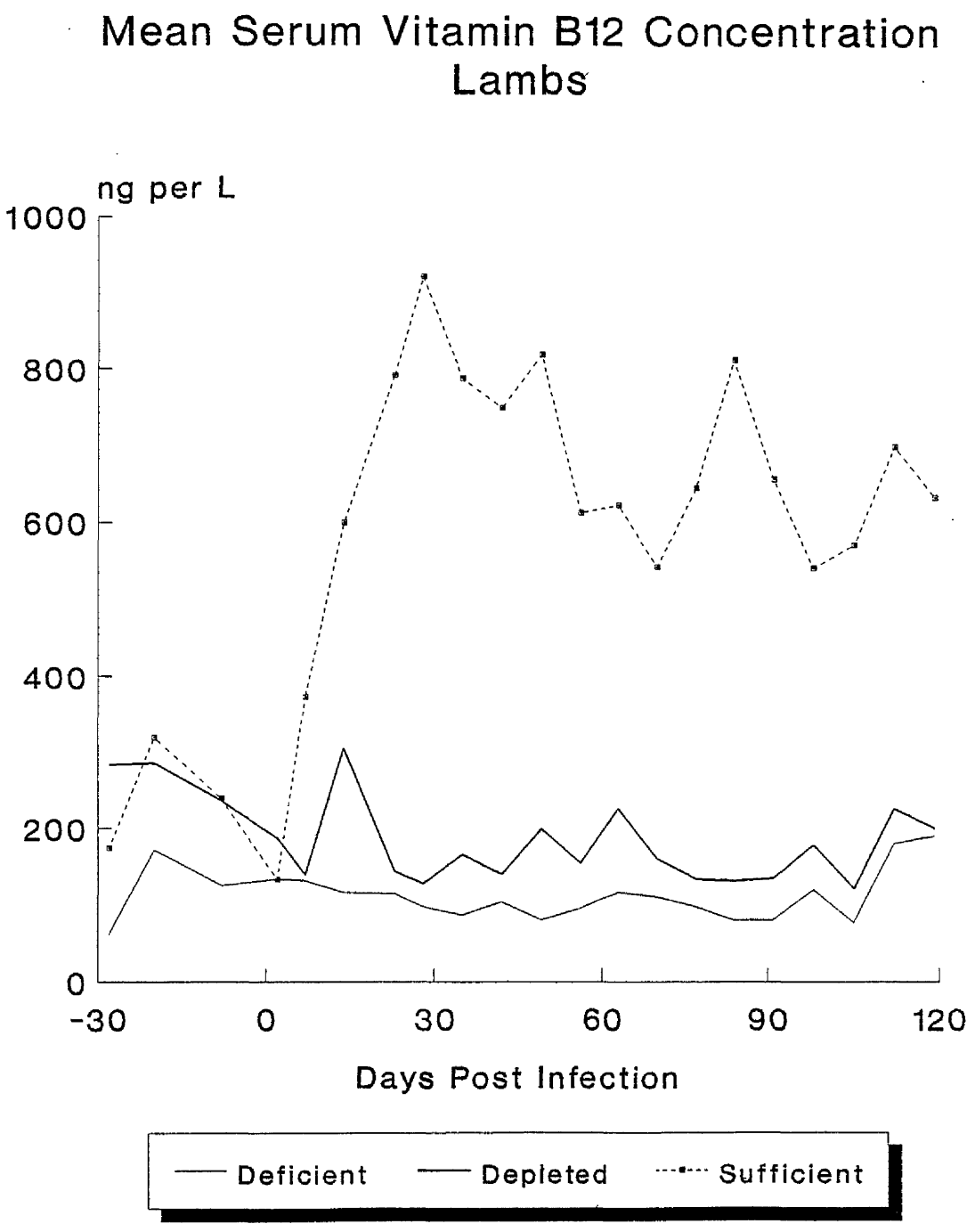
These values are tabulated in Table II, Appendix I, and are illustrated in Figure 7.

Statistical analysis showed no significant difference in serum Vitamin B_{12} levels between depleted and sufficient status groups before day 0. Levels at that time were lower than the 400 ng/L threshold defining deficiency in all groups and less than 200 ng/L in the deficient groups.

Mean serum Vitamin B_{12} concentrations of deficient lambs, groups 1 and 2, did not exceed 200 ng/L throughout the experiment. Sufficient lambs, groups 5 and 6, however, consistently maintained serum Vitamin B_{12} levels in excess of 400 ng/L throughout the period of infection. Supplementation of the sufficient group after day 7 resulted in serum Vitamin B_{12} levels significantly higher than non-supplemented (deficient and depleted) controls ($P < 0.001$).

Depleted lambs, groups 3 and 4, showed low serum Vitamin B_{12} levels (< 400 ng/L) during the pre-infection phase which were similar to those of sufficient lambs, but in the case of depleted lambs, failure to supplement after day 0 resulted in a gradual fall to levels in the deficient area (< 200 ng/L). Vitamin B_{12} levels in depleted lambs during the period of infection were greater than those found in deficient groups 1 and 2, although not significantly so.

FIGURE 7


Mean Serum Vitamin B12 Concentration
Lambs
ng per L
1000
800
600
400
200
0
-30
0
30
60
90
120
Days Post Infection
Deficient
Depleted
Sufficient

Methylmalonic acid concentrations:

As discussed previously, elevated methylmalonic acid (MMA) levels in serum resulting from the failure of methylmalonyl CoA mutase to convert MMA to succinyl CoA in hepatocytes has been used as an indicator of cobalt status. Serum MMA values used to predict cobalt status are listed in Table 6. In this experiment, although cobalt status was measured primarily by serum Vitamin B_{12} levels, MMA level was used as a secondary indicator to confirm cobalt status as illustrated in Vitamin B_{12} trends.

TABLE 6: MMA Concentration and Cobalt Status

COBALT STATUS	MMA VALUE (μMol/L)
Deficient	> 15
Marginally Deficient	5 - 15
Adequate	< 5

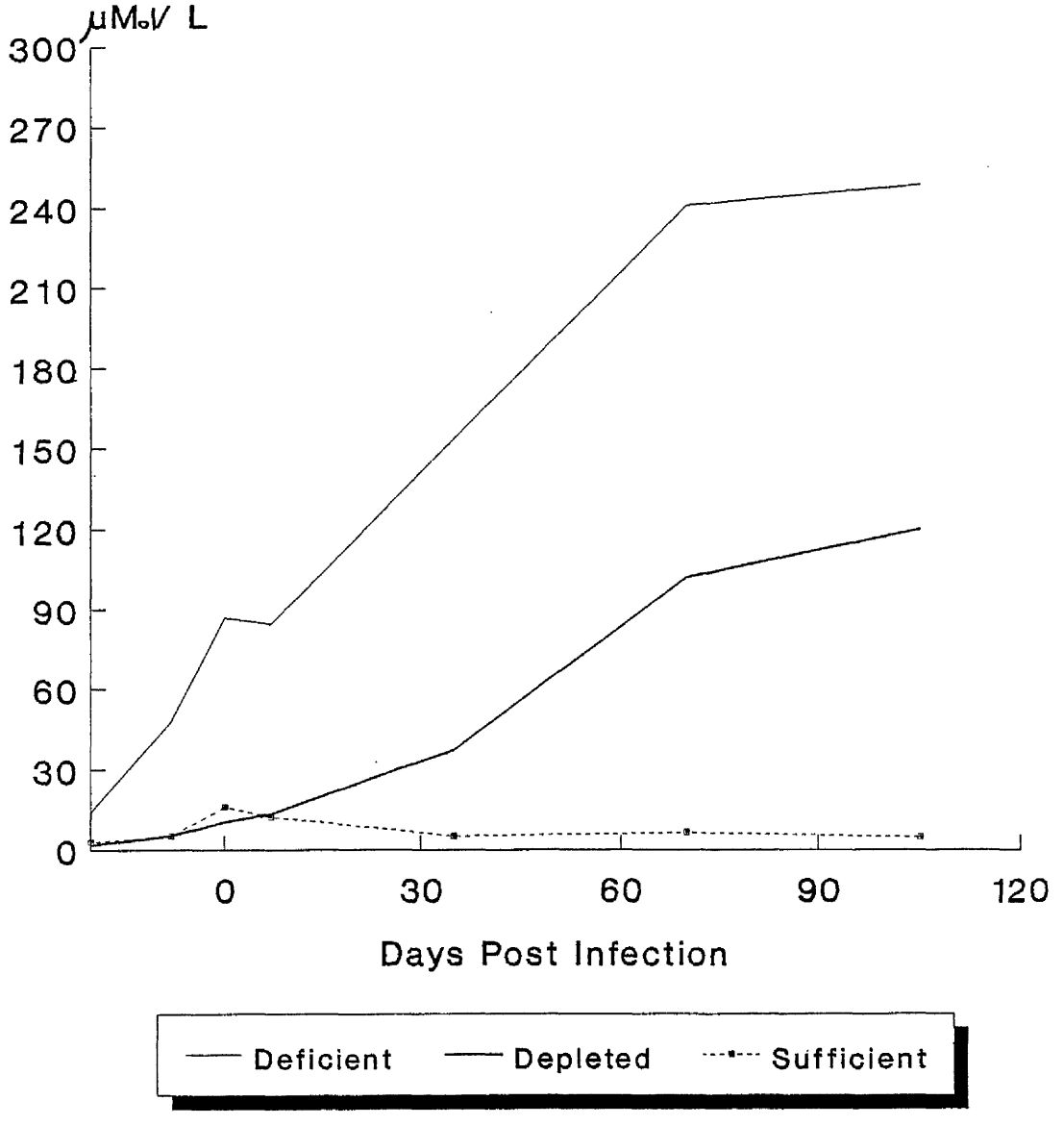
(MacPherson, personal communication)

MMA results in the present study, displayed trends in cobalt status essentially similar to Vitamin B_{12} levels examined in the previous section, ie no significant differences were found between infected and non-infected sub-groups of the same cobalt status and thus values used to construct Figure 8 (and listed in Table III, Appendix I) represent mean values from infected and non-infected sub-groups of each cobalt status.

Mean pre-infection serum MMA values were similar for both depleted, (groups 3 and 4) and sufficient (groups 5 and 6) lambs (1.83

FIGURE 8

Mean Serum MMA Concentration Lambs



and 2.86 μMol/L, respectively) but significantly higher in deficient animals, ($P < 0.01$) at 14.1 μMol/L, a value which approaches the upper limit defining marginal cobalt deficiency.

Mean serum MMA levels in depleted and sufficient lambs rose from the normal zone (< 5 μMol/L) to the marginally deficient zone (5-15 μMol/L) during the pre-infection stage until day 0, the start of infection, thereafter cobalt supplementation returned the sufficient groups (groups 5 and 6) to the normal range (< 5 μMol/L) where it remained for the duration of the experiment.

Deficient lambs, groups 1 and 2, had significantly greater serum MMA levels than either the depleted or sufficient lambs for the entire experiment ($P < 0.01$). Values for depleted lambs, groups 3 and 4, approximated to those of the sufficient group in the pre-infection period but as was found with serum Vitamin B_{12}, failure to supplement depleted lambs lowered their cobalt status and resulted in gradual elevation of serum MMA values to levels significantly greater than sufficient controls, ($P < 0.01$) and to a mean level exceeding 30 μMol/L by 35 days post infection.

Selenium and Vitamin E status:

Regular measurement of glutathione peroxidase (GSH-Px) activity demonstrated values consistently in excess of 100 U/ml, considerably greater than the level defining deficiency, ie 27 U/ml which indicates maintenance of normal selenium status. Samples assayed for Vitamin E determination showed levels greater than 1.0 μMol/L, the deficiency threshold, in all animals indicating

adequate status. Results are listed in table IV and V, Appendix I for mean GSH-Px and Vitamin E levels respectively.

Serum protein and albumin levels:

Serum albumin levels of animals in this experiment revealed mean values as listed in Table VI, Appendix I. No significant difference was detected between infected and non-infected sub-groups of the same cobalt status and the mean serum albumin levels which ranged between 27.4 and 38.2 g/L were close to the normal value, 33 g/L, and usually within the normal range, 23-34 g/L. Additionally, no significant difference in mean serum albumin levels was detected between the three cobalt status groups in this experiment.

Mean serum total protein levels from animals in this experiment are listed in Table VII, Appendix I and as with findings in serum albumin determination and with a single exception 98 days PI, no significant difference was noted between infected and non-infected sub-groups of the same cobalt status. No significant difference in serum total protein was detected between the three main status groups. As Table VII, Appendix I shows, all mean serum total protein levels were in the range 53.1-74.4 g/L, within the range regarded as normal for sheep, 60-75 g/L. From the results detailed above it is evident that total serum globulin levels (calculated by subtracting serum albumin from serum total protein), were also not significantly different either between infected and non-infected sub-groups of the same cobalt status or among the three cobalt status groups.

3.3.2 Clinical Findings

Deficient lambs suffered very high casualty rates as 15 of the original 17 animals succumbed to a number of diseases discussed below. This compared unfavourably with mortality in depleted lambs where 4 died from 21 starting the experiment. However all 22 sufficient lambs survived the duration of the experiment. These findings are summarised in Table 7 and full pathological data detailing time of death, requirement for euthanasia and a summary of main findings are listed in Table VIII, Appendix I.

TABLE 7: Summary of Mortality Rates - Experiment 1

GROUP NO.	NO. STARTING EXPT.	NO. FINISHING EXPT.	% MORTALITY
1	9	1	88.9***
2	8	1	87.5***
3	10	8	20.0
4	11	9	18.2
5	11	11	0
6	11	11	0

*** = $P < 0.001$ (Chi-Squared Analysis)

Statistical analysis using Chi-squared test (corrected for continuity) revealed a significantly higher mortality rate among deficient lambs. When compared to sufficient controls but mortality rate was unaffected by infection ($P < 0.001$). Mortality rate in depleted lambs was not significantly different from sufficient controls although considerably higher.

A number of post mortem findings were common to deficient and depleted lambs regardless of cause of death. These included 'poorness', emaciation, loss of weight, Ill-thrift, muscular weakness and incoordination. Causes of death in deficient and depleted lambs included hepatic degeneration, nephrosis, pneumonia, pasteurellosis and enterotoxaemia.

Hepatic degeneration:

All casualty lambs from the deficient group demonstrated some degree of hepatic degeneration either grossly or after histopathological examination. Livers of affected animals were pale or bronze in colour and usually demonstrated visible fatty change.

Histopathological examination revealed severe, extensive centrilobular fatty degeneration and necrosis in sections of liver from deficient lambs. Additionally, some deficient animals demonstrated proliferative changes in small bile ducts. Two casualty lambs from the depleted group showed similar gross and histopathological findings.

Biochemical analysis of blood samples taken from a number of animals immediately before euthanasia indicated a degree of hepatic dysfunction as measured by serum glutamic oxaloacetic transaminase (SGOT) activity. Normal levels, < 60 iu/L, were greatly exceeded and occasionally values in excess of 200 iu/L were recorded in affected lambs.

Spectroscopic analysis of liver cobalt status revealed very low levels, < 0.01 mg cobalt/kg, in most casualties although one liver sample from a depleted casualty showed 0.04 mg cobalt/kg. Liver copper levels in all animals were normal.

Neuropathology:

Analysis of fixed brains, spinal cords and eyes of four deficient and two depleted lambs revealed vacuolation resembling that described in hepatic encephalopathy in sheep. In one deficient lamb extensive vacuolation was demonstrated in the white matter and in the cerebellum of the brain. Vacuolation in areas of the spinal cord was also demonstrated in the same lamb. The remaining five lambs showed varying but lesser degrees of spongy transformation of parts of the central nervous system. Analysis of the central nervous system (CNS) of three further deficient lambs revealed varying degrees of vacuolation in both brain and spinal cord, although they were not so severely affected.

Examination of the CNS of two casualty lambs from the depleted groups, groups 3 and 4, showed similar though lesser spongy changes in the cerebellar white matter to that described in deficient lambs. Examination of fixed eyes from the same animals failed to reveal significant pathology in either deficient or depleted casualty animals.

Nephrosis:

In casualties from the deficient groups varying degrees of renal change were noted. Usually this resulted in pale swollen kidneys

which also appeared fatty and were occasionally friable and bronze in colour. One lamb in the deficient group showed sub-capsular haemorrhage in the renal cortex while a second had severe extensive nephrosis affecting the proximal convoluted tubules.

Blood urea nitrogen (BUN) levels in samples taken from deficient casualties prior to euthanasia were grossly elevated often exceeding twice the normal level of 6.6 mMol/L.

Among depleted casualty lambs, two were found to have sub-capsular haemorrhages in the renal cortex while renal histopathology from a third lamb revealed marked proximal tubular nephrosis.

Pneumonia:

Pneumonia was found in seven deficient casualty lambs. In two cases pneumonic areas of the apical lobes in both lungs were noted in addition to small areas of pneumonia and/or collapse in the cardiac lobes. Two further lambs had both pneumonia and septiciaemia, one of which had a lung abscess. *Pasteurella haemolytica* (Serotype A6) was isolated from four of seven affected lambs all of which received antibiotic treatment. Histopathological examination confirmed Pastuerella pneumonia in affected lambs.

One casualty lamb only from the depleted group was found to have organised areas of pneumonia affecting the right apical and cardiac lobes from which *P. haemolytica* was isolated.

Enterotoxaemia:

A deficient lamb which died suddenly in relatively good condition 83 days post-infection, had post mortem changes consistent with Pulpy Kidney Disease. Clostridial toxin was demonstrated in bowel filtrate and *Clostridium perfringens* (Type D) was isolated from the small intestine. The kidneys appeared swollen, soft and pale. Autolysis of the whole carcass was more rapid than that found in other casualties.

Miscellaneous:

Two deficient lambs were found to have some degree of ruminal acidosis (pH < 5.5) and associated abomasitis. One lamb suffered abomasal rupture and associated areas of enteritis caused by over-eating. Scour was found in another two deficient lambs which had associated enteritis in one case and abnormal gut flora in the other suggesting that the scour in the latter case was dietary in origin. A further deficient lamb was found dead after having haemorrhaged into the abdominal cavity via a number of tears in the liver capsule almost certainly due to trauma.

Two depleted lambs also had evidence of ruminal acidosis (pH < 5.5) and associated changes as described above in the deficient group. Additionally, one depleted lamb died as a result of haemorrhaging into the abdominal cavity via a tear in the liver capsule.

Parasitological findings in casualty animals:

Parasitological examination of five casualty lambs showed the following changes in post mortem. Gross examination demonstrated varying degrees of parasitic abomasitis in deficient lambs with areas of coalescent nodules resulting in a 'Morocco Leather' effect and lesions characteristic of *Ostertagia circumcincta* infection. Histopathological examination indicated changes in all infected casualties consisting of eosinophil infiltration of the abomasal epithelium and sub-mucosa. In a number of cases larvae were evident on the epthelial surface or in dilated glands. Details of worm burdens in casualty animals are shown in Table 8.

TABLE 8: Total Worm Counts From Infected Casualty Animals - Experiment 1

GROUP NO.	STATUS	LARVAE GIVEN	TIME OF DEATH (Days Post-Infection)	TOTAL WORM COUNT L4 + L5	Adults	Total
1	Def.	38000	44	2650	3600	6350
1	Def.	48000	55	2450	8200	10650
1	Def.	60000	68	2800	11499	14299
1	Def.	96000	109	1563	1825	3388
3	Depl.	60000	70	2413	15838	18251

3.3.3 Production Effects

No significant difference in liveweight was detected between infected and non-infected sub-groups of the same cobalt status, therefore mean liveweights for each of the status groups were

used to construct Figure 9 and are also listed in Table IX, Appendix I.

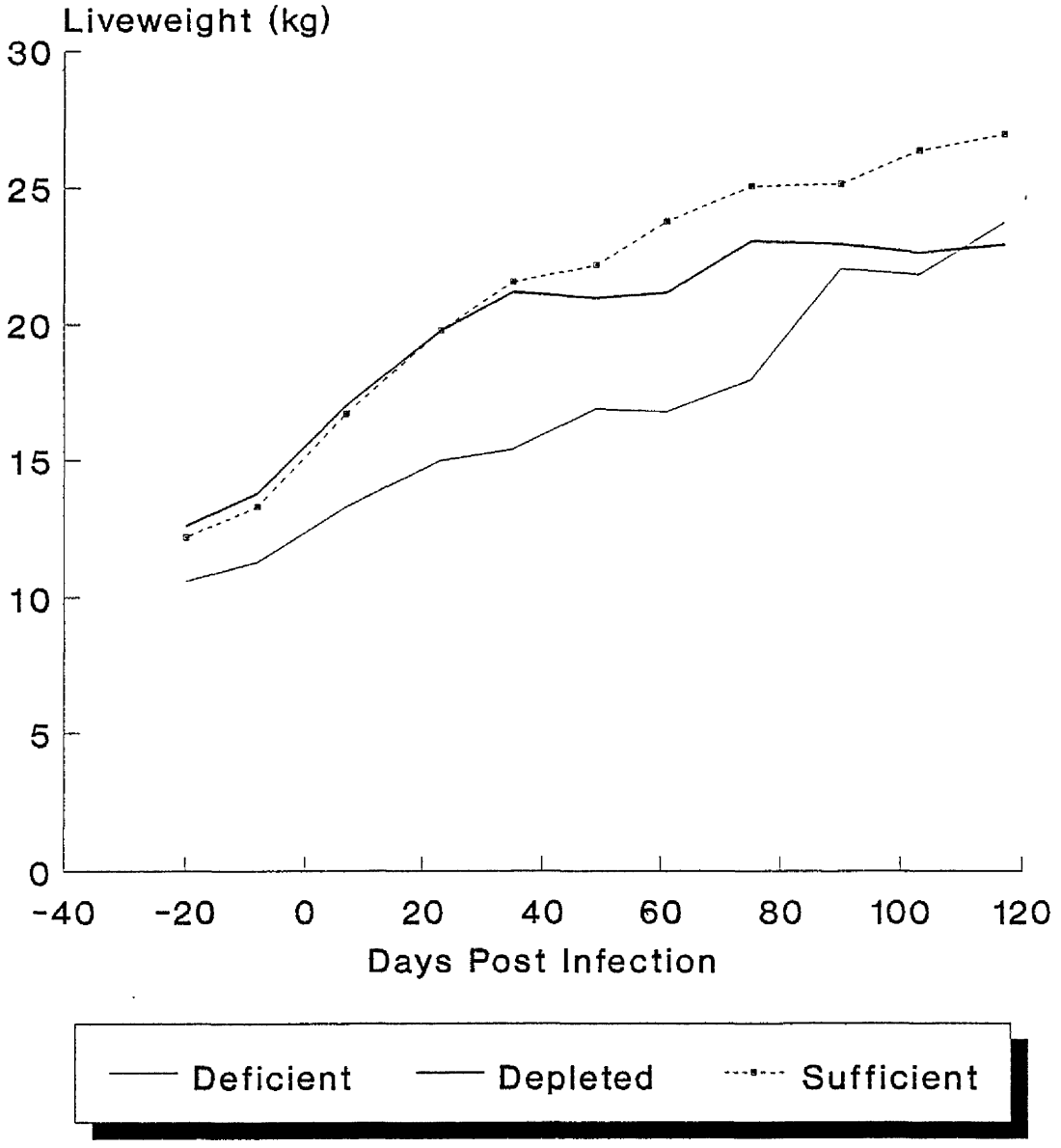
The liveweights of animals from all three status groups, were not significantly different at the start of the experiment and were in the range 10.6-12.6 kg. Dietary formulation and adjustment resulted in regular weight gain at the desired rate for the duration of the experiment in the sufficient groups, 5 and 6 (Figure 9).

Although no significant difference between status groups was detected in the pre-infection phase, the administration of a cobalt supplement to the sufficient groups from day 0 resulted in significantly increased liveweights ($P < 0.05$) by 7 days post-infection compared with deficient lambs. The weight difference between deficient and sufficient groups became increasingly significant ($P < 0.01$) as the experiment progressed until 90 days post-infection, when the high mortality rate among deficient lambs prevented further meaningful comparison.

By 7 days post-infection depleted lambs, groups 3 and 4, were also significantly heavier than deficient controls ($P < 0.05$) and the depleted group matched the performance of the sufficient group (groups 5 and 6), until 49 days post-infection when growth rate slowed. No significant difference between depleted and sufficient lambs was detected at 49 days post-infection but the poor weight gain of the depleted group thereafter which became frank weight loss by the end of the experiment, resulted in a

FIGURE 9

Mean Liveweight Lambs



significant difference ($P < 0.05$) by 105 days post-infection between these groups.

The findings illustrated in Figure 9 were further emphasised in Plate 1, which shows a rear view of deficient, depleted and sufficient lambs at the feeding trough. Deficient lambs (first and fourth from left), were considerably smaller than their sufficient counterparts (last three from right). The effects of chronic cobalt deficiency on depleted lambs (second and third from left), which were indistinguishable from deficient counterparts are discussed below.

Plate 1 demonstrates the maximum difference between the best sufficient lambs and the poorest deficient lambs. Plate 2 shows two lambs born on the same day, the lamb on the left to a cobalt sufficient, the other to a deficient ewe.

3.3.4 Parasitology

Faecal and blood samples for worm egg count (WEC) and plasma pepsinogen determination respectively, were taken every week from infected animals, groups 1, 3 and 5, but non-infected lambs were only sampled periodically to confirm their helminth-free status.

Worm egg counts:

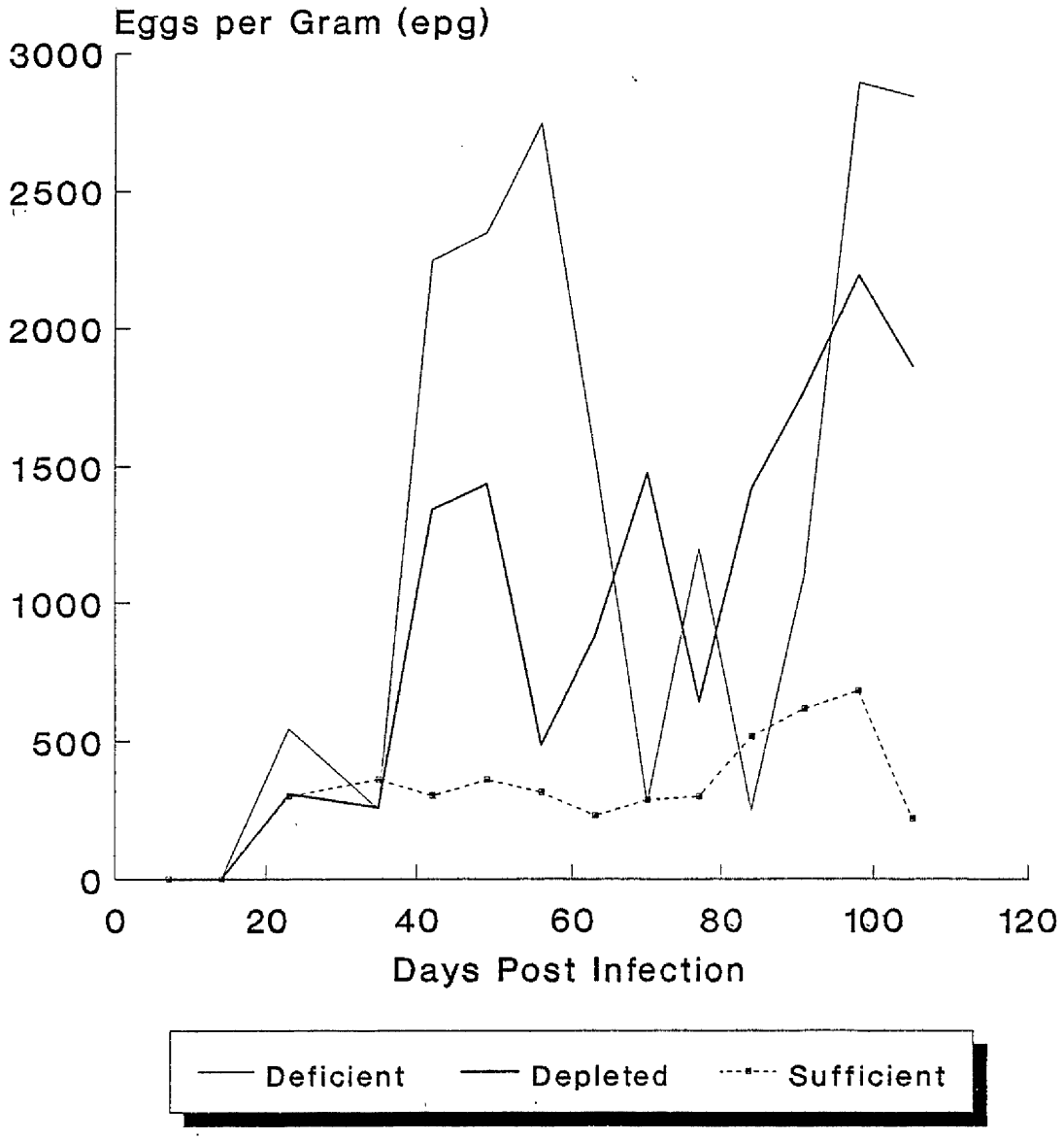
Mean worm egg counts for the three infected status groups, deficient, depleted and sufficient, are listed in Table X, Appendix I and illustrated in Figure 10. No difference was evident in time taken to patency among the groups, ie the time between first

PLATE 1: Deficient Depleted and Sufficient Lambs at the Feeding Trough (from left to right)

PLATE 2: The Effect of Chronic Cobalt Deficient in Lambs of the Same Age

FIGURE 10

Mean Worm Egg Counts Infected Lambs



administration of infective larvae and the appearance of strongyle eggs in faecal samples was not significantly different in any group of lambs.

Faeces from sufficient lambs, group 5, always had less than 700 eggs per gram (epg) for the duration of the experiment with a single exception 98 days post-infection (PI). Analysis of variance revealed that despite similar WEC's around the time of patency and in the early stages of the experiment, sufficient lambs had significantly lower WEC's ($P < 0.05$) from 63 days PI until the end of the experiment than either of deficient or depleted lambs, groups 1 and 3 respectively, with 2 isolated exceptions 70 and 84 days PI.

Deficient lambs showed very variable WEC's which on a weekly basis ranged from 500 epg at patency and reached a peak of 2500 epg, 56 days PI before falling to less than 500 epg around 70 days PI and rising thereafter. Limited emphasis could therefore be attached to WEC's found in the deficient groups due to that variability. The significant difference of early WEC's however remained until the end of the experiment despite the high mortality rate in the deficient group.

Worm egg counts in depleted, group 3, lambs were similar to those found in deficient lambs. After patency, WEC's similar to those found in both other groups were also found in depleted lambs, ie < 500 epg. However, in contrast to the deficient group, counts for depleted lambs remained at similar levels to the sufficient group until 35 days PI. Mean worm egg counts thereafter rose to

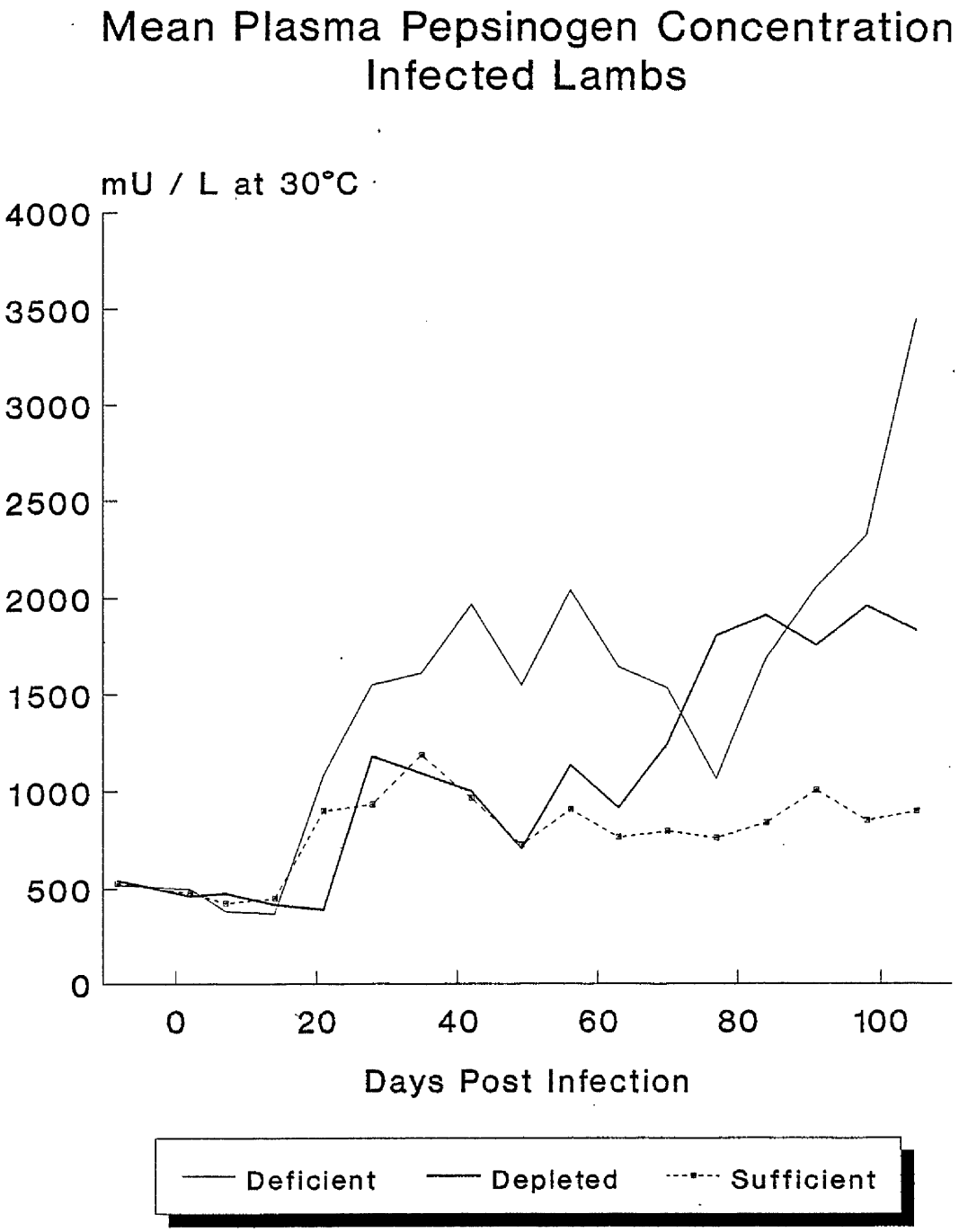
1300 epg by 49 days PI before falling to 500 epg by 56 days PI, before rising again. WEC's of the depleted group were significantly greater than deficient lambs 56 days PI ($P < 0.05$).

Plasma pepsinogen:

Mean plasma pepsinogen levels from non-infected lambs never exceeded 400 mu/l on any of the sample dates and as such indicated absence of infection when compared to experimentally infected equivalent animals. Similarly, no strongyle eggs were detected in faecal samples taken from non-infected control animals during any of the periodic sampling dates.

Pre-infection plasma pepsinogen levels and levels immediately prior to patency in all three status groups did not differ significantly (around 500 mU/L) but thereafter the expected rise in plasma pepsinogen associated with infection was demonstrated as Figure 11 shows. Statistical analysis of data whose mean values and standard errors are listed in Table XI, Appendix I, demonstrated significant elevation of plasma pepsinogen values of infected animals when compared to non-infected controls from 21 days PI ($P < 0.01$). Similar to that demonstrated in the previous section examining WEC, all three status groups showed similar plasma pepsinogen levels during, and in the first part of patency but both depleted, group 3, and sufficient, group 5, lambs remained around or below 1000 mU/L until 63 days PI while deficient lambs, group 1, rose to 1500 mU/L by 28 days PI and with one exception (77 days PI) remained in excess of those levels for the remainder of the experimental period. The difference between the deficient groups and the others was

FIGURE 11


Mean Plasma Pepsinogen Concentration
Infected Lambs
mU / L at 30°C
4000
3500
3000
2500
2000
1500
1000
500
0
0
20
40
60
80
100
Days Post Infection
Deficient
Depleted
Sufficient

significant by 28 days PI ($P < 0.01$) and remained so for the remainder of the experiment although the high mortality among deficient lambs prevented meaningful comparison with that in the latter part of the experiment.

After the initial rise to 1000 mU/L demonstrated by both the depleted and sufficient groups, sufficient lambs settled to a plasma pepsinogen level in the range 750-850 mU/L, where it remained for the rest of the experiment.

Interestingly, in the depleted group plasma pepsinogen trends were similar to those found for WEC's, whereby depleted lambs followed sufficient controls for the early part of the experiment before rising to levels similar to those found in deficient lambs. Thus, depleted lambs, group 3, remained around 1000 mU/L, a level similar to sufficient lambs, until 63 days PI when levels started to rise and became significantly higher than sufficient lambs ($P < 0.01$) by 77 days PI.

Total worm count (TWC):

High mortality in the deficient group during the experiment prevented meaningful comparison of TWC between this group and animals sacrificed 16 weeks PI. Details of worm burden in deficient infected casualties are recorded in Table 8. However, at the end of Experiment 1, representative samples of infected, depleted and sufficient groups, consisting of four lambs from each, were sacrificed to assess TWC. Worm burdens found in depleted and deficient groups are listed in Table 9. Mean total worm count (± SE) in depleted lambs was 32112 ± 632, which was

significantly higher ($P < 0.005$) than sufficient lambs with mean burdens of 10988 ± 2830.

TABLE 9: Total Worm Burdens Post Mortem

	TOTAL WORM COUNT				
GROUP NO.	ADULTS	L5	L4	TOTAL	MEAN (SE)
3	25350	4050	3250	32650	
3	25450	3200	2800	31450	
3	22800	4650	3300	30750	32112*** (632)
3	25250	5650	2700	33600	
5	11650	3100	3200	17950	
5	7400	2900	2800	13100	
5	1750	1800	1900	5450	10988 (2830)
5	3950	1500	2000	7450	

*** = $P < 0.005$ (Students T-test)

Although no significant difference in total numbers of developing larvae was noted in Table 9, expression of those counts as percentages of total worm burden, as in Table 10, revealed that a significantly greater percentage of the burden in depleted lambs were adult parasites compared to sufficient lambs ($P < 0.05$) and similarly that sufficient lambs had significantly greater proportions of developing stages than depleted equivalents ($P < 0.05$). In both depleted and sufficient groups however, there were significantly more adults than larvae, ($P < 0.01$). Worm egg counts carried out on faeces taken from sacrificed animals at post mortem examination are listed in Table 11 and showed significantly elevated ($P < 0.05$) counts in depleted lambs.

TABLE 10: Worm Counts as Percentages of Total Burden - Experiment 1

GROUP NO.	STATUS	% OF TOTAL WORM COUNT		
		ADULTS	L5	L4
3	Depleted	77.6	12.4	9.9
3	Depleted	80.9	10.2	8.9
3	Depleted	74.1	15.1	10.7
3	Depleted	75.1	16.8	8.0
MEAN (SE)		76.93^{a} (1.52)	13.63^{c} (1.46)	9.38^{c} (0.59)
5	Sufficient	64.9	17.2	17.8
5	Sufficient	56.5	22.1	21.4
5	Sufficient	32.1	33.0	34.9
5	Sufficient	53.0	20.1	26.8
MEAN (SE)		51.63^{b} (6.97)	23.10^{d} (3.45)	25.23^{d} (3.72)

Values with differing superscripts differed significantly ($P < 0.05$) or less

TABLE 11: Post Mortem Worm Egg Counts - Experiment 1

LAMB NO.	GROUP NO.	STATUS	WEC	MEAN ± SE
58	3		6450	
43	3	Depleted	2600	3775 ± 910*
91	3		3400	
101	3		2650	
98	5		150	
101	5	Sufficient	50	250 ± 108
50	5		550	
42	5		259	

* = $P < 0.05$ (Student's T-test)

Clinical biochemistry of sacrificed lambs:

Results from biochemical analysis of blood samples taken from sacrificed animals immediately before slaughter to enable BUN, SGOT and Non-Esterified Fatty Acid (NEFA) determination are listed in Table 12.

Blood Urea Nigrogen (BUN) levels in both groups were within normal limits and did not differ significantly from each other. SGOT levels also did not differ significantly but the results particularly for the depleted group were well above the normal limits. Depleted lambs showed significantly greater NEFA levels ($P < 0.01$) than sufficient equivalents but both levels still remained within the normal range (< 0.7 mequiv/L).

TABLE 12: Biochemistry From Sacrificed Animals - Experiment 1

GROUP NO.	STATUS	BUN ± SE (mM/L)	SGOT ± SE (Iu/L)	NEFA ± SE (Mequiv/L)
NORMAL VALUES:		< 6.6	< 60.0	< 0.7
3	Depleted	4.85 ± 0.64	197.5 ± 56.0	0.625* ± 1.11
5	Sufficient	4.45 ± 1.16	97.5 ± 20.2	0.125 ± 0.025

* = $P < 0.01$

The effect of deficiency on parasite growth:

Fifty male and fifty female adult worms from the abomasal washings from each of the sacrificed animals in this experiment were removed and measured, providing the results detailed in

Table 13. Isolated within group, significant differences in both female and male worm length ($P < 0.05$) were detected from both sufficient and depleted groups. Male worms were however significantly shorter than female equivalents from the same slaughtered animal. Manipulation of whole group data revealed mean worm lengths which are detailed in Table 14.

Analysis of variance of this data revealed no significant differences between female worms taken from depleted and sufficient lambs on a group basis. Mean female worm lengths for both groups fell within the range described previously. Male worms taken from the sufficient group were significantly shorter than those measured from the depleted group ($P < 0.01$). The ratio of female to male worms recovered post mortem from abomasal washings was as described in Table 15.

The percentage of female worms in the sufficient group ranged from 59-72 with a corresponding range of 28-41 for the percentage of males present and resulted in a mean female to male ratio of 66:34 in the abomasal washings taken from that group. Depleted lambs with percentages of females and males in the ranges of 68-72 and 28-32 respectively furnished a mean ratio of 70.5:29.5 which was not significantly different from the mean ratio found among sufficient lambs.

TABLE 13: Individual Mean Male and Female Worm Length - Experiment 1

LAMB NO.	GROUP NO.	STATUS	MEAN FEMALE WORM LENGTH (SE) mm	MEAN MALE WORM LENGTH (SE) mm
58	3	Depleted	10.50^{a} (0.189)	7.90^{c} (0.206)
43	3	Depleted	10.30^{a} (0.286)	6.275^{d} (0.160)
91	3	Depleted	11.14^{a} (0.227)	7.53^{c} (0.216)
101	3	Depleted	11.19^{a} (0.172)	7.41^{c} (0.221)
98	5	Sufficient	10.15^{a} (0.271)	6.775^{d} (0.155)
101	5	Sufficient	11.05^{a} (0.191)	6.725^{d} (0.229)
50	5	Sufficient	9.59^{b} (0.226)	6.00^{d} (0.134)
42	5	Sufficient	11.16^{a} (0.205)	6.99^{d} (0.152)

Values with differing superscripts differed significantly ($P < 0.05$)

TABLE 14: Group Mean Worm Length - Experiment 1

GROUP NO.	STATUS	MEAN FEMALE WORM LENGTH (SE) mm	MEAN MALE WORM LENGTH (SE) mm
3	Depleted	10.79^{a} (0.219)	7.28^{b} (0.200)
5	Sufficient	10.49^{a} (0.223)	6.62^{c} (0.168)

Values with differing superscripts differed significantly ($P < 0.01$)

TABLE 15: Mean Female:Male Ratio - Experiment 1

GROUP NO.	STATUS	RATIO	MEAN RATIO	'P'
3	Depleted	72:28	70.5:29.5	NS
3		68:32		
3		71:29		
3		71:29		
5	Sufficient	70:32	66.0:34.0	NS
5		65:35		
5		72:28		
5		59:41		

3.3.5 Haematology

Packed cell volumes:

Haematological examination of whole blood taken from a respresentative fraction of groups 1-6 was performed weekly and revealed the following results. Mean haematocrit packed cell volumes (PCV) for each group are detailed in Table XII, Appendix I.

Normal haematocrit PCV values for sheep were found in the range 22-40%. Table XII, Appendix I, illustrates that PCV determination on blood from animals in all groups in this experiment were at the lower end of the normal range. No significant difference was detected between infected and non-infected sub-groups in deficient lambs (groups 1 and 2), whose mean PCV values were in the range 23.5-31.3.

Table XII also demonstrates that, with the exception of an isolated incident 107 days PI ($P < 0.05$), no significant difference between infected and non-infected depleted sub-groups was found. Similar to that reported in the deficient group, PCV values for depleted lambs (groups 3 and 4) were in the lower part of the normal range of values for sheep ranging from 21.0-31.3%.

Sufficient lambs of infected and non-infected sub-groups (groups 5 and 6) demonstrated PCV values which were not significantly different from each other, except on an isolated date 65 days PI ($P < 0.05$), ie no anaemia attributable to infection was found in this or either of the two previous groups. Mean PCV values in the range 23.5-30.5, also in the lower part of the normal range were recorded in the sufficient group.

Examination of the PCV values between groups using analysis of variance demonstrated that levels of deficient lambs were significantly lower than sufficient lambs on three occasions, 14, 35 and 72 days PI. PCV values for deficient lambs were also significantly lower than depleted equivalents on an isolated occasion, 35 days PI. Comparison of PCV values between depleted lambs (groups 3 and 4), and sufficient counterparts (groups 5 and 6), revealed two isolated significant differences. Firstly, 65 days PI where the mean PCV for group 6 lambs (supplemented, non-infected), was significantly greater than any of the remaining five groups, and secondly, 107 days PI, when group 3 (depleted, infected) had a mean PCV value significantly greater than the three other groups assayed on that date.

Differential white cell count:

Differential white cell count carried out in this experiment revealed mean ranges of relative proportions listed in Tables XIII-XVIII, Appendix I and which can be compared to values found in normal ovine blood samples, listed in Table 16.

TABLE 16: Normal Differential White Cell Counts in Ovine Blood

CELL TYPE	PERCENTAGE OCCURRENCE
Neutrophils	23 - 35
Lymphocytes	40 - 75
Monocytes	2 - 16
Eosinophils	1 - 8
Basophils	0 - 1

Figures 12 and 13 illustrate the mean differential count found in groups 1 and 2, infected and non-infected deficient sub-groups respectively. Data was obtained on relatively fewer occasions than for sufficient controls because of the high mortality experienced in the deficient group.

Figure 12 revealed mean percentage neutrophils, lymphocytes and monocytes in the ranges 22.5-30.5, 34-54.75 and 6-13.75% respectively. Percentage eosinophils rose from 9%, 14 days PI to 23% by 21 days PI and remained in the range 17-33.25% for the remainder of the experiment. Percentage basophils reached a peak of

FIGURE 12

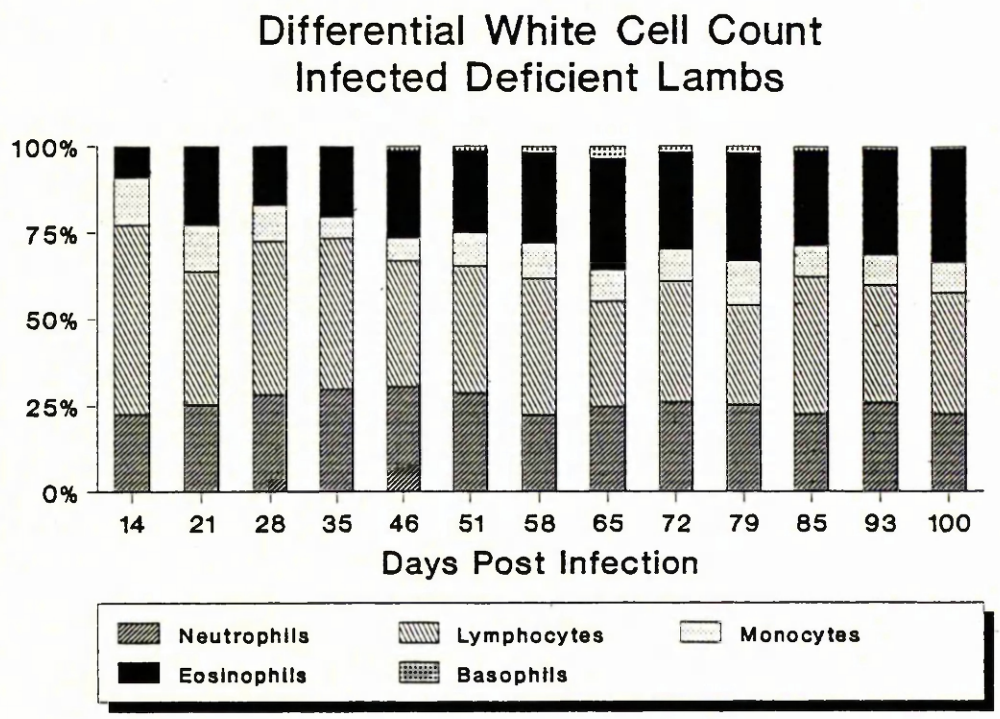
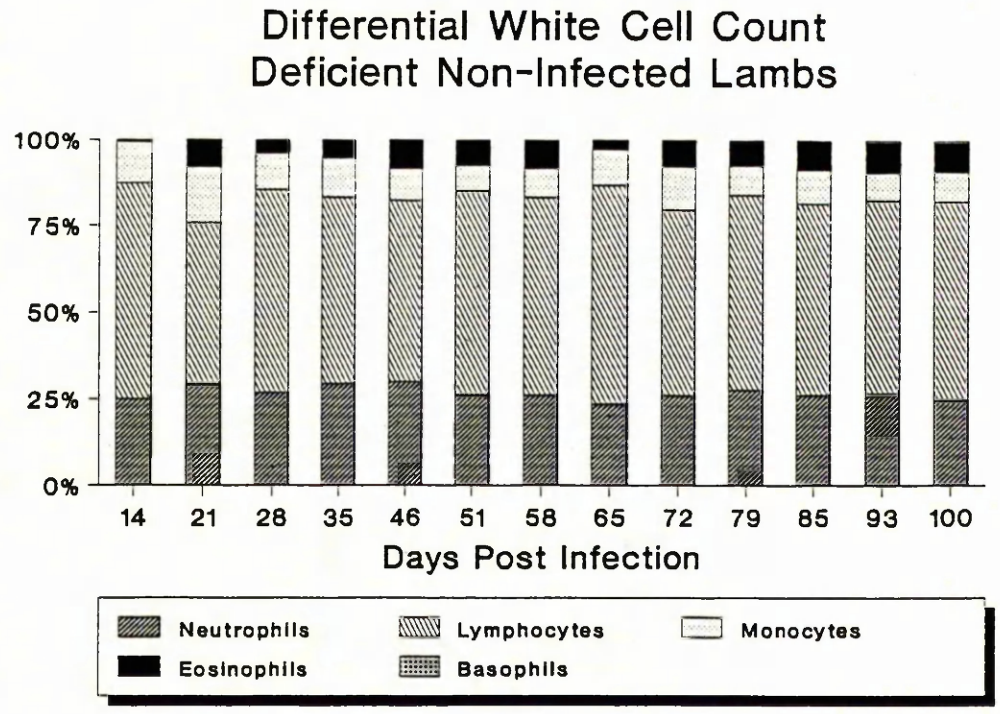


FIGURE 13



3.5% and although the frequency remained low, occurrence remained greater than in non-infected controls.

Figure 13 for group 2, deficient non-infected, revealed normal haematological ratios throughout the experiment. Mean percentage neutrophils was in the range 23.5-30% and mean percentage lymphocytes, and monocytes demonstrated similar normal values throughout the experiment and in the ranges 46.5-63.25, 7.25-16.25 and 0.9-9% respectively. Basophils were seldom found in blood smears prepared from animals in this group.

Figures 14 and 15 show the mean differential white cell counts for infected and non-infected depleted lambs, groups 3 and 4, on fewer occasions than the remaining four groups due to the nature of sampling frequency. Non-infected lambs, group 4, demonstrated white cell ratios within normal limits and in the ranges 29-31.5, 49.5-54, 8.25-12.5 and 3-11% for mean percentage neutrophils, lymphocytes, monocytes and eosinophils respectively. Very few basophils were noted in blood films from this group.

Infected depleted lambs, group 3, demonstrated mean percentage neutrophils and monocytes similar to non-infected equivalents and in the ranges 24.5-28.5 and 7-10.5% respectively. On all sample dates, this group demonstrated percentage eosinophils in excess of normal and in the range 25-34%. Mean percentage lymphocytes present was lower than in non-infected controls in the range 31.25-40.5% but was still within the normal limits listed in Table 16.

FIGURE 14

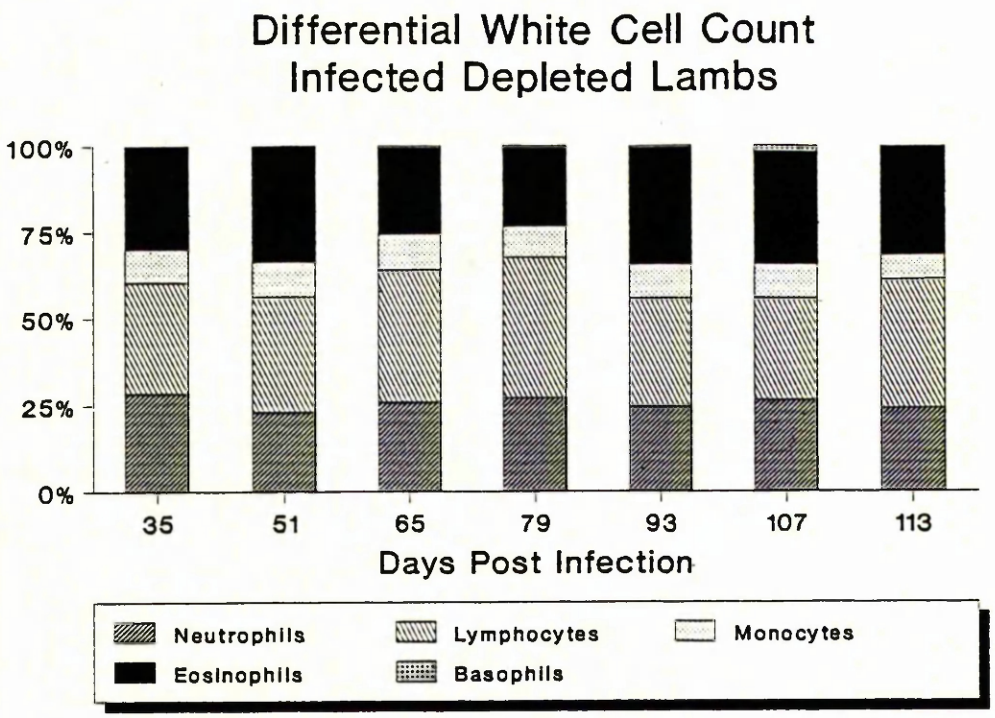


FIGURE 15

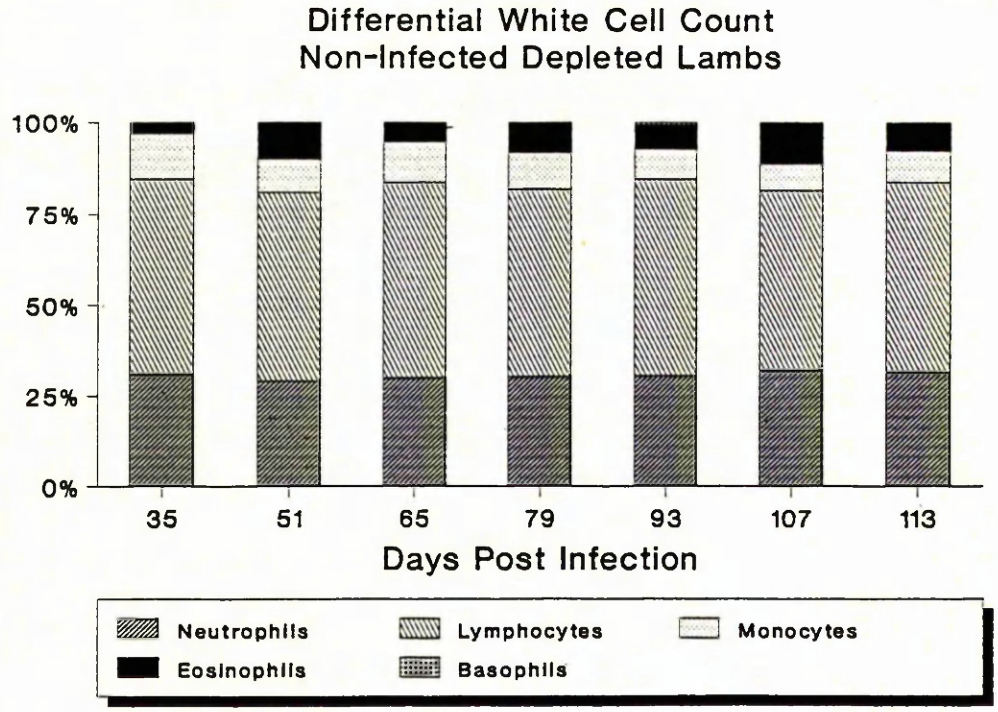


Figure 16 shows mean differential white cell count for group 5 lambs and revealed mean percentage neutrophil and monocyte levels in the ranges 23.5-31.75 and 6-12.5% respectively. Limited numbers of basophils were found in this group on seven occasions from fifteen sample dates. Percentage lymphocytes fell from 58%, 14 days PI to 30% 38 days PI but remained in the range 30-40% before returning and remaining at levels > 50% after 79 days PI. Eosinophilia among group 5 lambs was noted from 21 days PI when pre-patent level of 5.25% rose to 34.5% and remained in the range 26-32% until 65 days PI before gradually falling to normal thereafter and by the end of the experiment, 113 days PI, only 9% eosinophils were recorded.

Sufficient non-infected lambs, groups 6, showed mean percentage differential white cell counts as shown in Figure 17, which with percentages of neutrophil, lymphocyte, monocyte, eosinophil and basophil ranges of 26-32.5, 51-61.75, 8-12.75, 0.25-12 and 0-1 were all within the normal ranges for sheep.

Statistical analysis revealed no significant difference between comparisons of non-infected lambs of any of the three status groups. Comparison of percentage white cells in infected and non-infected sub-groups showed no significant difference in percentage neutrophils, basophils or monocytes.

There was significant elevation in percentage eosinophils in comparison to non-infected equivalents after patency, ie 21 days PI, among deficient and sufficent infected groups. This was usually at the expense of percentage of lymphocytes although all

FIGURE 16

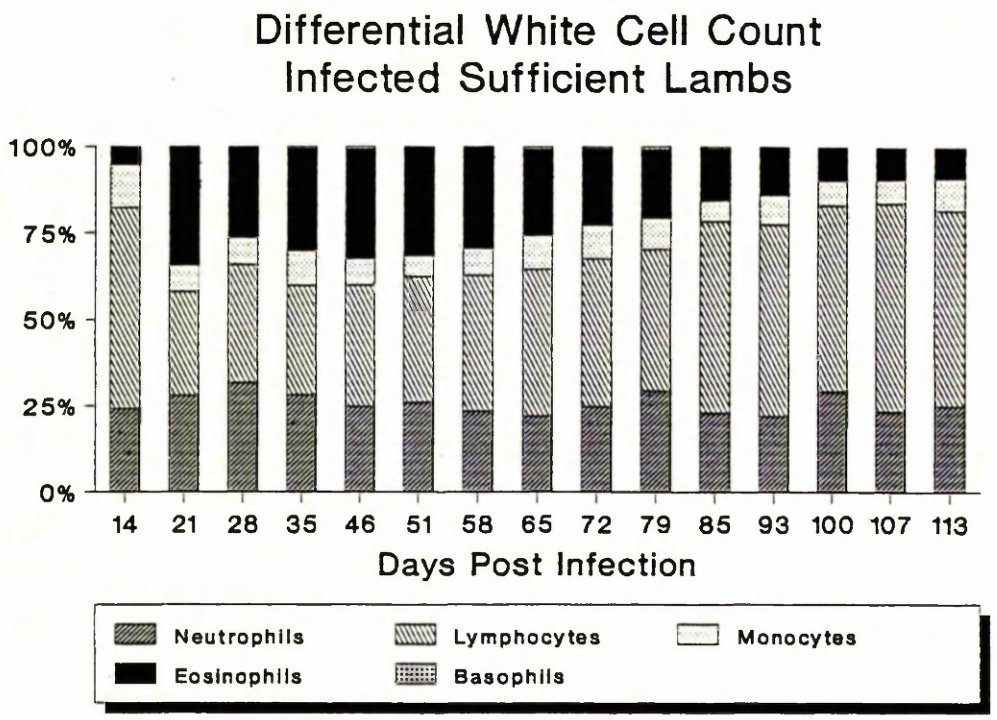
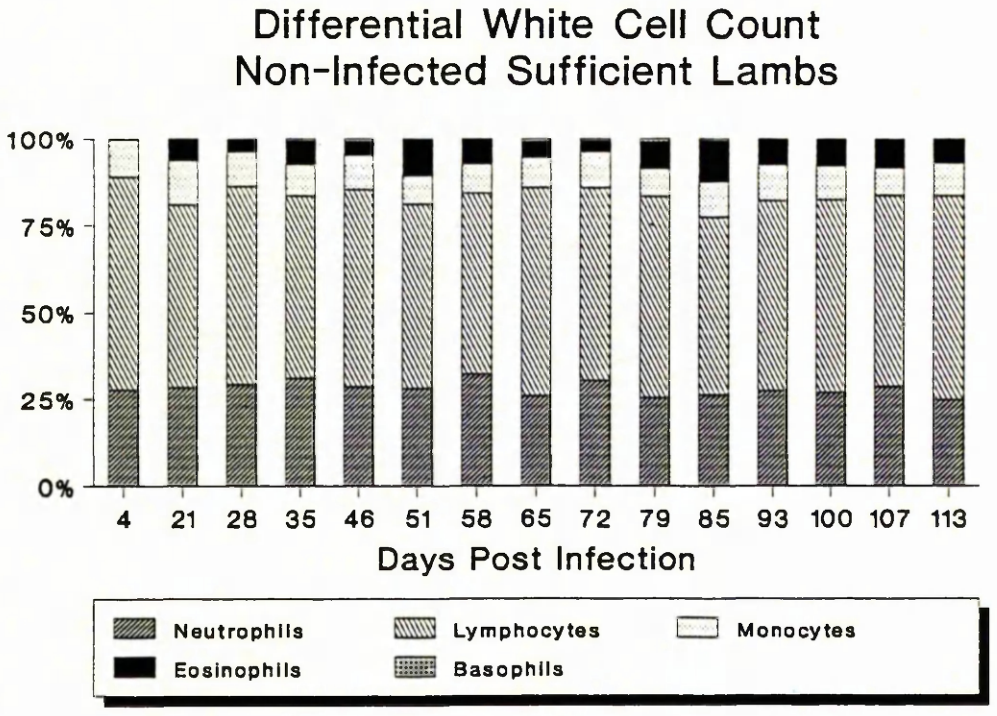


FIGURE 17



white cell percentages were generally lower than those found among non-infected equivalents ($P < 0.01$). No pre- and post-infection comparison of eosinophil percentage was possible among depleted infected lambs, group 3, due to the nature of blood sampling frequency.

Elevation of percentage eosinophils from normal among sufficient infected lambs, group 5, lost significance by 100 days PI in comparison with non-infected equivalents. Significant oesinophilia in comparison with non-infected controls remained among deficient and depleted infected counterpart ($P < 0.05$), since neither of their mean percentage eosinophils ever returned to normal during this experiment.

Deficient infected lambs mean eosinophil levels were significantly lower than depleted equivalent on two occasions 35 and 51 days PI ($P < 0.01$), although group 1 lambs' levels became significantly higher than sufficient and depleted equivalents from 65 days PI until the end of the experiment in the case of sufficient lambs ($P < 0.01$) but only until 85 days PI in the depleted instance ($P < 0.05$).

Comparison between depleted and sufficient infected lambs, group 3 and 5, revealed a significant difference, 93 days PI, which remained until the end of the experiment ($P < 0.05$).

3.3.6 White Cell Function Tests

Neutrophil function tests:

Neutrophil function tests as described in the analytical techniques chapter, were carried out on a respresentative sample of animals from each group in this experiment and provided the results shown in Table 17.

TABLE 17: Percentage *Candida* Killed = Killing Index (KI) - Experiment 1

DAYS POST INFECTION	DEFICIENT/ DEPLETED (SE)	SUFFICIENT (SE)	'p'
0	18.9 (1.63)	22.0 (1.46)	NS
14	28.6 (3.24)	31.6 (2.42)	NS
30	31.5 (1.00)	32.8 (3.38)	NS
44	23.5 (1.24)	36.1 (0.85)	$P < 0.05$
58	30.8 (2.23)	47.7 (1.71)	$P < 0.05$
72	33.0 (3.00)	44.4 (1.84)	$P < 0.05$
85	29.4 (1.43)	38.5 (2.67)	$P < 0.05$
100	29.8 (2.29)	42.5 (1.00)	$P < 0.05$
114	36.6 (2.25)	44.5 (0.82)	$P < 0.05$

Analysis of variance, with two isolated exceptions, 58 and 114 days PI, failed to reveal any significant difference in killing index (KI) between infected and non-infected sub-groups of the same cobalt status and consequently the date listed in Table 17

and illustrated in Figure 18 were based on mean values of infected and non-infected sub-groups of the same cobalt status.

In Figure 18, depleted and deficient lambs have been compared to sufficient controls. Both sufficient and deficient/depleted groups showed around 20% KI at the start of the experiment. Mean values for both groups rose to 31-32% by 30 days PI but thereafter the mean KI from sufficient lambs continued to rise while KI of neutrophils from deficient/depleted lambs remained in the range 23-33%. Statistical analysis revealed that by 42 days PI mean KI of neutrophils from sufficient lambs was significantly higher than the joint deficient/depleted group ($P < 0.05$), and remained so for the remainder of the experiment.

Nitroblue Tetrazolium reduction:

Table XIX, Appendix I, lists mean change in optical density (dOD) and standard errors for the six groups in this experiment. No significant differences between infected and non-infected sub-groups of the same cobalt status were found on any sample dates and therefore their combined mean values were used to construct Figure 19. A large degree of variation in detected dOD was evident on an individual animal basis and the only clear trend evident in Figure 19 was a downward one punctuated by intermittent rises in dOD. Statistical analysis however revealed isolated significantly higher dOD in the sufficient group (groups 5 and 6), 63 days PI.

FIGURE 18

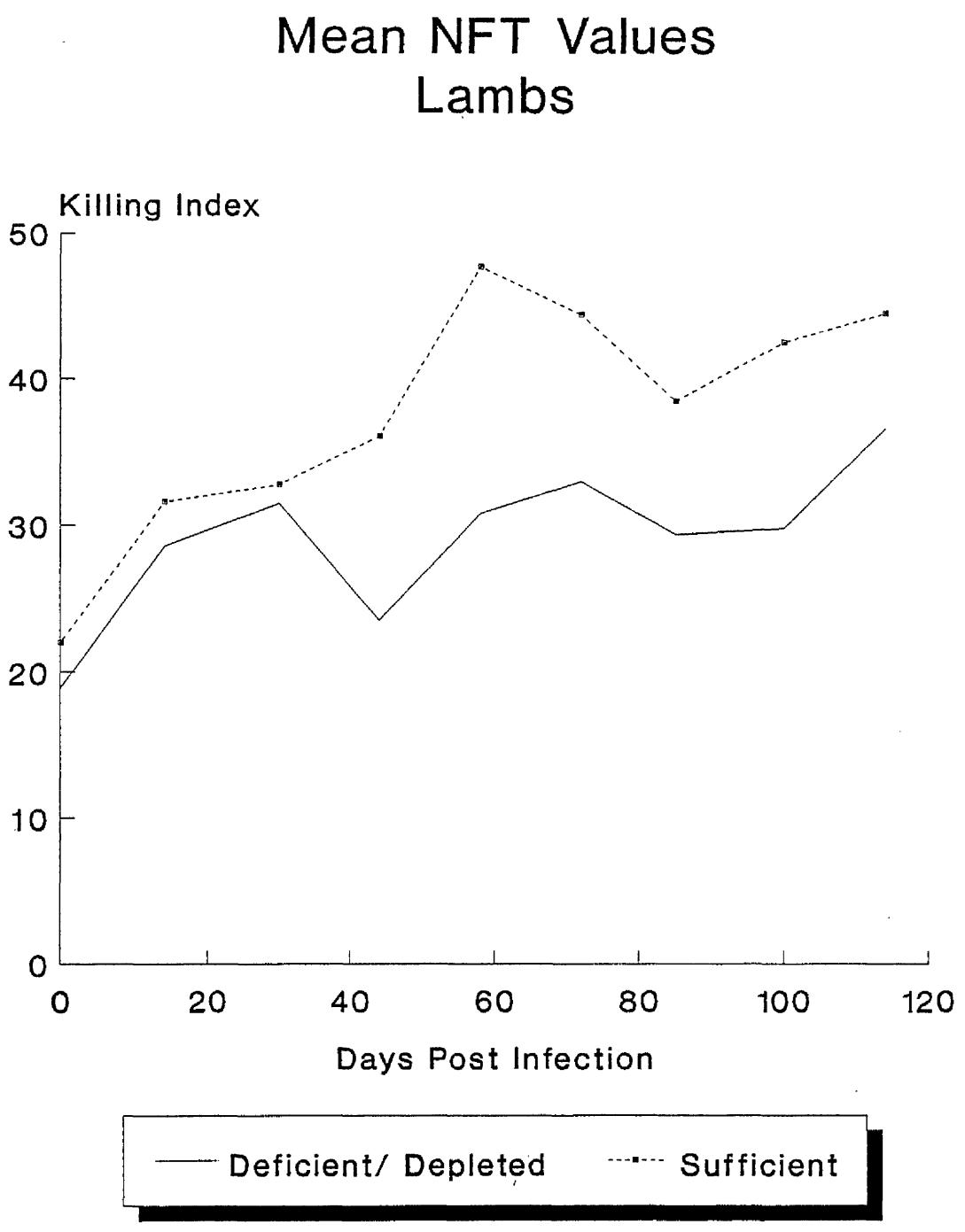
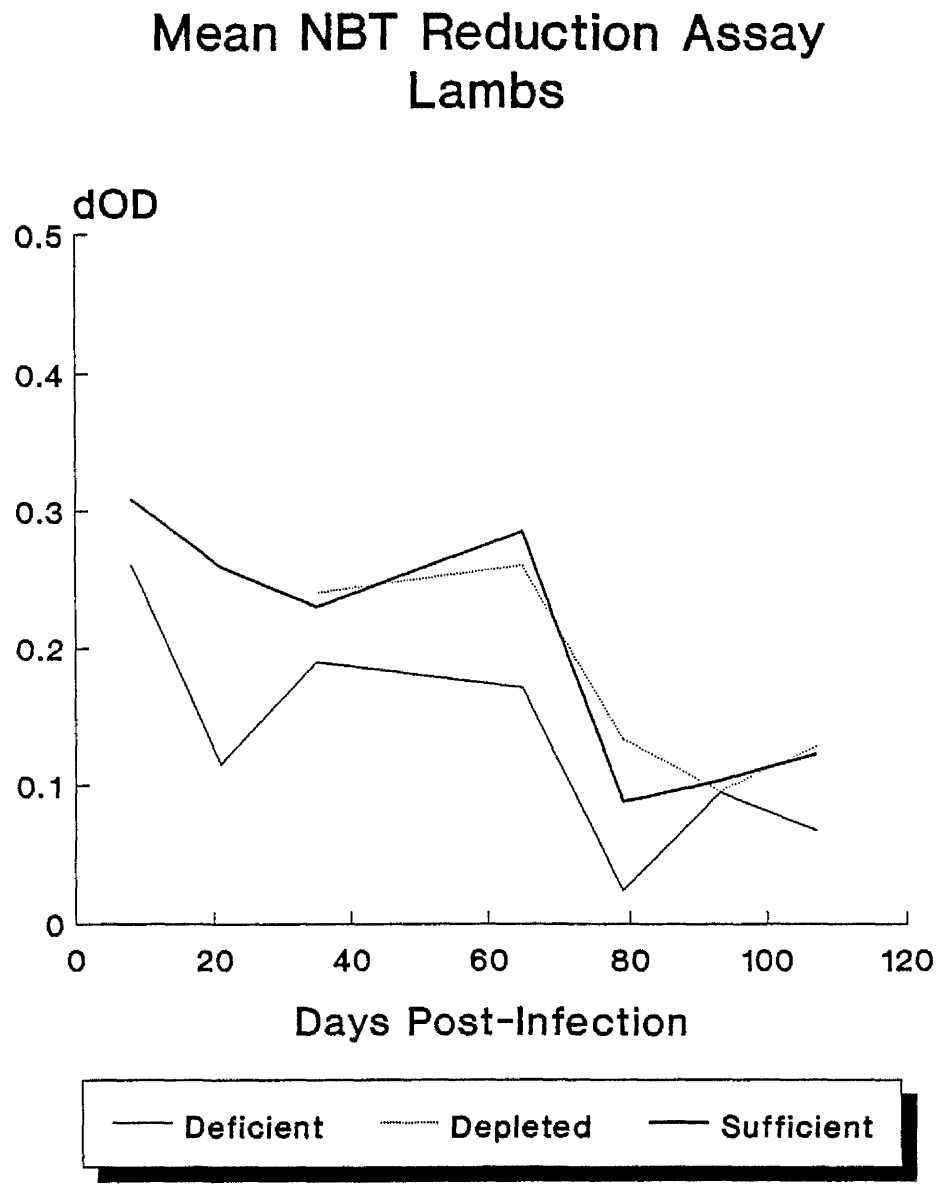

Mean NFT Values
Lambs
Killing Index
50
40
30
20
10
0
0
20
40
60
80
100
120
Days Post Infection
Deficient/ Depleted
Sufficient

FIGURE 19


Mean NBT Reduction Assay
Lambs
dOD
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
Days Post-Infection
Deficient
Depleted
Sufficient

3.3.7 Specific Immunity

Antibody response to *O. circumcincta*:

Figures 20-24 show mean antibody titre against *O. circimcincta* L3 antigen as measured using the ELISA technique detailed in the analytical techniques chapter. Mean results and standard errors are listed in Table XX, Appendix I. Figure 20 shows mean titre expressed as a percentage of a single pooled control serum sample (which was assayed on all ELISA plates) for deficient infected and non-infected sub-groups.

Pre-infection values (from 8 days pre-infection) of 29 and 30.36% of standard were found in non-infected and infected deficient lambs respectively. In both groups this value fell for the next forty days to a nadir of 5.16%, 23 days PI, for infected and 5.53% at 35 days PI for non-infected lambs. Thereafter the titres from both groups rose to around 43% by 49 days PI.

Non-infected lambs varied around that level (range 38.6-53.5%) for the remainder of the experiment. Infected lambs (group 1), however, continued to rise to a maximum of 99.45%, 63 days PI, remaining in the region of 90% for 21 days and falling thereafter. The high mortality rate experienced in the deficient groups (groups 1 and 2) make it difficult to attach meaningful significance to these results, however statistical analysis revealed that infected animals had significantly higher titres than non-infected lambs on a 'whole experiment' basis ($P < 0.05$) from 63 days PI until the end of the experiment.

FIGURE 20

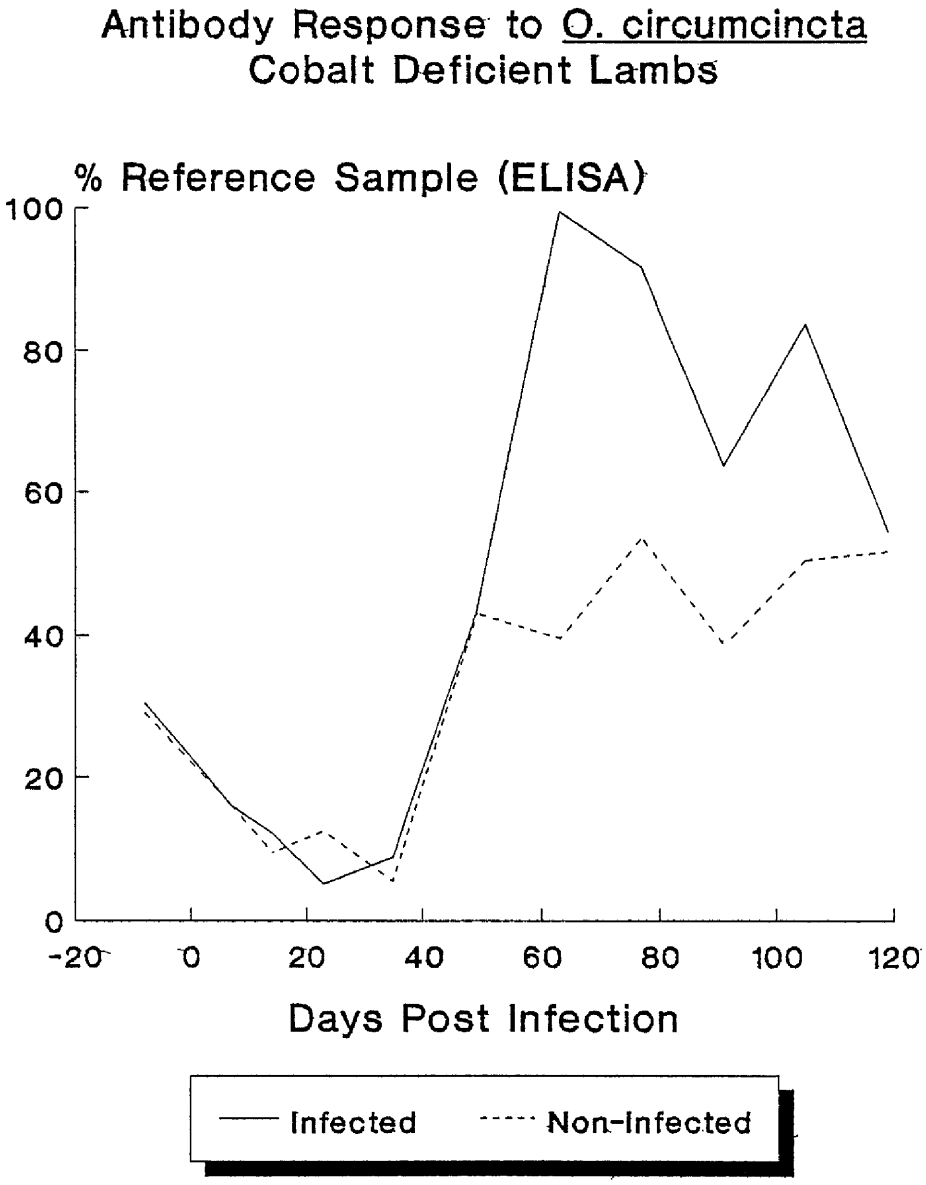

Antibody Response to O. circumcincta
Cobalt Deficient Lambs
% Reference Sample (ELISA)
100
80
60
40
20
0
-20
0
20
40
60
80
100
120
Days Post Infection
Infected
Non-Infected

Figure 21 shows a comparison of infected and non-infected sub-groups (groups 3 and 4) of the depleted lambs. Similar trends to those found in deficient lambs were evident in their mean titres. Initial titres of around 10% of standard in the non-infected sub-group fell progressively to 1.2%, 14 days PI, and did not exceed 4% until after 35 days PI when titres levelled around 36% (in the range 26-45%) for the remainder of the experiment. Infected lambs (ie group 3) did not show exactly the same pattern. Pre-infection titres of around 20% of control remained at that level and in the range 18.4-22.3% for the next 40 days and until 49 days PI when their value rose to 48% of standard and remained around the 50% level for the rest of the experiment, in the range 45.5-53.7%.

A small but clear difference in titre existed between infected and non-infected depleted lambs for the whole of the experiment and statistical analysis of variance revealed that this difference was significant from 63 days PI until the end of the experiment ($P < 0.05$).

Figure 22 shows mean antibody titre to *O. circumcincta* of infected and non-infected sufficient sub-groups (groups 5 and 6) in ths experiment. The initially relatively high titre gradually falling off was also evident in infected sufficient lambs in which the mean value of 32.3% fell progressively to reach its lowest level of 12.15%, 23 days PI. After remaining around that level for 14 days, the titre then rose to 45% by 49 days PI and remained around that high level and in the range 45-62% for the rest of the experiment.

FIGURE 21

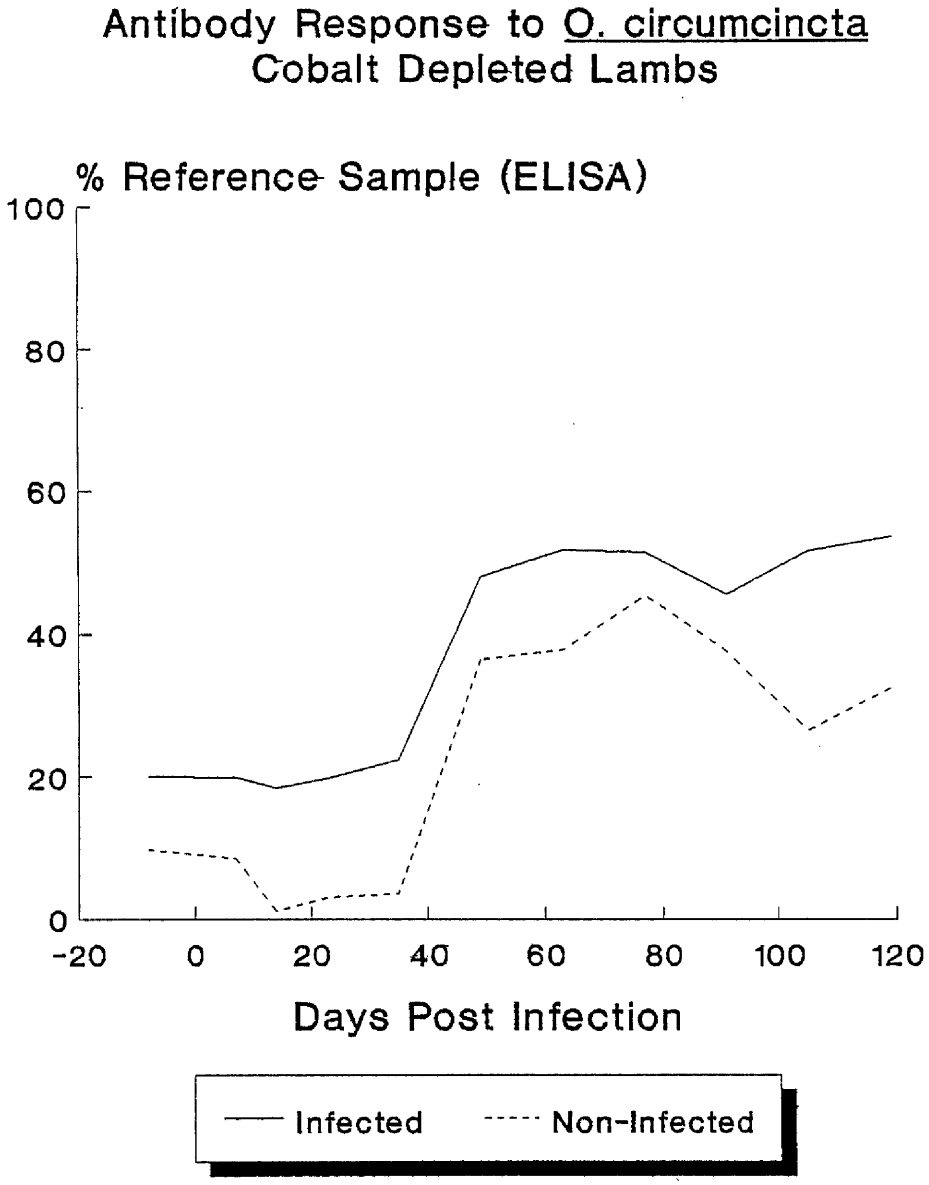
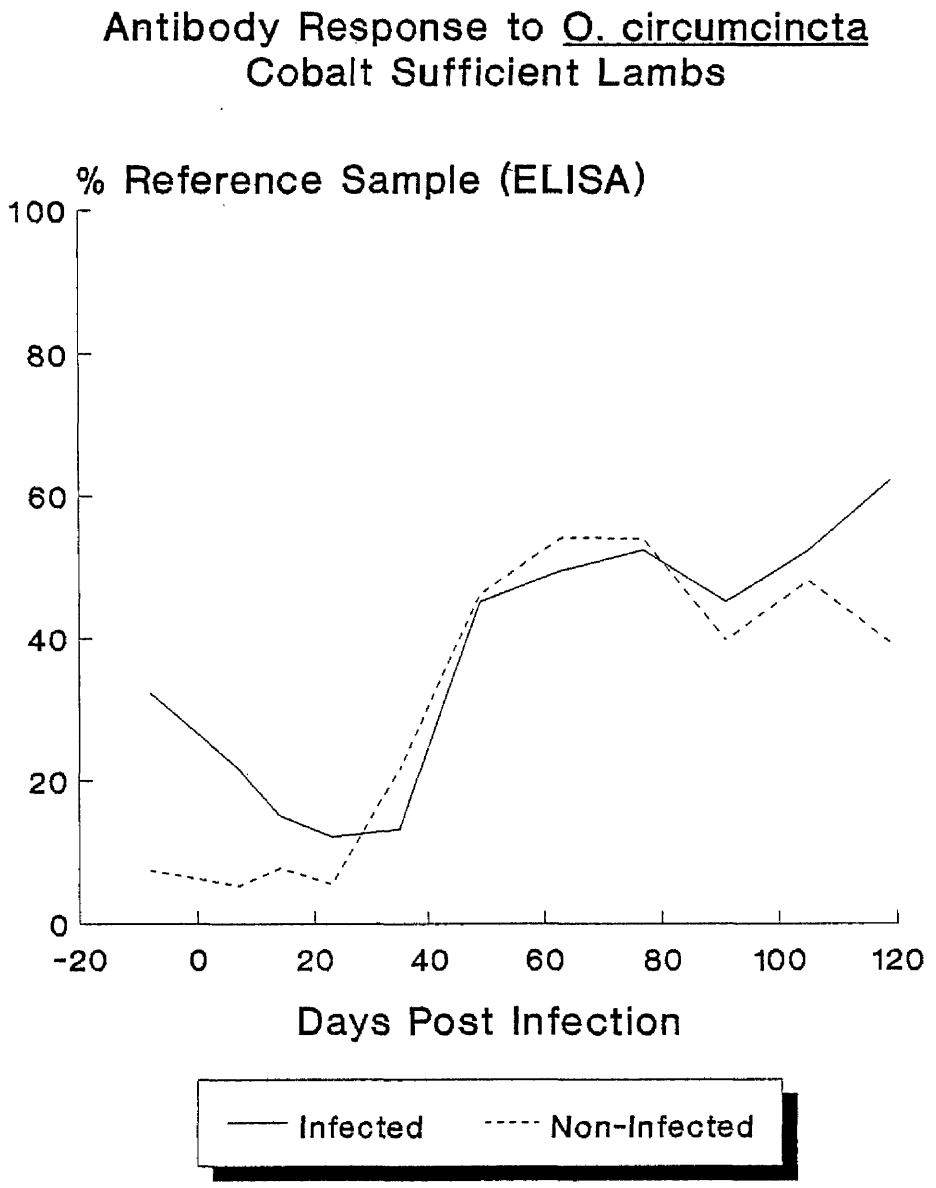

Antibody Response to O. circumcincta
Cobalt Depleted Lambs
% Reference Sample (ELISA)
100
80
60
40
20
0
-20
0
20
40
60
80
100
120
Days Post Infection
Infected
Non-Infected

FIGURE 22


Antibody Response to O. circumcincta
Cobalt Sufficient Lambs
% Reference Sample (ELISA)
100
80
60
40
20
0
-20
0
20
40
60
80
100
120
Days Post Infection
Infected
Non-Infected

Non-infected sufficient lambs however did not show the same trend. The initial high titre evident in other groups was missing and this groups' initial titre of 7.5% of standard was maintained for three further sample dates until 35 days PI when it rose to 21.5%. Mean titre for this group, group 6, continued to rise and was found to be 46% by 49 days PI, a level which was maintained for the remainder of the experiment in the range 39-54% of standard. Statistical analysis demonstrated that infected lambs had significantly greater titres than non-infected equivalents from 85 days PI until the end of the experiment.

Figure 23 shows the antibody titres from the three non-infected groups of the deficient, depleted and sufficient status groups in this experiment. The trend described earlier appears clearly in this graph. Most non-infected groups start at a pre-infection titre which falls then rises before levelling off around 40 days PI and remaining there for the rest of the experiment. Statistical analysis revealed no significant difference in titre between any of the three status groups.

Figure 24 shows mean titres to *O. circumcincta* antigen of infected sub-groups of deficient, depleted and sufficient status groups. The trend described in the previous four figures was very plain in this comparison. All infected sub-groups showed similar trends for much of the experiment, particularly during the post-patent period. Deficient lambs demonstrated greatly elevated titres by comparison from 63 until 105 days PI when the high mortality rate among deficient lambs prevented further

FIGURE 23

Antibody Response to O. circumcincta
Infected Lambs

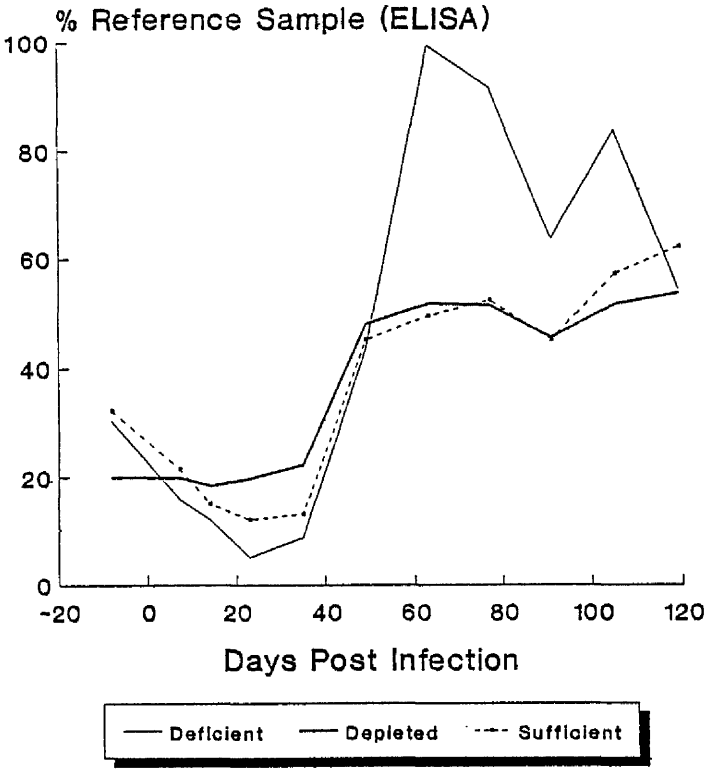
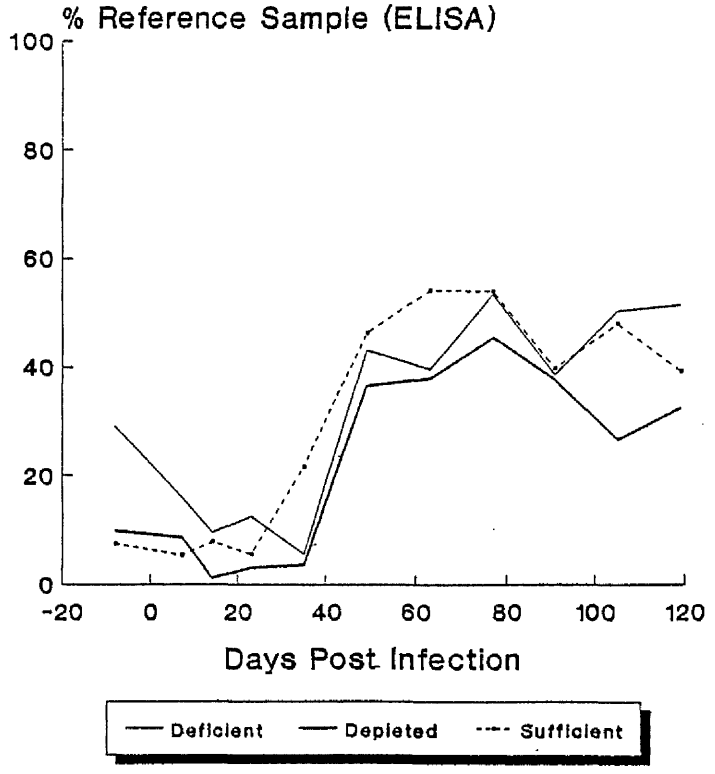


FIGURE 24

Antibody Response to O. circumcincta
Non-Infected Lambs



meaningful comparison and thus limited any conclusions which may be drawn from this result.

Statistical analysis revealed no significant difference in titre between infected groups which was attributable to cobalt status.

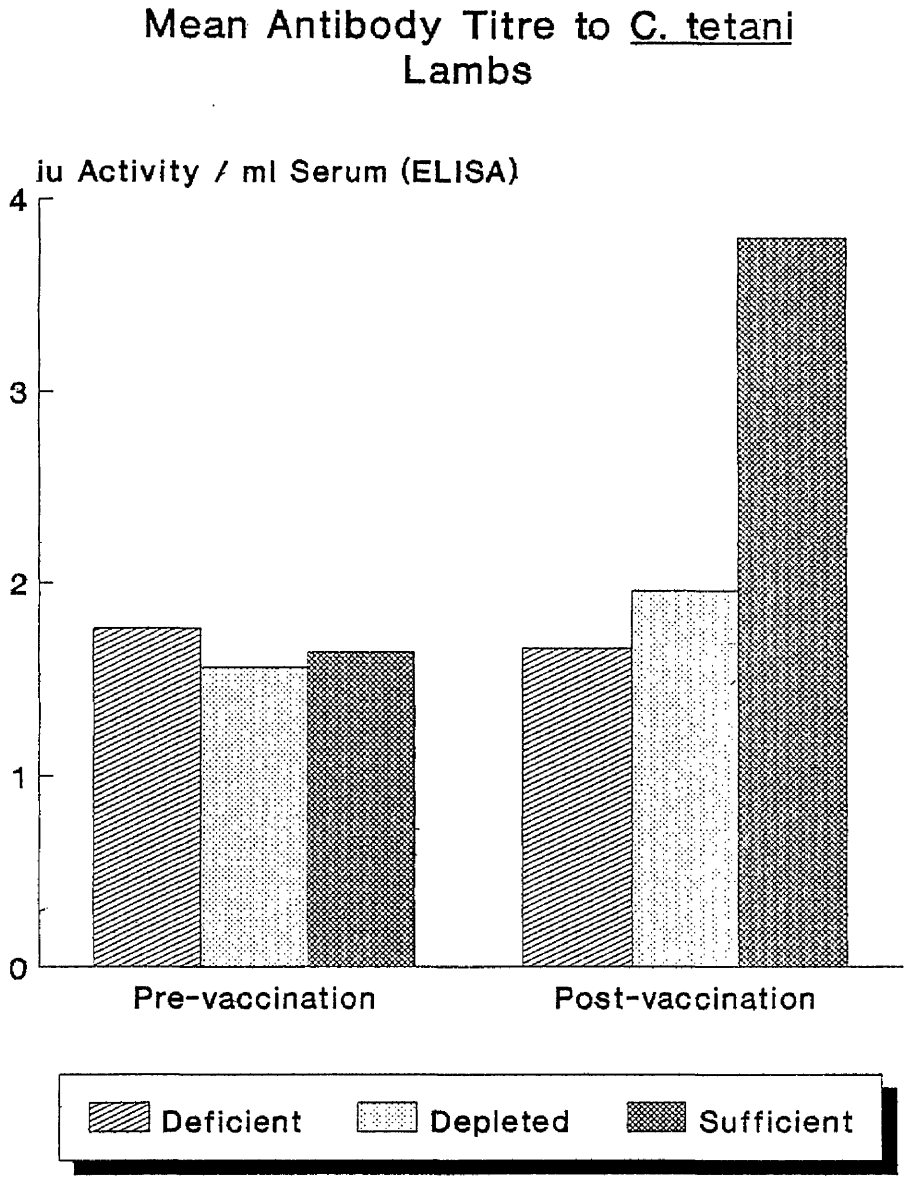
Antibody response to *Clostridium tetani* vaccination:

Serum samples from all relevant animals in this experiment were assayed to assess antibody level to *C. tetani* immediately before treatment with a multicomponent *Clostridial* and *Pasteurella* vaccine (Ovivac P., Hoechst Animal Health, Milton Keynes) and again during the anticipated 'peak' response to vaccinal challenge.

Precise details of procedures used in this assay are contained in the analytical techniques chapter. Figures 25 and 26 were drawn using data listed in Table XXI, Appendix I and thus show mean response to *C. tetani* antigen expressed as the protective titre against antigen of pre- and post-vaccination samples from the three status groups in this experiment.

Figure 25 shows a comparison of the pre- and post-vaccination titres of the three status groups. Deficient lambs had a mean pre-vaccination titre of 1.76 ± 0.316 iu activity per ml serum (iu/ml) and a post-vaccination titre of 1.66 ± 0.361 iu/ml which was not significantly different. Depleted lambs showed similar levels: mean pre-vaccination titre of 1.63 ± 0.262 iu/ml, a level which was not significantly different from the mean post-vaccination titre of 1.91 ± 0.255 iu/ml.

FIGURE 25


Mean Antibody Titre to C. tetani
Lambs
iu Activity / ml Serum (ELISA)
4
3
2
1
0
Pre-vaccination
Post-vaccination
Deficient
Depleted
Sufficient

Sufficient lambs however had pre-vaccination titre similar to other groups (1.69 ± 0.381 iu/ml) but showed a significantly elevated mean post-vaccination titre of 3.64 ± 0.536 iu/ml, ($P < 0.01$) when compared to their own pre-vaccination level. Figure 26 illustrates the same results with the three status groups compared individually as pre- and post-vaccination levels. The values listed in Table XXI, Appendix I and illustrated in Figures 25 and 26 demonstrate that no significant difference was detected in mean pre-vaccination titre among any of the three status groups.

Statistical analysis by Students t-test revealed that the mean post-vaccination titre of the sufficient status group was significantly higher than those of both depleted and deficient counterparts ($P < 0.01$) and that these did not differ significantly from each other.

3.4 DISCUSSION

3.4.1 Biochemical Considerations

Assignment to groups in the present study was made on the basis of maternal cobalt status, whereby sufficient lambs, groups 5 and 6, had adequate cobalt levels, and deficient lambs, groups 1 and 2, low cobalt levels. A gradual fall in mean serum Vitamin B_{12} from 290 ng/l, marginally deficient, to less than 200 ng/l after infection indicated depletion and probable eventual exhaustion of maternally derived cobalt reserves in the depleted groups, 3 and 4 as illustrated in Figure 7.

FIGURE 26

Mean Antibody Titre to C. tetani Lambs

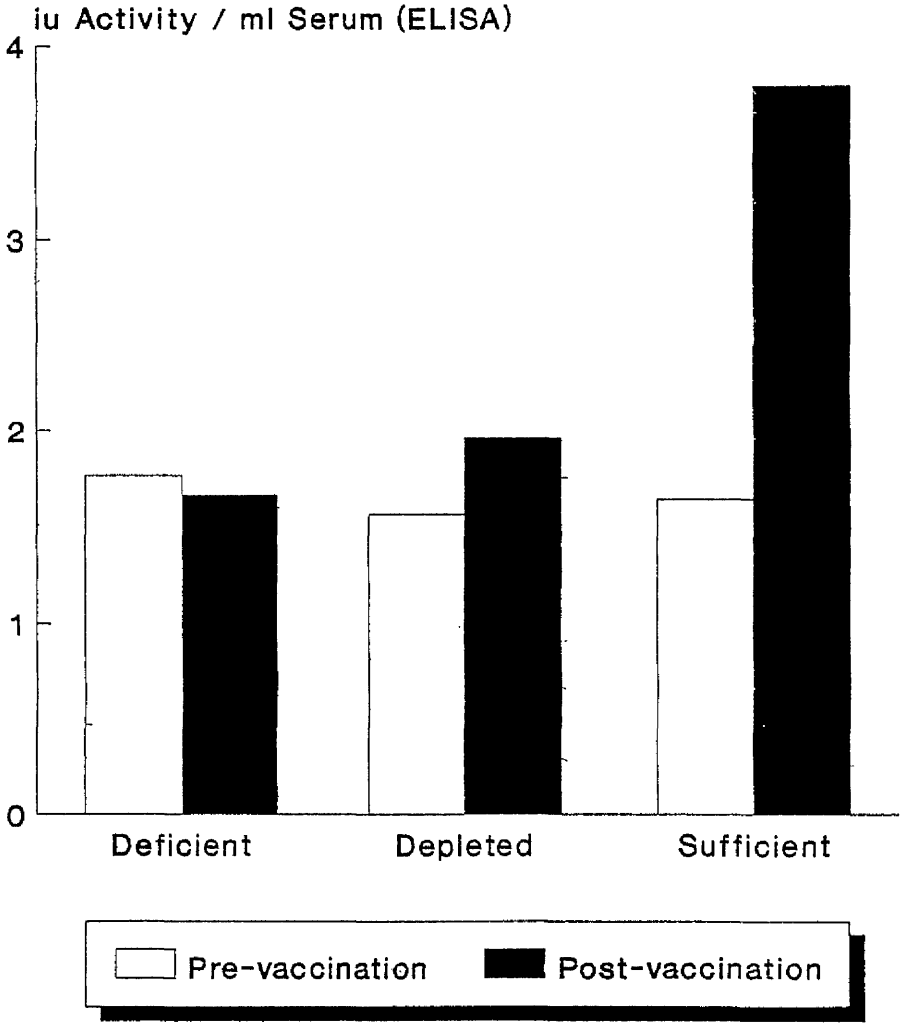


Figure 8 illustrates mean MMA levels of experimental groups and revealed a similar cobalt status trend although manifested in the reverse direction. By comparison with Table 5, sufficient lambs had high cobalt status as indicated by low mean MMA values and the low cobalt status of deficient lambs was confirmed by high MMA values. Depleted lambs had initially low MMA concentration similar to their sufficient counterparts and indicative of their cobalt sufficient state at that time but with the onset of cobalt deficiency they became elevated and remained so thereafter.

In contrast to previous reports of helminth mediated nutrient malabsorption, (Coop *et al*, 1977), albeit with non-cobalt deficient sheep, no significant difference in serum Vitamin B_{12} was detected between infected and non-infected sub-groups of the same cobalt status in this experiment. These findings also contrast in part with Weir *et al* (1948) who found that *H. contortus* infection in lambs lowered levels of Vitamins A and C in serum and with Downey (1966a) who reported lower levels of serum Vitamin B_{12} in chronically parasitised lambs when compared to non-infected controls. The two isolated instances in this experiment when significant differences were detected between infected and non-infected sub-groups of the same cobalt status could have been due to individual animal or normal biological variation since standard errors were similar in all groups.

It was noted in the early part of this experiment, ie before day 0, that all three status groups had Vitamin B_{12} levels less than 400 ng/l. However the age of the lambs (9-12 weeks) at the start of the experiment and thus their 'pre-ruminant' status negated

the use of conventional biochemical criteria defining deficiency. These findings are in agreement with Fisher (1988), who demonstrated that lambs from cobalt supplemented ewes had mean serum Vitamin B_{12} concentrations at 2 and 4 weeks post partum of 271 and 259 ng/l respectively, ie below levels considered adequate using normal biochemical criteria but similar to those found in the present study.

Hypoalbuminaemia which occurs in many helminthiases and is a common feature of ostertagiasis in cattle (Anderson *et al*, 1965) and sheep (Holmes and MacLean, 1971) was not found in this experiment. Mean plasma albumin levels for each group were always within the normal range and unaffected by either low cobalt or more importantly, infective status. This can be explained by consideration of the relative rates of *O. circumcincta* infection, V12. Holmes and MacLean (1971) used a single large (300000-900000 L3) dose which caused severe pathology and was considerably greater than the 6000 per week given in the present study. The normal albumin levels found in the present study are in agreement with the report of Coop *et al* (1977) that hypoalbuminaemia among *O. circumcincta* infected lambs was never severe even when performance was markedly affected by infection with up to 5000 L3 *O. circumcincta* per day.

Additionally, the static nature of plasma albumin level between samplings in the present study, also disagrees with a reported albumin hypercatabolism in *O. circumcincta* infected animals reported by Holmes and MacLean (1971), and Yakoob *et al* (1983), and again the larger *O. circumcincta* infection given by the

former authors provides the explanation. Comparison with the latter authors must be tempered since their studies examined immune sheep although they did use relatively much larger doses of 7000 per day intermittently (up to a total of 112000) in comparison with the present study.

Analysis of Table VII, Appendix I shows no significant difference in circulating plasma total protein levels in any of the six groups, where all values were within normal limits, attributable either to infection of low cobalt status. Thus hypoproteinaemia normally associated with *O. circumcincta* infection similar to that reported by Horak and Clark (1964), Holmes and MacLean (1971), and Yakoob *et al* (1983), was absent in the present experiment, and was probably due to the much lower infective rate used in the present study in comparison with other studies.

Calculation of serum globulin levels by subtracting serum albumin from serum total protein, showed that these values were similarly unaffected again either by infection or cobalt deficiency. This agreed with the findings of Coop *et al* (1977), that there was no significant difference in serum globulin levels between *O. circumcincta* infected lambs and control groups. Consequently albumin:globulin (a:g) ratio, although not listed, was not significantly affected by parasitic infection in contrast to previous reports (Horak and Clark, 1964) where large *O. circumcincta* infections of 240000-618000 L3 given in doses of 70000-312000 caused significant differences in a:g ratio again due to the relatively greater infective dose given to those sheep in comparison to the present study.

3.4.2 Pathological Findings

Downey (1965) reported enhanced mortality and greater pathogenicity in cobalt sufficient *H. contortus* infected sheep compared with cobalt deficient controls but the findings of the present study are in marked contrast to Downey's report. The most striking result from the present study was the extremely high mortality rate among deficient lambs. Depleted lambs suffered a much lower mortality rate but still well in excess of the zero rate in the sufficient group.

Helminth infection does not appear to have contributed significantly to these losses, although characteristic findings associated with *O. circumcincta* infection were recorded in relevant cases, since equal mortality rates were experienced in infected and non-infected status sub-groups which succumbed to a variety of pathogens. However, an increased susceptibility to infection with associated mortality may be related to low protein intake of cobalt deficient lambs. Similar findings were reported by Abbott *et al* (1988) in which increased mortality rate in *H. contortus* infected sheep fed low protein diets was experienced. Fisher and MacPherson (1986) and Fisher (1988) also described higher mortality rates and requirement for treatment for increased prevalence of neonatal diseases in lambs from cobalt deficient ewes.

Deficient and depleted lambs in the present study required more frequent and repeated antibiotic treatment for pneumonia although such treatment was only partially successful since nine from fifteen casualty lambs had organised areas of pneumonia post

mortem. *P. haemolytica* (Seroptype A6) recovered from organs of a number of deficient casualty lambs confirmed immune impairment in deficient lambs since all animals had previously been vaccinated on two occasions with this serotype of *P. haemolytica* as part of their multi-component vaccination regime (Ovivac-P, Hoechst Animal Health, Milton Keynes). It was interesting therefore to note that Tui *et al* (1954, after Gershwin *et al*, 1985) reported greater susceptibility to and incidence of upper respiratory tract infection in animals receiving low protein diets. Low protein intake associated with inappetance of cobalt deficient lambs may have had a similar effect in the present study.

Further evidence of immune compromise in cobalt deficient animals was provided by a confirmed case of enterotoxaemia (Pulpy Kidney Disease) despite this being a component of the multi-component clostridial vaccine (Ovivac-P, Hoechst Animal Health). Most emphatic of all however, was the significantly lower post-vaccination titre to *C. tetani* in depleted and deficient lambs when compared to sufficient controls as discussed in Section 3.9.9.

3.4.3 Hepatic Encephalopathy and White Liver Disease (WLD)

Varying degrees of hepatic degeneration was a consistent feature in deficient and depleted casualty animals in this experiment. Histopathological examination of affected livers from these lambs revealed moderate to severe centrilobular fatty degeneration and bile duct proliferation. Grossly livers were enlarged, fatty and friable and biochemical analysis of blood samples from lambs

euthanased *in extremis* confirmed liver dysfunction. Affected lambs demonstrated severe ill-thrift and spectroscopic determination of liver cobalt content confirmed severe cobalt deficiency. These findings indicated the presence of a condition resembling ovine white liver disease (WLD) similar to that originally reported in New Zealand by Cordes and Gardener (1971) and later found in sheep worldwide (Wensvoort and Herweijer, 1975, Ulvund and Overas, 1979, Sutherland *et al*, 1979 and McLoughlin *et al*, 1984, 1986). The condition is characterised by pale, swollen livers and including varying degrees of parenchymal fatty change, bile duct and lymphocytic proliferation in the portal area and ceroid pigmentation (McLoughlin *et al*, 1984). White liver disease is associated with low serum and liver Vitamin B_{12} levels and low dietary cobalt availability (Sutherland *et al*, 1979) and results in ill-thrift, anaemia and photosensitivity (McLoughlin *et al*, 1984).

Neuropathological examination revealed possible cytotoxic processes of pathological significance in six of fourteen lambs examined. Two deficient lambs showed spongy transformation of white matter in both brain stem and spinal cord possibly resulting from hyperammonaemia as characterised in hepatic encephalopathy (Walker and Schenker, 1970, Strombeck *et al*, 1975 and Keane and Blackwell, 1983). Inferences from these findings must be tempered in the light of the report of Wells and Wells (1989) of vacuolation in white matter resulting from prolonged holding of brains in a fixing solution of 70% alcohol since the central nervous systems (CNS) from casualty lambs in this experi-

ment were held in a 10% formalin fixing solution for an extended period of time.

The involvement of cobalt deficiency in neurological disorders has been previously recorded (Hartley *et al*, 1962, MacPherson *et al*, 1976, Garton *et al*, 1981 and Fell *et al*, 1985). However no evidence of a link between WLD and hepatic encephalopathy has been previously demonstrated. The present studies provide a tenuous foundation for such a claim although when severe WLD was demonstrated grossly and histopathologically, neuropathological vacuolation and associated spongy change in the CNS were not always evident.

Clear evidence of the influence of maternal cobalt status on subsequent increased occurrence of WLD and hepatic encephalopathy in lambs from deficient ewes was obtained in the present study and confirms the reports of Garton *et al* (1981) whereby neuronal atrophy and degeneration in the cerebral cortex and brain stem of clinically deficient ewes probably had a role in reduced numbers and broadening of cerebral gyri of their lambs. The same group (Fell *et al*, 1985) later concluded however that CNS neurological defects were probably secondary to the clinical effects of cobalt deficiency on the liver and abnormal neuropathology could therefore be described as resulting from hepatocerebral disease. This theory supported the findings of the present study that there may be a link between WLD and hepatic encephalopathy.

3.4.4 Production

Previous authors have demonstrated that *O. circumcincta* infection causes a marked reduction in utilisation of nutrients whereby infected lambs failed to grow as well as pair-fed non-infected controls (Coop *et al*, 1977) and the same authors later speculated that malabsorption, alteration of digestibility and induction of inappetance were possible causes (Coop, 1981). Contrasting results were found in the present study since no reduction in liveweight, or gain, was demonstrated in any of the infected groups, when compared to their respective controls, regardless of cobalt status.

There are two possible explanations for this difference. Firstly the dose rate adopted in the present study (6000 per week) was less than the rate of 18000 per week which Coop *et al* (1977) reported as causing reduction in feed intake and reduction in body weight. Alternatively the nature of the cobalt deficient diet fed in the present study which caused inappetance and reduction in feed intake, previously described in cobalt deficiency (Underwood, 1977, 1981), may have masked underlying production effects attributable to *O. circumcincta* infection.

The cause of inappetance in cobalt deficient sheep lies in their inability to remove energy-supplying propionate from circulation (Marston *et al*, 1972) with a consequent reduction in energy gain from that source (Peters *et al*, 1983a,b). Thus, prolonged high levels of propionate in blood can lead to a reduction in voluntary food intake, mediated by nervous mechanisms responsive to circulatory matabolite concentration, which control appetite

(MacDonald *et al*, 1981). Hence the main symptoms of clinical cobalt deficiency are indistinguishable from starvation (Underwood, 1981). MacPherson (personal communication) reported a selected preference for fibrous feedstuffs and roughage, usually hay over concentrate among deficient sheep and cattle and similar selective inappetance was found in this experiment. Concentrate put before all lambs to facilitate photography was refused by deficient and depleted lambs which would only come to the trough after Timothy hay was offered. Plate 3 illustrates that deficient and depleted lambs, on the left, consumed hay but showed little interest in the concentrate being eaten by their sufficient counterparts.

Thus results listed in section 3.3 show that cobalt status was the main determinant of production in this experiment as deficient lambs in the present study showed poor weight gain by comparison with sufficient controls similar to that predicted by Underwood (1981).

An interesting production effect was noted among depleted lambs, groups 3 and 4, whereby their growth rates matched sufficient controls for the first part of the experiment until 50 days PI when they slowed before finally changing direction and becoming manifest as frank weight loss. The fact that this finding was common to both infected and non-infected equivalents further confirms that cobalt status was the main determinant of production and agrees with previous studies showing a weight deficit of up to one-third (Smith and Marston, 1970a,b) when comparing cobalt deficient sheep to sufficient counterparts and similar

PLATE 3: Illustration of Inappetance in Deficient Lambs and Selective Preference for Hay Against Concentrate

reports of a liveweight loss after the introduction of a cobalt deficient ration to cattle (MacPherson, 1973) and sheep (MacPherson, 1976).

The probable explanation of the trend among depleted lambs lies in the original experimental design where depleted lambs came from cobalt adequate ewes by suffered a gradual exhaustion of maternally derived cobalt reserves. This suggestion is supported by the measurement of biochemical parameters - particularly the progressive rise in serum MMA concentration. Additional subjective evidence of the development of a full cobalt deficiency syndrome causing production impairment among depleted lambs is shown in Plate 3, taken 85 days PI where it can be seen that depleted lambs appear unthrifty and selectively inappetant, refusing concentrates.

3.4.5 Parasitology

Comparison of plasma pepsinogen levels of infected and non-infected lambs of the same cobalt status demonstrated elevation among infected lambs associated with the 'leak lesion' and consequent increased permeability to macromolecules such as pepsinogen into the bloodstream similar to that reported by Holmes and MacLean (1971) in lambs infected with *O. circumcincta*.

Mean plasma pepsinogen levels of sufficient infected lambs settled around or below 1000 mU/l and indicated a possible absence of the 'self cure' reaction first reported by Stewart (1953) to *H. contortus* infection. The apparent absence of immunity was confirmed by the continued presence of worm eggs in

faeces throughout the experiment, by TWC at sacrifice and by the absence of stunting of any worms recovered from abomasal washings from sufficient and depleted lambs, since all of these manifestations were stated by Urquhart *et al* (1986) to be indicators of immunity to helminth infection.

The relatively young age of lambs in this experiment (3 months) may have caused age related unresponsiveness to parasitic infection. Smith *et al* (1985b) cited well documented evidence that lambs aged 6 months or less were more susceptible to infection (Manton *et al*, 1962, Urquhart *et al*, 1966 and Dineen *et al*, 1978) and quoted evidence that immunisation of lambs aged less than 6 months with *H. contortus* or with irradiated *H. contortus* or *T. colubriformis* larvae did not make them resistant to homologous challenge although resistance was present in similarly challenged older sheep. Smith (1985b) also cited Soulsby (1981) who opined that age related unresponsiveness to parasitic infection was difficult to understand since even foetal lambs could mount protective immune responses to viral and bacterial challenge. Additionally, Smith *et al* (1985a) were able to show significantly less well developed cellular and IgA components of gastric lymph responses in 4.5 month old lambs when compared to 10 month old equivalents both of which were challenged with 50000 *O. circumcincta*.

It was clear that an inverse relationship existed in the present study between cobalt status and pathogenicity of *O. circumcincta* infection. Sufficient lambs, of high cobalt status had low levels of infection as measured by pathological damage, the

associated rise in plasma pepsinogen and TWC at sacrifice. Although no direct comparison at sacrifice was possible, deficient lambs, of low cobalt status, had progressively increasing burdens and consistently greater pathological damage as measured by plasma pepsinogen level. Depleted lambs' cobalt status fell throughout the experiment, presumably reaching a point when maternally derived cobalt reserves became exhausted, and showed a relatively low initial level of pathogenic infection which increased with falling cobalt status, resulting in a significantly higher burden at sacrifice than similarly infected, sufficient controls.

An inverse relationship was also found between WEC and cobalt status whereby relatively low WEC among high cobalt, sufficient lambs contrasted with higher, but variable, counts among deficient lambs. As was previously found WEC of depleted lambs increased as cobalt status fell. Total worm count data from sacrificed lambs reflected the degree of infection at that time, however comparison of TWC with plasma pepsinogen and WEC data (since percentage of females was not significantly different in any group) would indicate that the ability to limit reinfection or establishment was impaired in the deficient group and subsequently in the depleted group after exhaustion of their cobalt reserves.

Previous studies examining parasitic infection and micronutrient deficiency produced contradictory reports. Downey (1965, 1966a) concluded that *H. contortus* and *T. axei* infection produced less deleterious effects in cobalt deficient hosts as measured by

mortality and resistance to infection. He further stated that nutrient malabsorption, as measured by lower serum Vitamin B_{12}, occurred in infected lambs. These findings however contradicted earlier reports by Threlkeld *et al* (1956) in which cobalt deficient *H. contortus* infected lambs had greater worm burdens and longer, more mature and more gravid female parasites than supplemented equivalents. Downey (1966b) however negated his previous findings in experiments with parasite naive cobalt deficient lambs infected with *O. circumcincta* singly or superimposed on a longstanding *T. axei* burden. Cobalt deficient lambs infected with *O. circumcincta* only, had higher WEC's and haemoglobin concentrations but lower weight gains than similarly infected supplemented controls. Higher TWC at necropsy was also evident and Downey (1966b) therefore concluded that the effects of mild to moderate *O. circumcincta* infestation and low cobalt intake were additive, a view confirmed by similar findings in the present study.

The level of TWC's found by Downey (1966b) were lower than those in the present experiment, ie 3906 vs. 10000 among sufficient lambs and 6150 vs. 30000 for deficient/depleted counterparts. This is explained by the different dosing regime adopted by Downey (1966b), ie 15000 L3 *O. circumcincta* at 12 weeks of age, 17000 at 15 weeks of age and 32000 at 35 weeks of age and the present studies in which 6000 L3 *O. circumcincta* were given per week as 3 x 2000 L3 doses for 16 weeks, ie a total of 96000 larvae.

Worm egg count data from Downey (1966b) was confirmed by findings in the present study but that same author speculated that variation in egg counts after 25 weeks of age suggested that some animals had undergone 'self cure' and developed immunity. This suggestion is contrary to the findings in the present study where 'self cure' was not evident since TWC revealed that a significantly higher proportion of recovered worms were adult in all cases, even though comparison of status groups showed significantly more larval stages in cobalt sufficient lambs than in deficient and depleted controls. This was despite the fact that Downey (1966b) indirectly supported the contention of greater establishment in cobalt deficient lambs as found in the present study when he suggested that WEC trends among cobalt deficient infected lambs indicated that they harboured greater numbers of worms over most of the period of observation.

An explanation of the observed differences in the establishment and pathogenic effects of helminth infection in deficient, and depleted compared to sufficient lambs may involve selective inappetance and a consequent associated deficiency in protein intake. Symons and Steel (1978) stated that it was generally accepted that animals on an elevated plane of nutrition expressed better resistance to infection and to disease. Studies have shown that dietary protein influences gastrointestinal nematode establishment and expression of parasitic diseases including ovine haemonchosis (Abbott *et al*, 1985a,b). Additionally Abbott *et al* (1986a,b) showed that three month old lambs given a low protein diet and infected with a single acute dose of 350 *H. contortus* per kg bodyweight, suffered more severe clinical

disease than equivalent lambs given a higher protein diet despite having similar worm burdens and gastrointestinal plasma losses.

Further studies in ovine haemonchosis (Abbott *et al*, 1988) revealed that continuous low trickle infection (200 *H. contortus* larvae per head thrice weekly) in lambs fed a high protein diet allowed the development of immunity in contrast to lambs fed low protein diets infected under an identical regime, none of which developed resistance and which had a significantly greater worm burdens at slaughter. The findings of those authors supported the contention of increased susceptibility to infection in cobalt deficient and depleted lambs which may, in the present study, have been compounded by inadequate dietary protein intake due to inappetance. Abbott *et al* (1988) however reported equal pathogenicity of infection with both high and low protein diets although higher faecal egg counts and reduced survivial occurred in the low protein group as in the present study.

Studies by Bown *et al* (1986) in which post-ruminal casein infusion reduced mean faecal egg output and parasite burden at slaughter in *T. colubriformis* infected sheep (3000 larvae per day x 12 weeks) confirmed a role for protein intake in resistance to parasitic infection. No direct quantitative comparison of the effects of varying protein intake between groups of lambs in the present study was possible due to the difficulties and demands of using suitable pair-fed control lambs in an experiment of this size. The contribution of low protein intake to increased pathogenicity of the helminth infection in the present study cannot however be ignored.

3.4.6 Haematology

PCV data (Table XII, Appendix I), showed no significant difference between infected and non-infected sub-groups of the same cobalt status other than on an isolated occasion 107 days PI. These findings disagree with those of Horak and Clark (1964) who reported a marked decrease in PCV level of *O. circumcincta* infected sheep. This was probably due to the comparatively low *O. circumcincta* infection rate adopted in the present study (6000 L3 per week). Cobalt status of the various groups may also be important since only supplemented lambs which received a cobalt supplement regularly had mean PCV values significantly higher than either deficient or depleted lambs on a total of five occasions, 14, 35, 65, 72 and 107 days PI. Thus normochromic, normocytic anaemia in cobalt deficient sheep as reported by Smith and Loosli (1957), Gawthorne *et al* (1966) and MacPherson *et al* (1976) was confirmed in deficient and depleted lambs in this experiment and may have masked any parasitic effect on PCV.

Differential white cell counts in non-infected lambs of varying cobalt status remained within normal ranges and did not differ significantly from each other on any sampling occasion. Tizard (1986) stated that "eosinophils, attracted to the site of helminth invasion by the eosinophil chemotactic factor of anaphylaxis (ECF-A) were mobilised from the body's pool of eosinophils and their resultant release in large numbers into the circulation was the reason for characteristic circulatory eosinophilia associated with helminth infestations". Persistent eosinophilia was noted among *O. circumcincta* infected lambs of all three infected groups in this experiment in agreement with early

recordings of peripheral blood eosinophilia by Andrews (1962, after Miller, 1986a), later confirmed in sheep infected with trichostrongyles (Gallacher, 1963, after Watson, 1986) and further described by Huxtable and Rothwell (1975) in *T. colubriformis* infected sheep.

Eosinophilia noted in infected lambs in the present experiment seemed however to be extended in duration in all experimental groups as reported in section 3.3.5 of this chapter. Maintained eosinophilia, particularly among depleted and deficient lambs may be explained by the absence of the 'self cure' phenomenon, as first described by Stewart (1953) in *H. contortus* infected sheep. Relatively higher plasma pepsinogen levels, worm egg counts and TWC's in casualty cobalt deficient lambs and slaughtered depleted lambs compared to sufficient controls indicate the continued establishment of the incoming larval challenge among deficient and depleted lambs and presumably resulted in the persistent eosinophilia previously described by Tizard (1986). Helminth establishment in sufficient controls was reduced in terms of lower TWC, plasma pepsinogen and worm egg counts. Previous studies examining the effect of cobalt deficiency on parasitic infection in sheep did not include haemotological examination and thus no comparisons can be made with previous studies.

3.4.7 Non-Specific Immune Effect

Boyne and Arthur (1979) first demonstrated impaired killing ability in neutrophils of micronutrient (selenium) deficient ruminants (cattle). Subsequent investigation revealed similar leukocyte malfunction in copper deficient cattle and sheep (Boyne

and Arthur, 1981, 1986 and Jones and Suttle, 1981). Fisher and MacPherson (1986) revealed impaired candidacidal activity in neutrophils from cobalt deficient ewes and Wright *et at* (1982) and later MacPherson *et al*, (1987) recorded similar findings in cobalt deficient cattle. Similar impaired candidacidal activity was found in netrophils from cobalt deficient lambs in this experiment although the initial difference in killing index (KI) between sufficient and deficient groups was not as large as the 50% cited by Fisher and MacPherson (1986) in ewes. A rise in KI in both deficient lambs and sufficient counterparts early in the experiments may be explained by the continued development of functional immunity, similar to that described by Tizard (1986) in lambs in the present study, which were aged 3-4 months at the start of the experiment. This may also explain the absence of a significant difference between KI's of sufficient and deficient lambs in the first part of the experiment.

Subtle changes in test conditions, such as the impairment reported by Murata *et al* (1987) in bovine netrophils from animals subjected to road transportation and lairage stress, may also have occurred between sampling dates in the present study and may explain the observed variation in KI experienced both within and between groups on a week to week basis.

Stinnett (1983), working with human subjects, cautioned that 'overkill' of pathogens by polymorphonuclear leukocytes (PMN) must surely limit inferences drawn from KI deficits of less than one half of log activity and that subjects not meeting that criterion were probably not compromised in terms of immune response.

The same author also stated however that the NFT test remained a useful primary indicator of cellular immune function. Using this criterion, candidacidal impairment in cobalt deficient lambs in the present study would not be regarded as 'compromised' but extrapolation here, is limited by species differences.

Fisher (1988) speculated that since neutrophils were important factors in phagocytic and lytic activity, reduction in candidacidal acitivity in neutrophils of cobalt deficient ewes would have an adverse effect on host immune response. The same study supported the findings of reduced candidacidal activity in cobalt deficient and depleted lambs found in the present experiment.

Depression in KI in deficient ewes prior to sub-clinical cobalt or Vitamin B_{12} mediated depression of energy input or balance, in the absence of liveweight effects, led Fisher (1988) to propose that a deficit in cobalt or Vitamin B_{12} supply to the neutrophil may be the cause of impaired activity. Boyne and Arthur (1985a) had previously noted in selenium deficient calves that the hexose monophosphate shunt (HMPS) may have been inhibited. This was measured by glucose-6-phosphate dehydrogenase activity and reduced oxygen consumption which caused attenuation of the phagocytic respiratory burst and consequently reduced free radical generation of hydrogen peroxide (H_2O_2) and superoxide (O_2^-). The same authors measured superoxide production in the conversion of NBT to formosan during phagocytosis *in situ* in neutrophils in a blood film which resulted in the inclusion of blue formosan granules in the neutrophil and using this technique demonstrated significant impairment in selenium deficient calves.

Measurement of NBT conversion in the present experiment did not demonstrate any clear trends in dOD either between status groups or due to infection. Isolated significant results which occurred were probably due to biological or individual animal variation since standard errors in those groups were similar. The hypothesis that impaired superoxide production may not be the mechanism causing functional impairment of killing ability in neutrophils from cobalt deficient animals was indirectly supported by Boyne and Arthur (1985a) who contended that GSH-Px and glutathione reductase in selenium deficient ruminants may be needed to generate NADPH from which superoxide and other free radicals are derived. Suttle and Jones (1989) proposed that in the case of selenium deficient animals, freeze-trapping of free radicals derived from stimulation and their assay by electron spin reasonance (ESR) may resolve the issue.

3.4.8 Immunity to *Ostertagia circumcincta* Infection

The measurement of IgG antibody to a crude antigen of third larval stage *O. circumcincta* in this experiment revealed several interesting phenomena in both infected and non-infected groups of the same cobalt status and infected groups of varying cobalt status. In most groups falling ELISA titres which recovered in the early part of infection, can be explained in terms of decreasing levels of maternally derived antibody (providing passive immunity) consistent with the earlier findings of Smith (personal communication).

Comparison of infected and non-infected sub-groups of the same cobalt status must be viewed with the knowledge than non-infected

lambs also showed an elevation in measured titre against *O. circumcincta*, greater than their initial maternally derived level. Since those lambs were proven free of infection as indicated by plasma pepsinogen and the absence of strongyle eggs in faeces this indicated a non-specific immune response in those lambs.

This can possibly be explained by the crude nature of the larval antigen used in this assay which might have encouraged measurement of a non-specific component of the immune response for example to *E. coli* infection possibly due to shared antigens (Smith, personal communication).

However serology did indicate that titres from infected lambs were significantly elevated in comparison to non-infected controls after 63 and 85 days PI suggesting that the immune response as measured in this assay contained a significant specific component indicative of infection. Early heightened serological response of cobalt deficient infected lambs which, from parasitological data, appeared to allow a greater establishment of incoming larvae than sufficient controls, suggested a greater antigenic stimulus in deficient lambs responsible for the early peak detected in that group. These findings confirm previous reports that ELISA measurement of titres against *O. ostertagia* in infected cattle did not measure immunity per se but were reflective of the degree of pathogenic infection (Mitchell, personal communication).

A similar magnitude of response to *O. circumcincta* antigen in depleted and sufficient lambs in the present study despite the

clear differences in worm burdens between the two groups is in agreement with the findings of Yong *et al* (1985) who detected no significant difference in peripheral antibody response to *Trichostrongylus* antigens in copper deficient and supplemented lambs in their experiment.

The findings in the present study confirm the views of Sinski (1975) that serum antibody against nematode infection may not be directly related to any "resistance phenomena", although he conceded that reaginic antibodies responsive to *O. circumcincta* excretory-secretory (ES) antigens were found in serum by Hogarth-Scott (1969). Sinski and Bezubik (1980) later confirmed Sinski's earlier (1975) suggestion and concluded that resistance may not be related to serum antibody titres particularly where infection occurred via mucosal surfaces. Smith *et al* (1981) implicated immune mechanisms operating in the abomasal mucosa in acquired immunity to *O. circumcincta* and later confirmed the importance of mucosal IgA, IgG and IgM isolated from the abomasal lymph gland in this response. A later series of studies not only measured differences between infected and non-infected sheep in this way, but also demonstrated differences in the local immune response but also illustrated the adoptive passive transfer of immunity to *O. circumcincta* infection to parasite naive sheep using lymphocytes (Smith *et al*, 1983a,b,c, 1984, 1985a, 1986, 1987).

In the present study the effectiveness of the cellular immune response in cobalt deficient lambs may be impaired as indicated by NFT results. This broad contention is supported by Yong *et al* (1985) who proposed that copper deficiency could have a role in

cell mediated immunity to *Trichostrongylus* spp. since lymphocyte stimulation indices (LSI) were impaired in copper deficient lambs in comparison to normal controls.

3.4.9 Response to Vaccination

Functional immunity was also assessed using the serological response to *C. tetani* toxin as a measure of the response to vaccination. Low maternal cobalt status did not adversely affect the transfer of passive immunity since pre-vaccination titres of all lambs were similar. This contrasts with the findings of Fisher (1988) that immunoglobulin transfer from cobalt deficient ewes was significantly lower than sufficient controls. This may be due to the fact that lambs in the present study received maternal transfer of antibodies resulting from a specific priming vaccination whereas the report of Fisher (1988) assessed transfer of non-specific total immunoglobulins.

Post-vaccination titres of *C. tetani* were elevated in comparison to pre-vaccination levels significantly so for non-supplemented lambs, but were nonetheless indicative of the effectiveness of vaccination in all lambs. Significant elevation in titres of sufficient lambs, however, in comparison with both depleted and deficient lambs, clearly demonstrated an impaired response to vaccination in cobalt deficient lambs and underlines the importance of cobalt supplementation. It is important however, to realise that none of the vaccinated lambs from any status group could be defined as 'at risk' according to the criteria defined by Kerry and Craig (1979) (Table 18).

TABLE 18: Classification of Vaccinal Status

CLASSIFICATION	VACCINATION STATUS (iu/ml)
At risk	< 0.1
Poor responder	0.1 - 1.0
Normal	> 1.0

(After Kerry and Craig, 1979)

An interesting comparison in this assay suggested that the vaccine response of depleted lambs was significantly lower than sufficient controls. This contradicted previous findings in terms of production, pathogenicity of *O. circumcincta* infection and immunity, that depleted lambs matched sufficient counterparts until 50 days PI and thus suggested that protective immune responses in the present study were affected even by marginal cobalt deficiency. The duration of 'normal' vaccinal status in depleted and deficient lambs requires further study.

CHAPTER 4

THE INTERRELATIONSHIP BETWEEN SELENIUM DEFICIENCY, VITAMIN E DEFICIENCY, *Ostertagia circumcincta* INFECTION AND IMMUNE FUNCTION IN SHEEP

4.1 INTRODUCTION

Many studies exist examining the general role of selenium and Vitamin E deficiencies in the immune response (Reviews: Tengerdy *et al*, 1984, Spallholz, 1981, Kiremidijian-Schumacher, 1987). Similarly studies from Tiege *et al* (1977, 1978 and 1982), Nockels (1979), Tengerdy *et al* (1981), Colnago *et al* (1984) and Smith *et al* (1984) have highlighted the effects of selenium and Vitamin E deficiencies on the immune response to a number of diseases in a variety of species. Suttle and Jones (1989) however reported that, at that time, no published evidence existed proving that selenium status altered susceptibility of ruminants to parasitic infection. No studies have been carried out to date in ruminants examining concurrent selenium and/or Vitamin E deficiency under sustained helminth infection similar to that found in field conditions. Therefore, the experiment detailed below was designed to examine the effects of selenium and Vitamin E deficiencies in sheep infected with *Ostertagia circumcincta* under simulated field conditions on the pathogenesis of infection and on immune response, including response to vaccination.

4.2 MATERIALS AND METHODS

4.2.1 Experimental Animals

Ewes:

Ewes, similar to those detailed in Chapter 2, of normal selenium and Vitamin E status were used to provide lambs for experimentation. In contrast to Chapter 3, no attempt was made to examine

or determine the effects of low maternal selenium or Vitamin E status on lamb performance or immune response. 53 ewes produced 67 lambs similar to those described in Chapter 2 from two successive oestrus cycles of 52 and 15 lambs respectively although only the older group was used in the experiment described below.

Lambs:

Biochemical analysis revealed that an initially high dietary selenium concentration left lambs at the start of this experiment with a relatively high selenium status. A conventional 2 x 2 factorial design was adopted to examine the effects of and interactions between infection and supplementary Vitamin E in lambs in Phase I as shown in Table 19.

At that time all lambs were of a high selenium status but were deficient in Vitamin E. Thus the role of Vitamin E in parasitic infection was examined through the administration of a supplement to the first group of 16 lambs (**Vitamin E supplemented**), which was then sub-divided into infected and non-infected sub-groups (1 and 2 respectively), as listed in Table 19. The second status group termed **Non-supplemented** lambs were similarly divided into infected and non-infected sub-groups (3 and 4 respectively) but received no supplement and were Vitamin E deficient during Phase I.

TABLE 19: Summary of Experimental Groups - Phase I

Group 1 (8 lambs)	-	Vitamin E Supplemented Infected
Group 2 (8 lambs)	-	Vitamin E Supplemented Non-Infected
Group 3 (18 lambs)	-	Non-Supplemented Infected
Group 4 (18 lambs)	-	Non-Supplemented Non-Infected

All lambs received a diet low in both selenium and Vitamin E in Phase I to reduce blood and tissue selenium levels. The Vitamin E supplement given to Groups 1 and 2 initially consisted of alpha-tocopheryl acetate (Rovimix E50, Roche Products, Welwyn Garden City) absorbate (1 mg absorbate = 0.5 iu Vitamin E) given at the rate 44 mg per head per day (= 22 iu Vitamin E per head per day) and mixed in with their normal mineral supplement. This was subsequently changed 20 days PI to 88 mg absorbate per head per day plus a weekly 300 mg absorbate gelatin capsule given orally.

Group sizes were unequal initially when the number of non-supplemented lambs was approximately twice that of supplemented lambs to allow the non-supplemented groups, 3 and 4, to be sub-divided into appropriate groups during Phase II. In Phase II, the control groups (1 and 2) remained supplemented with Vitamin E as before and additionally received a selenium supplement of 3 mg (as sodium selenite) per head. At the beginning of this phase non-supplemented, infected and non-infected sub-groups were ranked in order of GSH-Px activity. Those with the lowest con-

centrations and thought to be the most likely to become selenium deficient were then supplemented with Vitamin E. The upper half of the rank were thought relatively unlikely to become selenium deficient and were given the selenium supplement detailed above but were allowed to remain Vitamin E deficient. Group assignments for Phase II are summarised in Table 20.

TABLE 20: Summary of Experimental Groups - Phase II

Group 1 (7 lambs)	-	Vitamin E Supplemented Infected
Group 2 (8 lambs)	-	Vitamin E Supplemented Non-Infected
Group 3A (8 lambs)	-	Vitamin E Supplemented **Selenium Deficient** Infected
Group 3B (7 lambs)	-	Selenium Supplemented **Vitamin E Deficient** Infected
Group 4A (8 lambs)	-	Vitamin E supplemented **Selenium Deficient** Non-Infected
Group 4B (8 lambs)	-	Selenium Supplemented **Vitamin E Deficient** Non-Infected

Normal husbandry procedures were applied to all animals in this experiment, including vaccination against clostridial disease at 8 and 13 weeks of age (Ovivac-P, Hoechst Animal Health). Assignment to groups in Phase I was on a restricted random basis such that sex and weight differences between groups were minimised.

4.2.2 Diet and Ration Formulation

Ewes which provided lambs for this experiment were fed a diet adequate in selenium and Vitamin E (Super Ewebol, BOCM Silcock, Renfrew, containing 0.2 mg selenium/kg and 22 iu Vitamin E/kg). Also the pre-experimental phase diet fed to the lambs was relatively high in selenium and Vitamin E (Elite Lamb Weaner, MacGregors Animal Feeds, Edinburgh), containing 0.2 mg selenium/kg and 10 iu Vitamin E/kg. During the experimental period, however, all lambs were fed a ration deficient in both selenium and Vitamin E, which consisted of Timothy Hay (0.036 ± 0.006 mg Se/Kg (DM) and trace amounts of Vitamin E), and locally grown barley, (0.0187 ± 0.008 mg Se/Kg (DM) and 4.5 ± 0.84 iu Vitamin E/kg (DM)). The protein source employed was intitially prairie meal but as it had unacceptably high levels of selenium, (0.231 ± 0.07 mg Se/Kg (DM)) it was replaced by urea fed at the rate of 4.34g per head per day. Hay and water were offered *ad libitum* during the experiment and concentrate was supplied twice daily at a rate chosen to meet the protein and energy requirements for 0.1 kg daily liveweight gain (ARC, 1980). Minerals were also added to concentrate mixtures using the same guidelines. Dietary composition was monitored and rations were adjusted as necessary to maintain the same level of production.

4.2.3 Experimental Parameters

All lambs were approximately 12 weeks of age at the start of the experiment, 27 July 1988, (= Day 0). Infected lambs received 6000 L3 *O. circumcincta* per week as 3 x 2000 L3 oral doses. Dosage was based on a 15 kg lamb eating 3.5 kg grass per day (wet

weight) contaminated with 300 L3 *O. circumcincta* per kg (wet weight) (Armour, personal communication). The first phase of the experiment continued for 11 weeks and Phase II continued for a further 5 weeks. Thereafter, 12 animals selected for slaughter for TWC assessment were kept in metabolism cages for a further 2 weeks to permit collection of faecal material for larval collection. Details of Experiment 2 are summarised in Table 21.

4.2.4 Monitoring Procedures

Lambs were penned three times per week for sample collection and administration of larvae. Liveweight was measured fortnightly, fresh faecal samples were taken weekly from the rectum to assess worm egg count and weekly blood samples were used for biochemical and serological monitoring procedures including GSH-Px determination, Vitamin E assay, measurement of creatine kinase activity (CK) and determination of plasma pepsinogen levels.

TABLE 21: Summary of Experimental Parameters

Date of 1st Infection	:	27 July 1988
Age of Lambs	:	12 weeks
Duration of Experiment Phase I Phase II	:	16-18 weeks 11 weeks 5 weeks + 2 weeks for faecal collection
Infective Dose	:	3 x 2000 L3 *O. circumcincta* per week
Date of Sacrifice	:	6 December 1988
Selenium Supplement	:	3 mg Se as sodium selenite
Vitamin E Supplement	:	44 iu Vitamin E per head per day (as alpha-tocopheryl acetate absorbate) + 150 iu Vitamin E (as above) in gelatin capsule

White cell function tests were determined in alternate weeks on occasions other than normal blood sampling times. PCV determination and differential white cell counts were carried out on blood samples taken for white cell tests. Statistical analysis in this experiment was as listed in Chapter 2. All monitoring procedures carried out in this experiment are summarised in Table 22.

TABLE 22: Summary of Monitoring Procedures

Biochemistry	-	Vitamin E (weekly) GSH-Px (weekly) Vitamin B_{12} (periodically) Creatine Kinase Activity (weekly)
Clinical Pathology	-	Post Mortem Examination Histopathology Liver Enzyme, BUN and NEFA Assessment Liver Copper and Cobalt Determination
Production	-	Liveweight (fortnightly)
Parasitology	-	Plasma pepsinogen (weekly) Worm Egg Count (weekly) Total Worm Count (at sacrifice) Larval Collection from Faeces
Haematology	-	Differential White Cell Count (weekly) PCV Determination (weekly)
Immunity	-	White Cell Function Tests - Neutrophil Function Tests (alternate weeks) - NBT Reduction (alternate weeks) ELISA - *O. Circumcincta* antigen (fortnightly) - *C. tetani* antigen (2 weeks post second vaccination)

4.3 RESULTS - PHASE I

4.3.1 Biochemistry

Vitamin E:

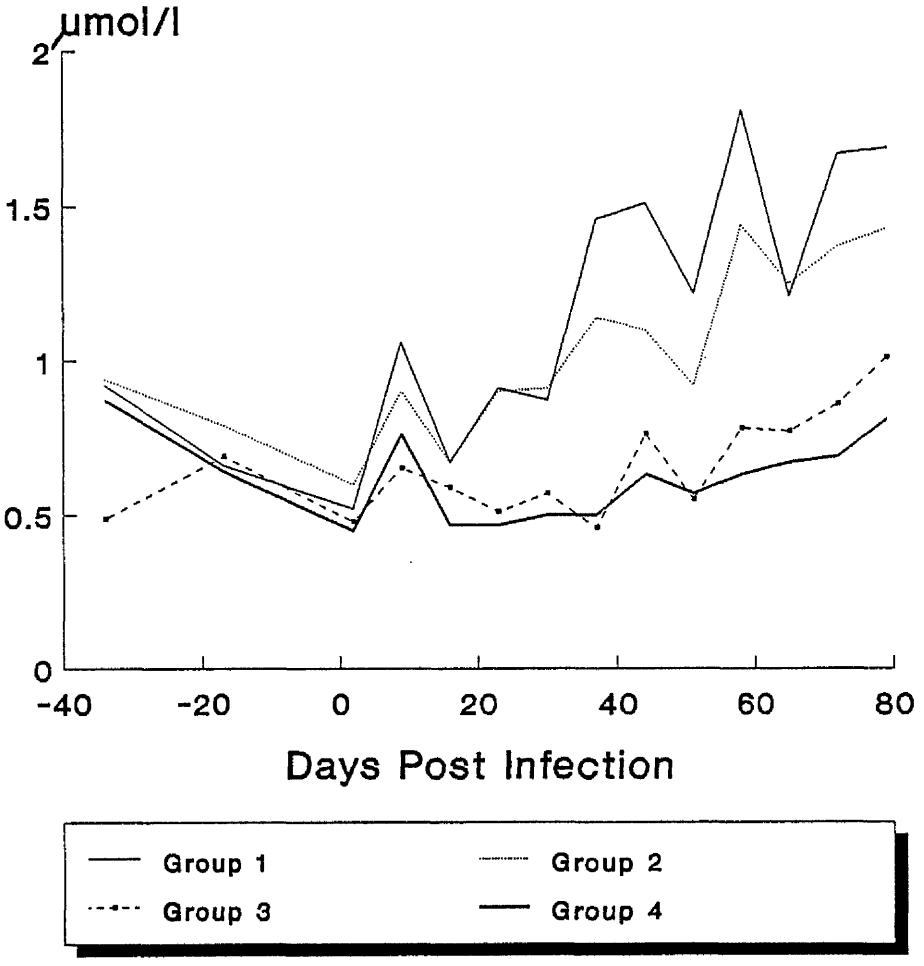
Group mean Vitamin E concentrations of lambs are illustrated in Figure 27 and listed in Table II, Appendix II. All lambs had mean Vitamin E levels less than 1.0 µmol/l, the lower limit of normality, before day 0, ranging from 0.49-0.94 µmol/l. Mean Vitamin E levels of Groups 3 and 4 remained less than 1.0 µmol/l throughout this phase of the experiment and ranged from 0.45-0.86 µmol/l.

Initial supplementation of Groups 1 and 2 increased mean Vitamin E concentrations to values exceeding 1.0 µmol/l in Group 1 and to a mean value of 0.9 µmol/l in Group 2, by 9 days PI which fell thereafter. A change in method of supplementation however caused mean Vitamin E levels to exceed 1.0 µmol/l after 30 days PI and with a single exception at 51 days PI for Group 2, they remained above this level for the duration of Phase I with mean values ranging from 1.1-1.81 µmol/l.

The sustained elevation in mean Vitamin E level in the supplemented Groups (1 and 2) caused their values to be significantly greater than non-supplemented controls (3 and 4) from 37 days PI until the end of this part of the experiment ($P < 0.05$). No significant difference in mean Vitamin E level attributable to infection was found in any status group.

FIGURE 27

Mean Vitamin E Concentration Lambs



Phase I

Group 1: Vitamin E Supplemented, Infected Lambs

Group 2: Vitamin E Supplemented, Non-Infected Lambs

Group 3: Non Supplemented, Infected Lambs

Group 4: Non Supplemented, Non-Infected Lambs

GSH-Px:

Figure 28 shows the mean GSH-Px levels in the four groups with means and standard errors listed in Table III, Appendix II. It was evident, even in the pre-infective phase, that GSH-Px levels in all groups were very much in excess of the lower normal limit of 27 U/ml. Initial levels in all groups ranged from 136-187 U/ml and still exceeded 100 U/ml 30 days PI. However, the selenium status of all groups declined progressively thereafter except for an isolated peak 51 days PI in Groups 1 and 2. Mean GSH-Px levels did not fall below 27 U/ml limit, the lowest recorded value being 39 U/ml in Group 3, 72 days PI.

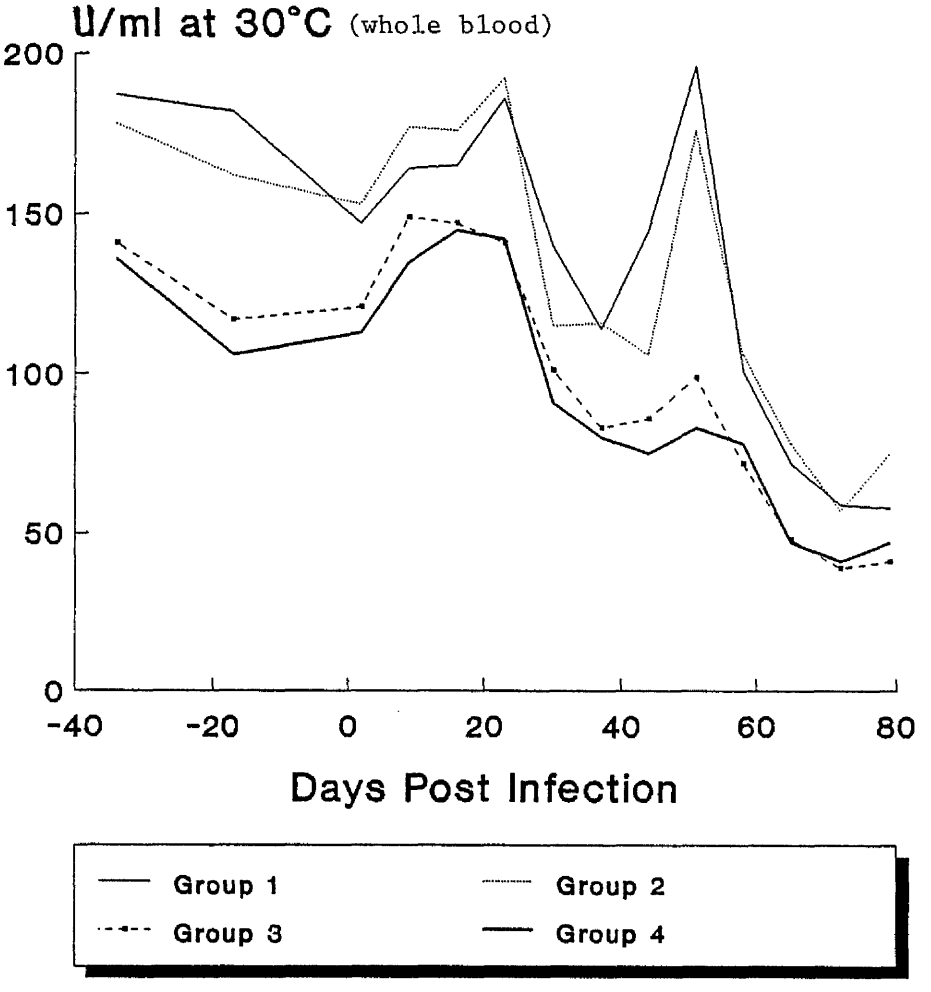
Mean GSH-Px values of non-supplemented lambs were significantly lower ($P < 0.05$) than Vitamin E supplemented controls for the whole of Phase I with two exceptions at 16 and 44 days PI although, as stated above, animals did not become deficient during this phase. No significant difference in mean blood GSH-Px level attributable to *O. circumcincta* infection was found at any time in this experiment in either status group, supplemented or non-supplemented.

Creatine kinase:

All lambs, Groups 1-4, showed CK activity greater than normal (< 50 iu/l at 30°C), in the pre-infective phase and for the duration of the experiment. In Groups 1 and 2, initial levels of 303 and 448 iu/l respectively increased to 715 and 650 iu/l at 9 days PI but thereafter gradually fell to levels usually less than 200 iu/l. Groups 3 and 4 however, had initial mean levels of 1310

FIGURE 28

Mean Glutathione Peroxidase Activity
Lambs



Phase I

Group 1: Vitamin E Supplemented, Infected Lambs

Group 2: Vitamin E Supplemented, Non-Infected Lambs

Group 3: Non Supplemented, Infected Lambs

Group 4: Non Supplemented, Non-Infected Lambs

and 1143 iu/l respectively, and although varying thereafter in the range 433-2662 iu/l were always greater than supplemented controls as shown in Figure 29 and listed in Table IV, Appendix II.

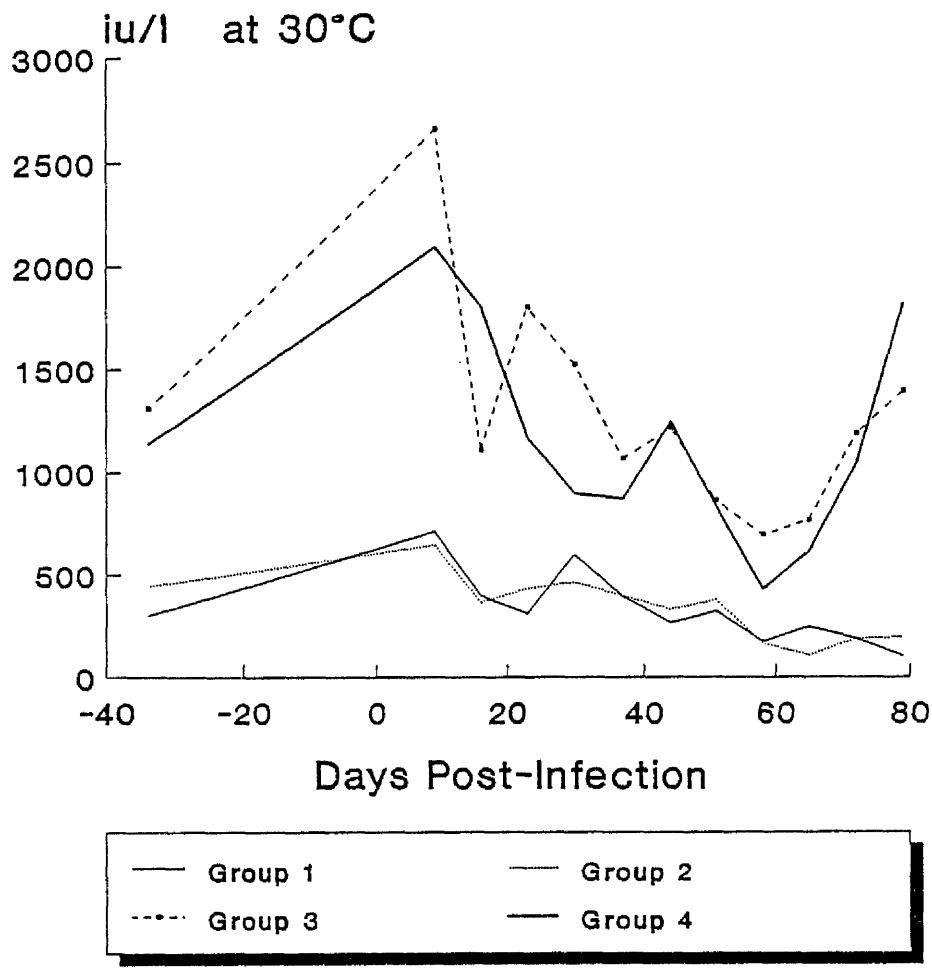
Statistical analysis revealed that mean CK levels in Vitamin E supplemented lambs were always significantly lower ($P < 0.05$) than non-supplemented controls except on two isolated instances in the early part of the experiment, 16 and 30 days PI. Additionally, no significant difference in CK activity attributable to infective status was found.

Cobalt status (Vitamin B_{12} and MMA):

Mean Vitamin B_{12} concentrations listed in Table V, Appendix II, indicated adequate cobalt status in all groups, ie > 400 ng/l, in the early part of the experiment with values ranging from 655-740 ng/l at 9 days PI. Vitamin B_{12} level thereafter fell to a mean of 473 ng/l for all animals (range 379-632) by 30 days PI. Later determination showed a reduction in mean values in all groups below the 400 ng/l threshold by 51 days PI and remained in the marginal zone for the remainder of this phase of the experiment. Serum MMA levels of all groups indicated normal cobalt status in the early part of the experiment but marginally deficient status later with mean values of 5.97 µmol/L (range 4.10-7.56 µmol/L) 35 days PI and 9.11 µmol/L (range 7.38-10.36 µmol/L) 63 days PI for all lambs. Statistical analysis however, revealed no significant difference in mean MMA concentration between any of the groups in Phase I. Mean MMA concentrations and standard errors are listed in Table VI, Appendix II.

FIGURE 29

Mean Creatine Kinase Activity Lambs



Phase I

Group 1: Vitamin E Supplemented, Infected Lambs

Group 2: Vitamin E Supplemented, Non-Infected Lambs

Group 3: Non Supplemented, Infected Lambs

Group 4: Non Supplemented, Non-Infected Lambs

4.3.2 Clinical Findings

Deaths occurred in both supplemented and non-supplemented groups and summaries of *post mortem* findings and other relevant details are listed in Table VII, Appendix II. Only one casualty occurred in the supplemented infected group. This occurred 10 days PI and the affected lamb was euthanased *in extremis* despite having been previously treated for white muscle disease (WMD) (Plate 4). *Post mortem* examination revealed extensive gross lesions consistent with WMD although the heart muscle remained unaffected. Plated 5, 6 and 7 illustrate and describe the muscle changes found in this animal. Histopathological examination confirmed the presence of widespread lesions associated with sub-acute muscular dystrophy.

Typical pathological changes associated with *O. circumcincta* infection were noted grossly in the abomasum and confirmed by histopathological examination. Pale, swollen kidneys were noted, as illustrated in Plate 8 and histopathological examination revealed nephrosis, with dilation of kidney tubules. Selenium status was normal (GSH-Px = 153 U/ml) but Vitamin E concentration was low (0.88 µmol/l).

Five deaths occurred in the non-supplemented status groups, comprising four infected lambs and one lamb from the non-infected sub-group. Two Group 3 lambs were found dead 12 days PI and showed lesions suggestive of myopathy. Serum Vitamin E concentration was low in both cases 0.35 and 0.82 µmol/l respectively and CK levels were also greatly elevated at up to 30000 iu/l. Both lambs showed evidence of pulmonary congestion and

PLATE 4: WMD Affected Lamb, Unable to Stand. Note 'Knuckled-over' Fetlock

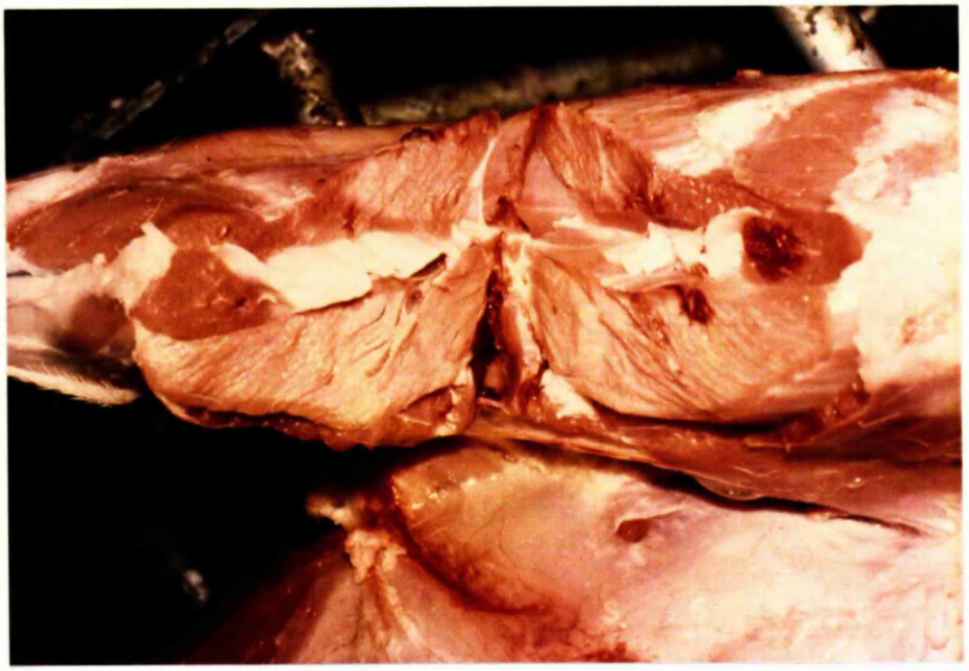


PLATE 5: Affected Hind Limb Muscles Showing Pallor

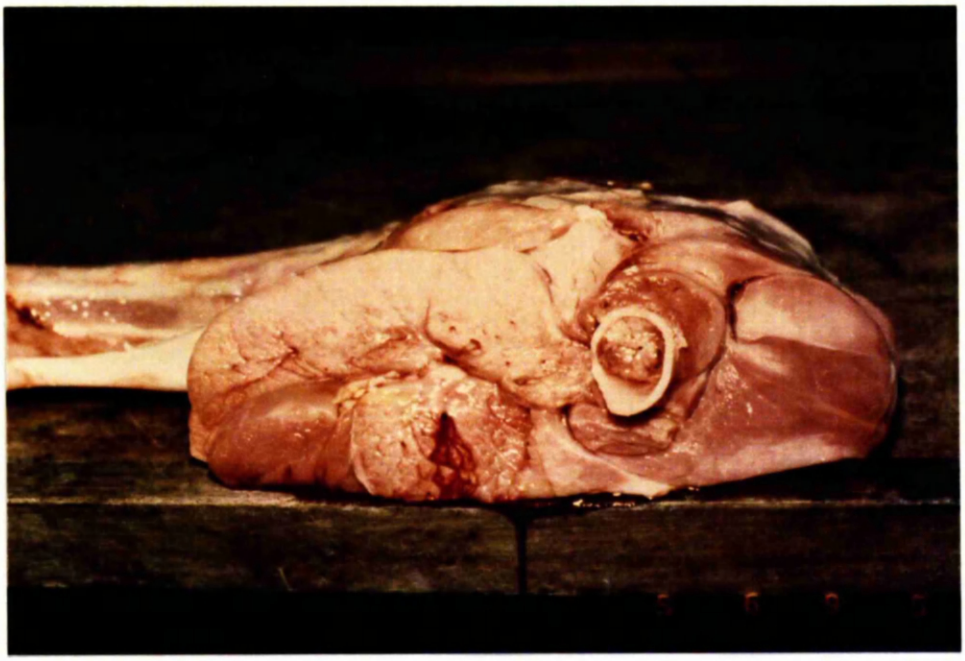


PLATE 6: Cross Section of Hind Leg Showing Contrast Between Normal and Affected Muscle Groups

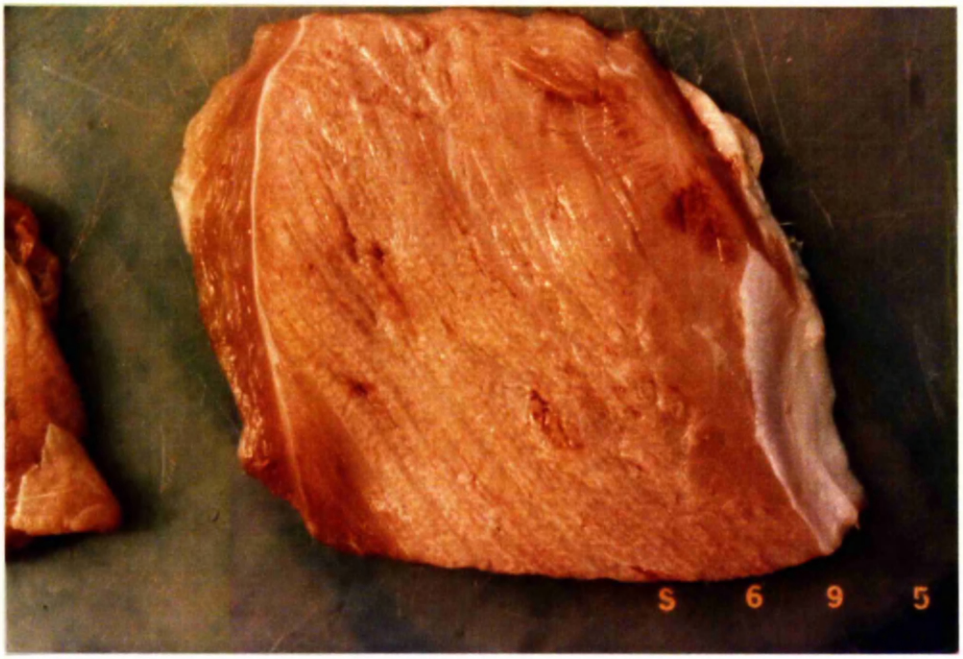


PLATE 7: Longitudinal Section of Affected Muscle Showing Striations and Pallor

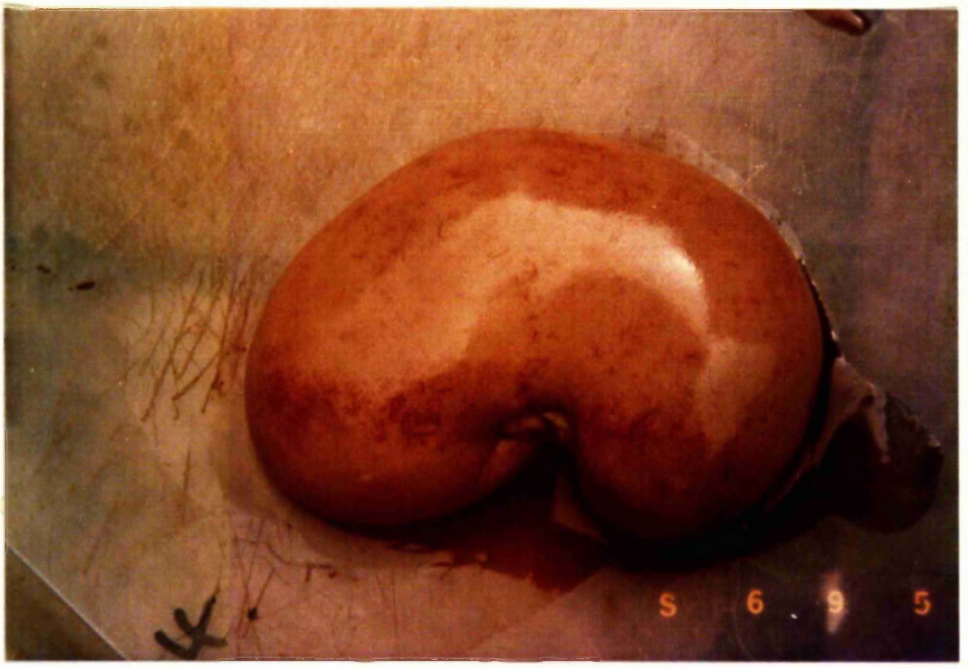


PLATE 8: Nephrosis Noted in Vitamin E - WMD Casualty

one had pale soft kidneys although both revealed abnormally high BUN levels of 23.0 and 25.2 mmol/l. *O. circumcincta* infection had not become patent and although early, characteristic signs of infection were found no estimation of total worm burden was carried out.

A third casualty lamb from Group 3, which died 24 days PI and also showed pale hind limb musculature but although CK was slightly elevated at 300 iu/l, Vitamin E concentration was normal, 2.29 μmol/l. Hepatic congestion and anaemia were noted *post mortem* and biochemical analysis revealed elevated SGOT activity while liver copper and cobalt levels were normal, 2099 μmol/kg and 0.52 μg/g respectively. The abomasal mucosa showed evidence of parasitic infection but no worm burden estimation was carried out. Beta and epsilon *Clostridial* toxins, consistent with enterotoxaemia, were recovered from gut contents. The fourth casualty, which was from the non-supplemented, non-infected sub-group, Group 4, died 26 days PI and was found to grossly resemble a case of enterotoxaemia but *Clostridial* toxin could not be demonstrated in bowel filtrate. Biochemistry indicated uraemia (BUN = 17.8 mmol/l), elevated CK activity (336 iu/l) and raised SGOT levels, 71 iu/l.

The fifth casualty of the non-supplemented status group occurred 90 days PI and showed pneumonia in the apical and ventral lobes of both lungs from which *P. haemolytica* (Serotype A2) and *Mycoplasma ovipneumoniae* were isolated. Other tissues appeared normal and biochemical analysis revealed that liver copper levels were normal at 2811 μmol/kg while liver cobalt was marginal at

0.1 µg/g. Although five casualties from the non-supplemented group compared very unfavourably with a single death among supplemented lambs, statistical analysis by Chi-squared and Corrected Chi-squared test (for continuity) revealed that the results could have occurred without the influence of low Vitamin E status.

Cough indices:

An outbreak of pneumonia in experimental groups was monitored by clinical scoring of affected animals. Lambs were assessed on two occasions and the mean scores listed below in Table 23. Full data is contained in Table VIII, Appendix II. Mean cough indices were not significantly different between any groups on either of the two comparison dates and no significant within group difference was detected between those dates.

TABLE 23: Mean Cough Index

GROUP	STATUS	MEAN INDEX SCORE (SE)	
		109 DAYS PI	112 DAYS PI
1	Supplemented Infected	4.29 (0.75)	3.71 (0.42)
2	Supplemented Non-infected	3.25 (0.96)	3.75 (0.86)
3	Non-supplemented Infected	2.86 (0.38)	2.50 (0.62)
4	Non-supplemented Non-infected	2.50 (0.45)	3.21 (0.45)

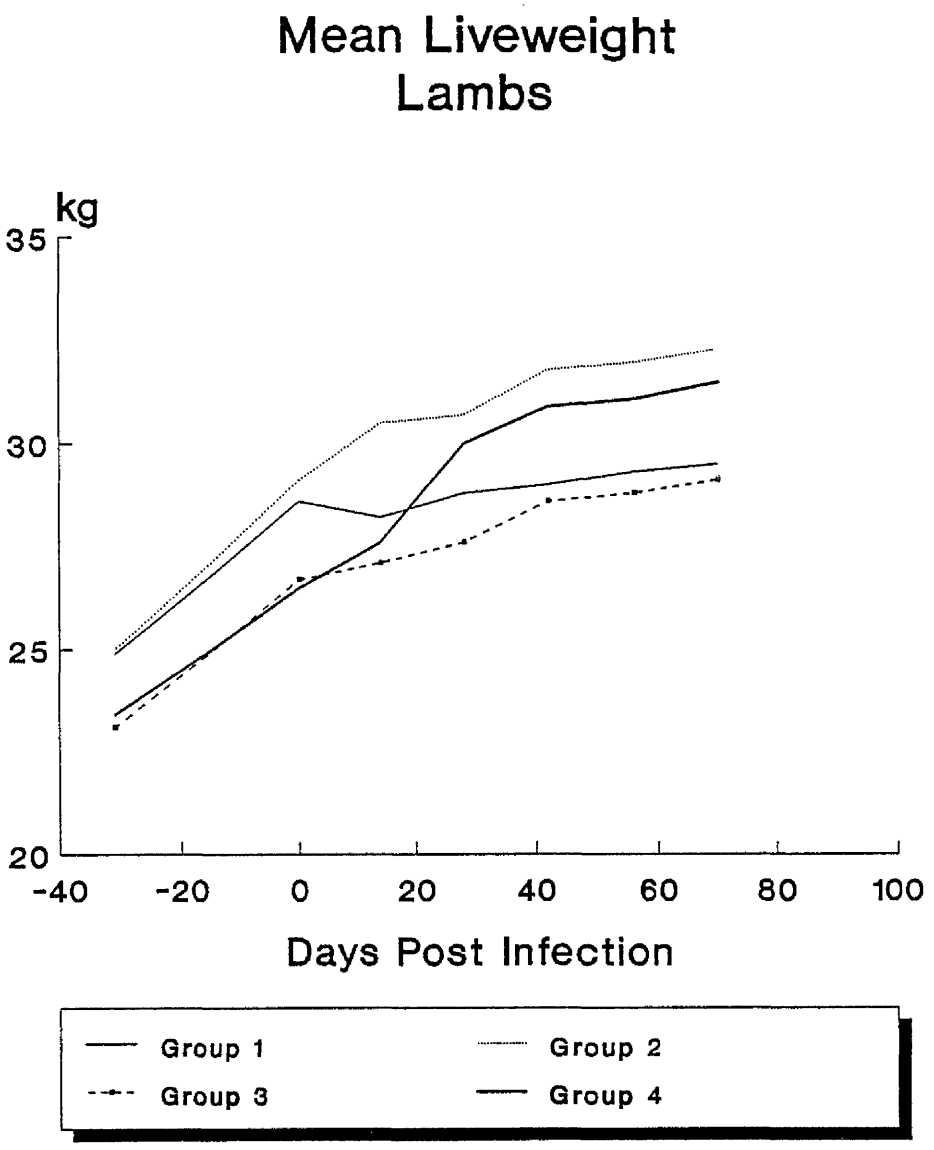
4.3.3 Production

Figure 30 shows mean liveweight of the four groups in Phase I of this experiment. All Groups were of similar, not significantly different, mean liveweight in the pre-infection phase ranging from 23.1-25 kg 31 days pre-infection and rising to the range 26.5-29.2 at day 0. After infection however, only non-infected supplemented lambs, Group 2, gained weight consistently reaching 30.5 kg, 14 days PI and thereafter continued to increase by 0.5 kg per week. Although Group 1, infected supplemented lambs, closely matched non-infected controls (Group 2) in terms of mean liveweight in the pre-infective phase, they remained 2.0 kg lighter than non-infected equivalents after infection and throughout Phase I.

Similar trends were exhibited among non-supplemented lambs, Groups 3 and 4, whereby infected lambs matched their non-infected equivalents ranging from 23.1-27.1 and 23.4-27.6 kg respectively in the pre-infection phase and early part of patency but thereafter showed a similar 2.0 kg deficit when compared to non-infected controls.

Mean liveweights from supplemented lambs exceeded their non-supplemented counterparts in the pre-infective stage but after infection, the mean liveweights of Group 4, non-supplemented, non-infected, continued to rise whereas the mean Group 1 liveweight (supplemented infected) fell away to a level similar to Group 4 around 19 days PI when a 'crossover' occurred and thereafter mean liveweights of Group 1 lambs remained consistently below Group 4. Statistical analysis revealed that no significant

FIGURE 30



Phase I

Group 1: Vitamin E Supplemented, Infected Lambs

Group 2: Vitamin E Supplemented, Non-Infected Lambs

Group 3: Non Supplemented, Infected Lambs

Group 4: Non Supplemented, Non-Infected Lambs

difference in liveweight attributable either to supplementation with Vitamin E or *O. circumcincta* infection was found in this phase of the experiment.

4.3.4 Parasitology

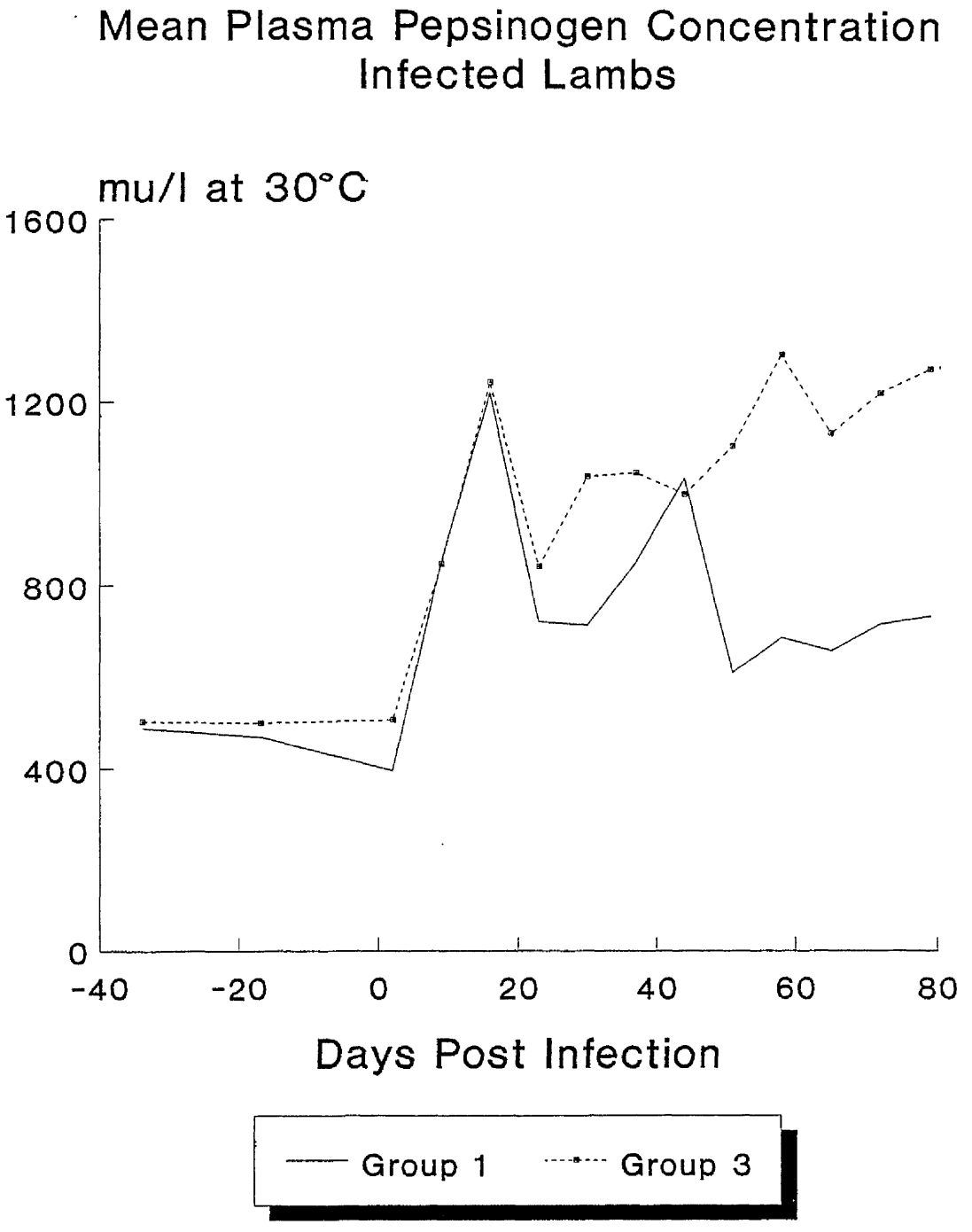
Periodic assessment of non-infected lambs to detect accidental infection measured as either a rise in plasma pepsinogen or in the appearance of strongyle eggs in faeces showed that no such infection took place.

Plasma pepsinogen:

Figure 31 shows mean plasma pepsinogen concentration of infected lambs, Groups 1 and 3. Pre-infection levels were similar to those found in non-infected lambs at around 500 mu/l (range 395-506 mu/l), subsequently these levels rose to around 850 mu/l by 9 days post infection and continued to rise to 1200 mu/l 16 days PI, the first peak in both infected groups. Values of supplemented infected lambs, Group 1, fell thereafter to around 700 mu/l and fluctuated around that level during Phase I except for an isolated value of 1034 mu/l, 44 days PI. Non-supplemented infected lambs, Group 3, fell from a similar initial peak of 1200 mu/l, 16 days PI to a mean of 842 mu/l, 23 days PI but thereafter rose to levels around 1000 mu/l until 44 days PI and maintained mean plasma pepsinogen concentrations > 1100 mu/l for the remainder of Phase I.

Statistical analysis revealed that mean plasma pepsinogen values from infected lambs were significantly greater than non-infected

FIGURE 31



Group 1: Vitamin E Supplemented, Infected Lambs

Group 3: Non Supplemented, Infected Lambs

controls from 9 days PI and for the remainder of Phase I ($P < 0.05$). Comparison of mean plasma pepsinogen levels between Groups 1 and 3 showed that no significant difference occurred until 51 days PI when pepsinogen values became significantly higher in the non-supplemented group and remained so throughout Phase I ($P < 0.05$). Mean values for each of the groups and standard errors are listed in Table X, Appendix II.

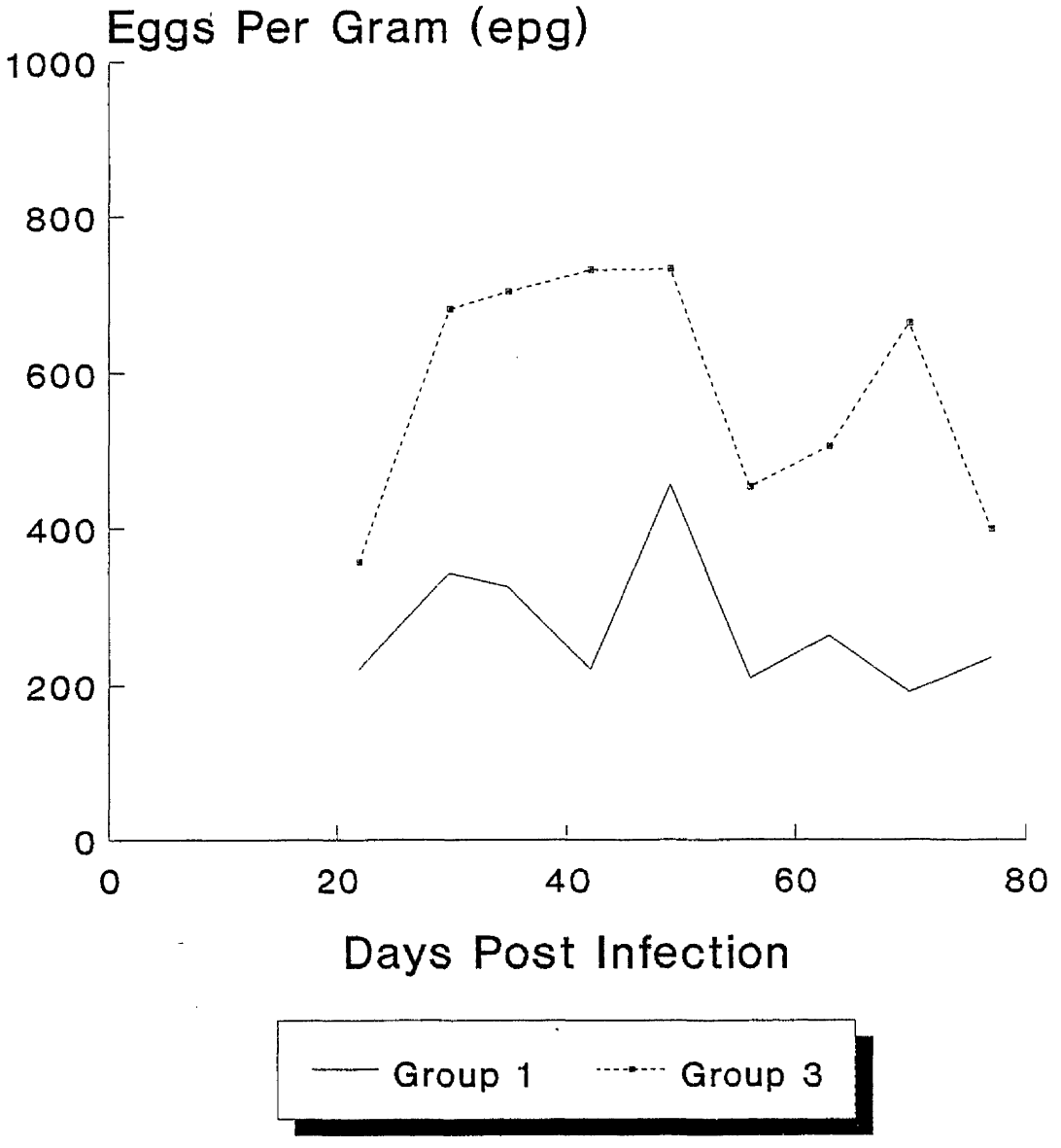
Worm egg count:

Daily faecal sampling of infected lambs immediately after the start of infection revealed that mean pre-patent periods of infected lambs were not significantly different at 18.6 ± 0.53 days in the supplemented group and 17.5 ± 0.73 for non-supplemented controls.

Figure 32 shows mean faecal worm egg counts of lambs in this experiment, and full data with standard errors appears in Table XI, Appendix II. Although initial WEC's were similar, mean counts for supplemented lambs fluctuated in the range 192-457 epg but never exceeded 500 epg while non-supplemented values remained consistently > 650 epg with two exceptions (< 500 epg) on 56 and 77 days PI. It was evident from Figure 32 that mean WEC's of non-supplemented lambs consistently exceeded those of supplemented lambs but statistical analysis showed that this difference was only significant on two occasions, 42 and 70 days PI ($P < 0.05$).

FIGURE 32

Mean Worm Egg Count
Infected Lambs



Phase I

Group 1: Vitamin E Supplemented, Infected Lambs

Group 3: Non Supplemented, Infected Lambs

Total worm counts:

Total worm count estimation was carried out on a representative sample of lambs from the two groups examined in this phase, however no analysis of those results is reported here since description of results, including the proportion of adults:larvae, worm length and sex ratios is contained in Section 4.4.3 (Total worm count (TWC)).

4.3.5 Haematology

Results thus far have only concerned Phase I but the haematology section below examines both Phases I and II since it was not possible to carry out haematological examination of white cells from all six groups in Phase II.

Packed cell volume:

Mean packed cell volumes from all animals in this experiment are listed in Table XII, Appendix II. Supplemented lambs, Groups 1 and 2, had mean values in the range 29-39%, all of which would be regarded as normal. Non-supplemented lambs had mean values of similar magnitude and in the same range. Statistical analysis revealed no significant difference in mean PCV levels on any sample date which could be attributable to infection in either Phase I or II. Similarly, no significant difference in mean PCV level resulting from Vitamin E supplementation was found other than on two isolated occasions 31 and 51 days PI ($P < 0.05$). In Phase II, mean PCV levels for Vitamin E deficient, Group 3B, and Vitamin E supplemented lambs remained in the same range and those of non-supplemented were significantly different from supple-

mented control lambs on only a single occasion 86 days PI ($P < 0.05$).

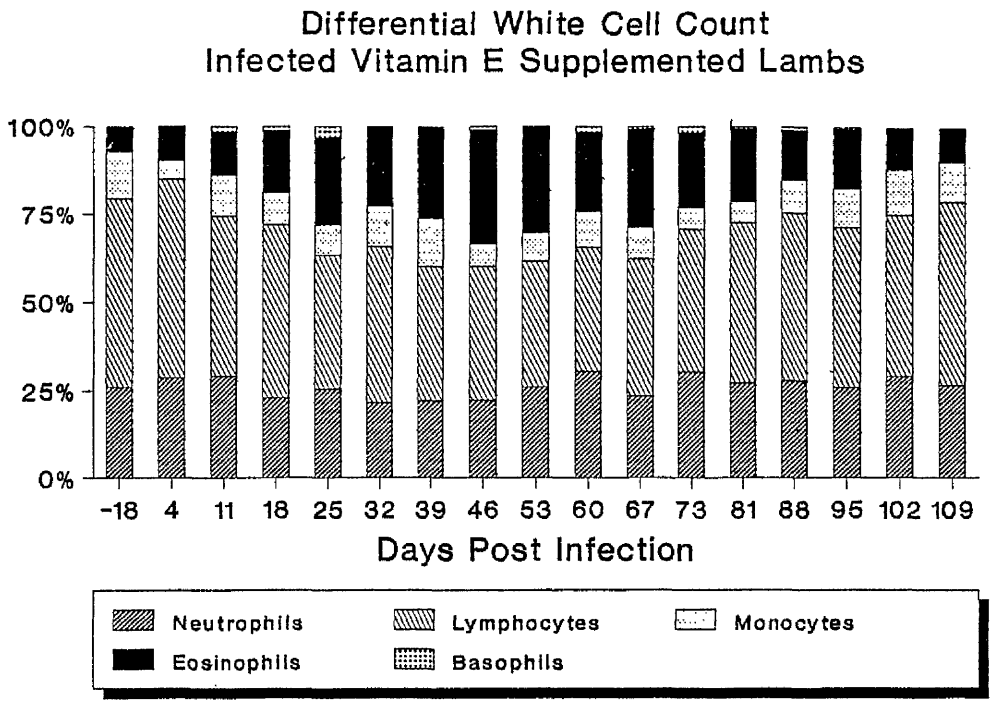
Differential white cell counts:

Differential white cell counts carried out in this experiment produced the results listed in Table XIII-XVI, Appendix II, and illustrated in Figures 33-36. Figures 33 and 34 show mean results from infected and non-infected supplemented sub-groups.

Infected supplemented controls (Figure 33) showed percentage occurrences of 21.5-30.5 and 6.0-13.75 for neutrophils and monocytes respectively. Percentage eosinophils remained below 13% until 11 days PI but rose to 25% by 18 days PI and remained in excess of 20% (occasionally > 30%), in the range 20.5-32.5, until the end of Phase I. Percentage lymphocytes fell from a prepatent level of > 50% to a low of 35%, 60 days PI although still at the lower end of the normal range (as listed in Table 15, Chapter 3). During Phase II the percentage of lymphocytes in Group 1 lambs gradually rose from a mean value < 40% to 52.25%, 109 days PI, a level similar to that found in that group at the start of the experiment. Restoration of percentage lymphocytes was accompanied by a drop in the percentage eosinophils from around 20% at the end of Phase I to 9.75%, 109 days PI. Few basophils in blood smears from this group were found ranging from 0-3.25%, although occurrence among infected lambs was greater than in non-infected counterparts.

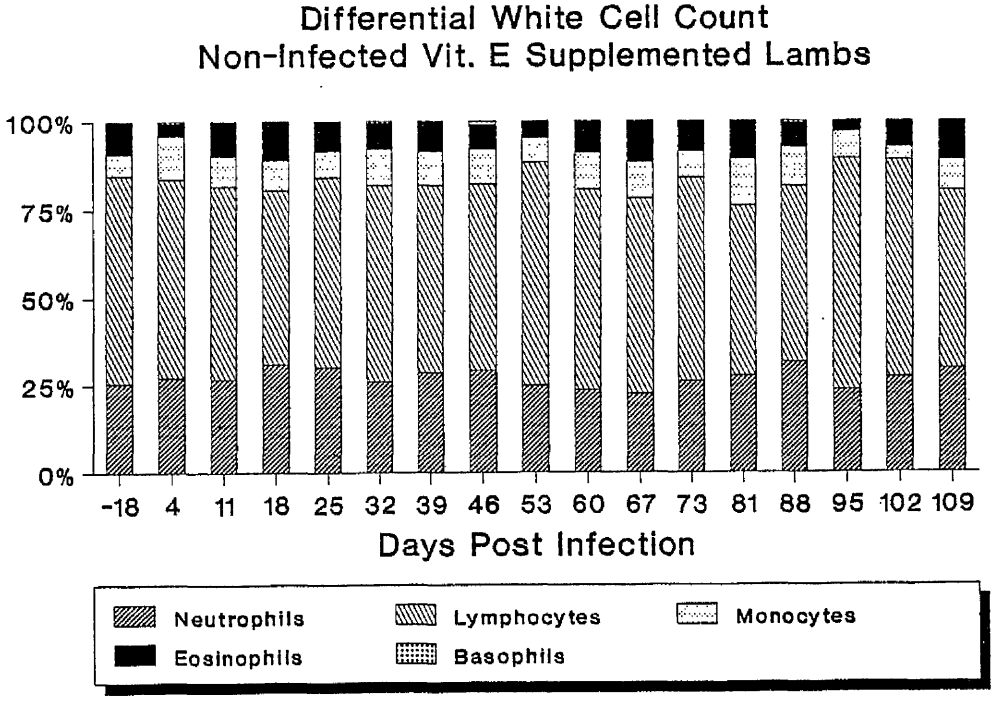
Statistical analysis revealed that percentage eosinophils in infected supplemented lambs was significantly higher ($P < 0.05$)

FIGURE 33



Phases I and II

FIGURE 34



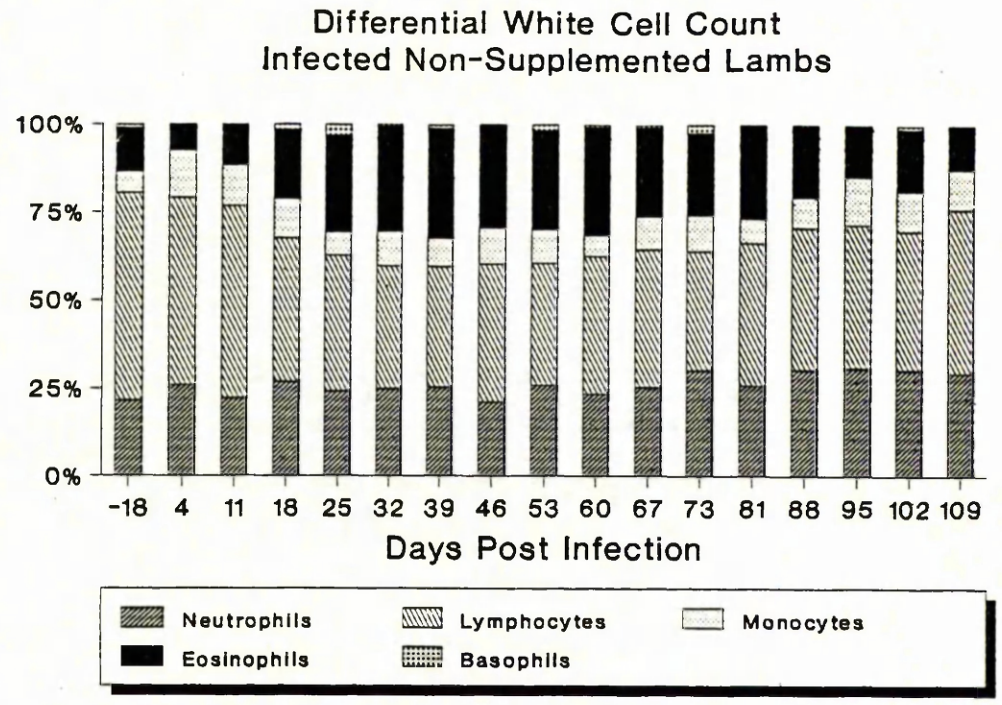
Phases I and II

than non-infected controls from 18 days PI until 95 days PI when the reduction in percentage eosinophils in Group 1 lambs restored their level to normal. Correspondingly a significant difference in mean percentage lymphocytes of supplemented infected lambs over non-infected controls from 29 days PI declined and was not significant by 81 days PI. No significant difference from normal was detected at any time in any other white cell fraction. Mean differential white cell count and standard errors are listed in Table XIII, Appendix II.

Non-infected supplemented lambs, Group 2, had mean percentages in the ranges, 22.5-31.5, 48.5-65.75, 3.75-13.25, 3.25-11.5 and 0-1 for neutrophils, lymphocytes, monocytes, eosinophils and basophils respectively as illustrated in Figure 34 and which remained within these (normal) ranges for the whole of Phase I and Phase II. Mean differential white cell counts and standards are listed in Table XIV, Appendix II.

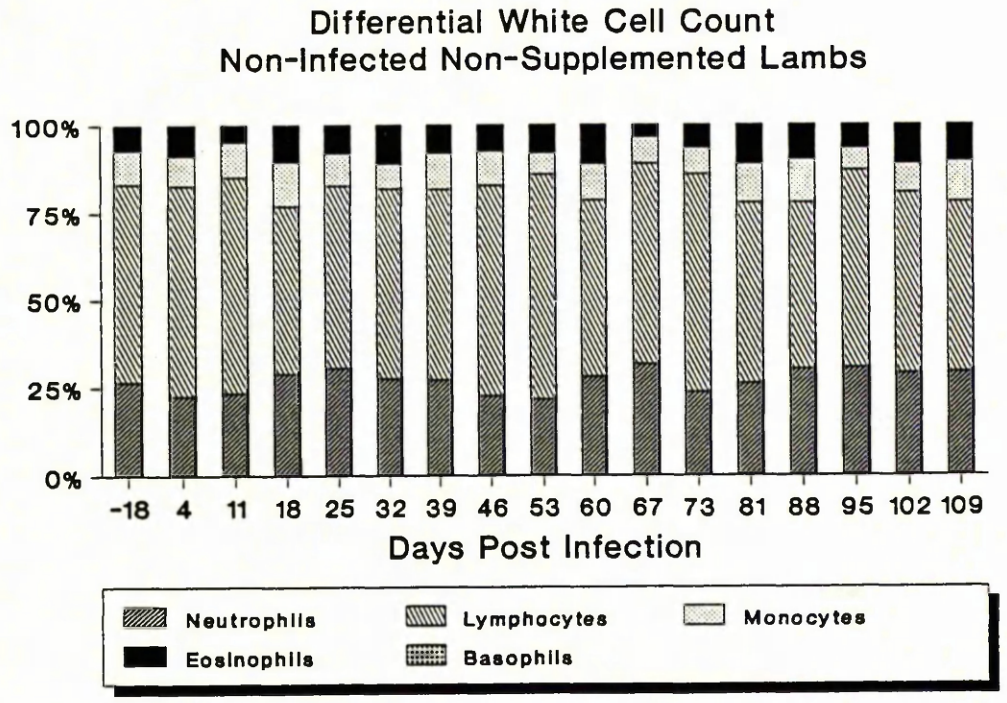
Infected, non-supplemented lambs showed similar trends in Figure 35 to their supplemented controls where eosinophilia was noted from 18 days PI through Phase I (ranging from 19.0-31.5) and persisted until 102 days PI (Phase II). Mean percentage lymphocytes fell from > 58% during the pre-patent period to a nadir of 34%, 39 days PI but recovered to > 40% by 81 days PI although the mean level at the end of Phase II was still less than the pre-infection level. An increased, if infrequent occurrence of basophils was noted among Group 3 lambs when compared to non-infected counterparts, Group 4. Mean differen

FIGURE 35



Phases I and II

FIGURE 36



Phases I and II

tial white cell count and standard errors for Group 3 lambs are listed in Table XV, Appendix II.

Differential white cell counts of non-supplemented, non-infected lambs, Group 4, were similar to their supplemented equivalents and showed mean percentage neutrophils, lymphocytes, monocytes, eosinophils and basophils within the normal ranges being 21.5-31.5, 47.75-64.5, 6.0-12.5, 4.75-11.25 and 0-0.5 per cent respectively. These results are shown in Figure 36 and mean data and standard errors are listed in Table XVI, Appendix II.

Statistical analysis revealed that infected non-supplemented lambs had a significant eosinophilia which was absent in their controls from 18 until 102 days PI and significantly lower percentage lymphocytes from 25 until 102 days PI during Phases I and II ($P < 0.05$). Comparison of levels of eosinophilia between supplemented and non-supplemented infected lambs, Groups 1 and 3/3B, showed that eosinophila among non-supplemented lambs was significantly greater than supplemented equivalents on three occasions during Phase I, namely 32, 39 and 60 days PI and on three other occasions during Phase II, 81, 88 and 102 days PI ($P < 0.05$).

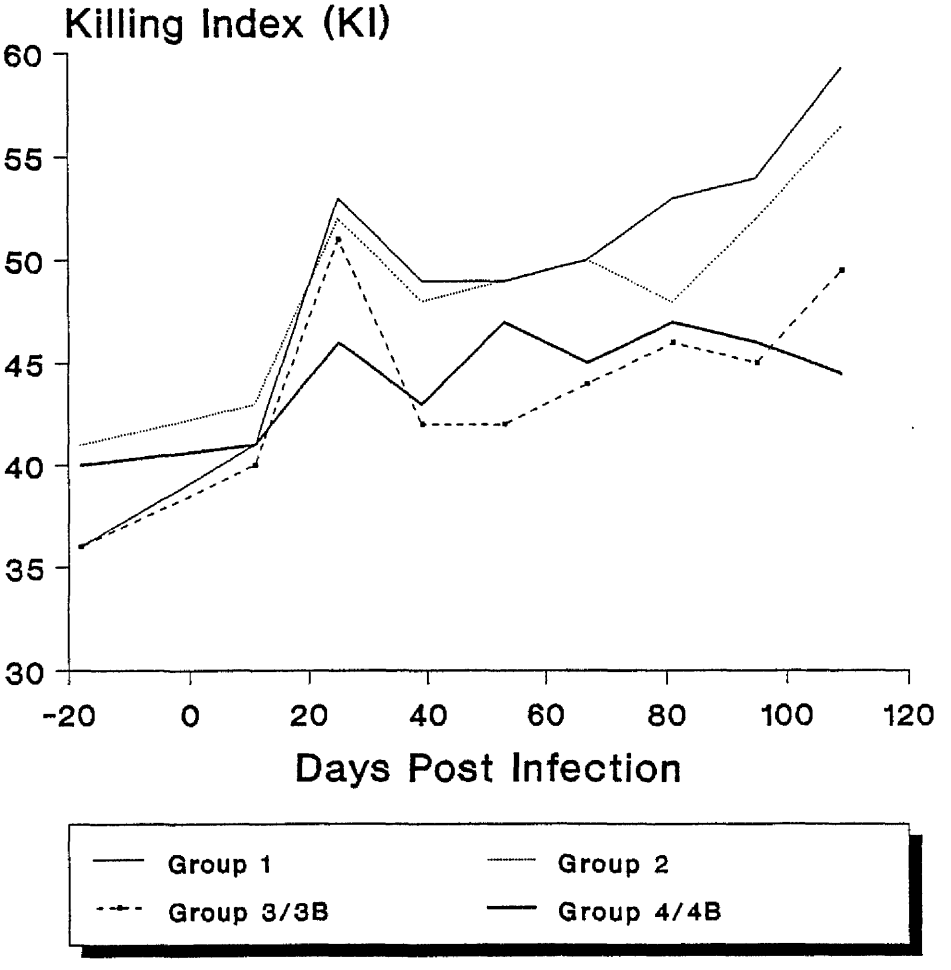
4.3.6 White Cell Function Tests

Neutrophil function tests:

Mean pecentage kill of *C. albicans*, or killing index (KI) for each of the four groups in Phase I are shown in Figure 37. KI of supplemented lambs, Groups 1 and 2, rose from pre-infection

FIGURE 37

MEAN NFT VALUES
Lambs



Phases I and II

Group 1: Vitamin E Supplemented, Infected Lambs

Group 2: Vitamin E Supplemented, Non-Infected Lambs

Group 3/3B: Non Supplemented, Infected Lambs

Group 4/4B: Non Supplemented, Non-Infected Lambs

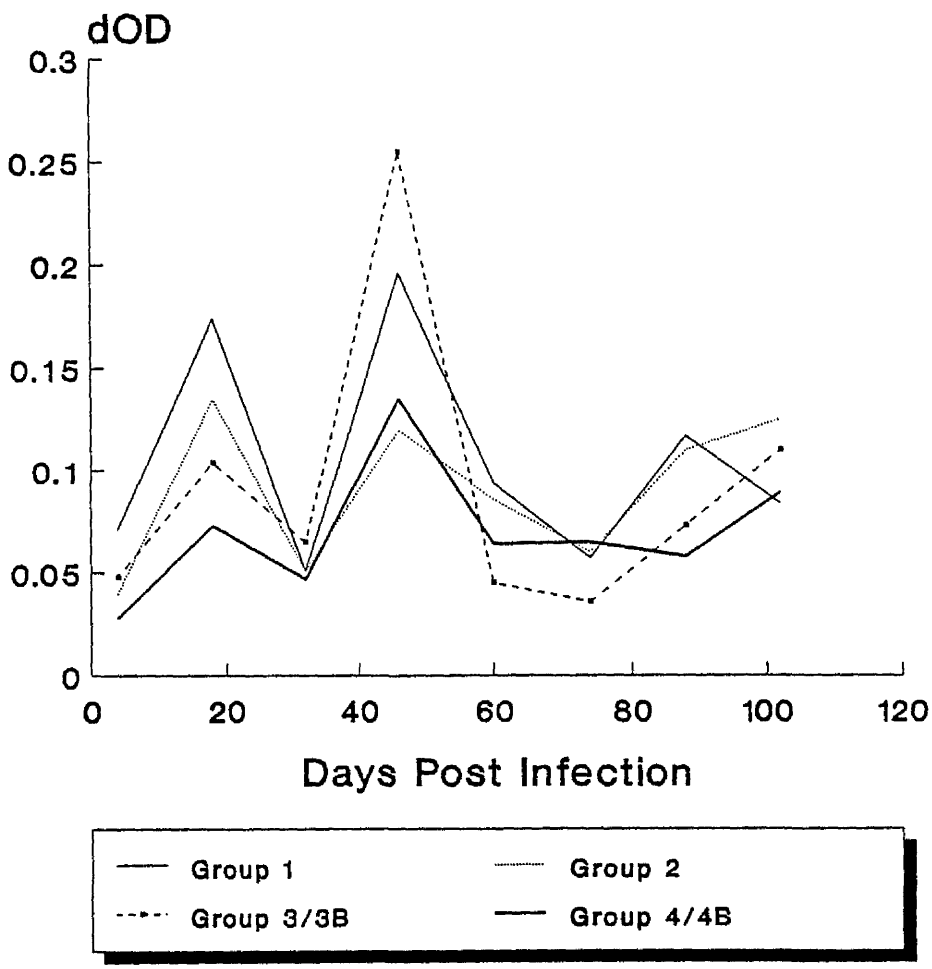
levels of 36-41% to around 50% for the remainder of this phase of the experiment and both infected and non-infected lambs showed similar levels. KI of infected and non-infected, non-supplemented controls also rose from pre-infection levels of 36-40% to values in the mid-forties. In both supplemented and non-supplemented status groups, no significant difference attributable to infection was found. Comparison of supplemented and non-supplemented equivalents from Figure 37 demonstrated a clear difference between the two groups which was significant from 20 days PI ($P < 0.05$) for the duration of Phases I and II except for a single occasion 67 days PI. Mean KI's and standard errors for Phase I results are listed in Table XVII, Appendix II.

NBT reduction:

Mean change in optical density, dOD and standard errors for Groups 1-4 are listed in Table XVIII, Appendix II and are illustrated in Figure 38. dOD's from all groups were extremely variable despite initially similar values 4 days PI and were very low (ranging from 0.028-0.196) in all cases. Values of non-supplemented lambs were signifiantly lower than supplemented controls on two occasions 18 and 60 days PI during Phase I ($P < 0.05$). Infected lambs of both status groups had significantly higher dOD's than non-infected counterparts ($P < 0.05$) on only a single date 46 days PI.

FIGURE 38

Mean NBT Reduction Assay
Lambs



Phases I and II

Group 1: Vitamin E Supplemented, Infected Lambs

Group 2: Vitamin E Supplemented, Non-Infected Lambs

Group 3/3B: Non Supplemented, Infected Lambs

Group 4/4B: Non Supplemented, Non-Infected Lambs

4.3.7 Specific Immunity

Antibody response to *O. circumcincta*:

Figure 39 shows the mean antibody titre to an *O. circumcincta* L3 antigen as measured using the ELISA methodology detailed in Chapter 2. Mean titres and standard errors, expressed as a percentage of the same positive reference serum used in Chapter 3, are listed in Table XIX of Appendix II.

All groups showed low initial titres of < 20% of reference during the pre-infective phase and until 9 days PI. Mean titre of non-infected lambs (Groups 2 and 4) remained < 30% for the duration of Phase II except for a single value of 33.6% in Group 4, 79 days PI. Titres of infected lambs rose beyond 30% by 37 days PI and continued to rise thereafter to peak around 74%, 77 days PI in Group 1 and 67.5, 65 days PI in Group 3.

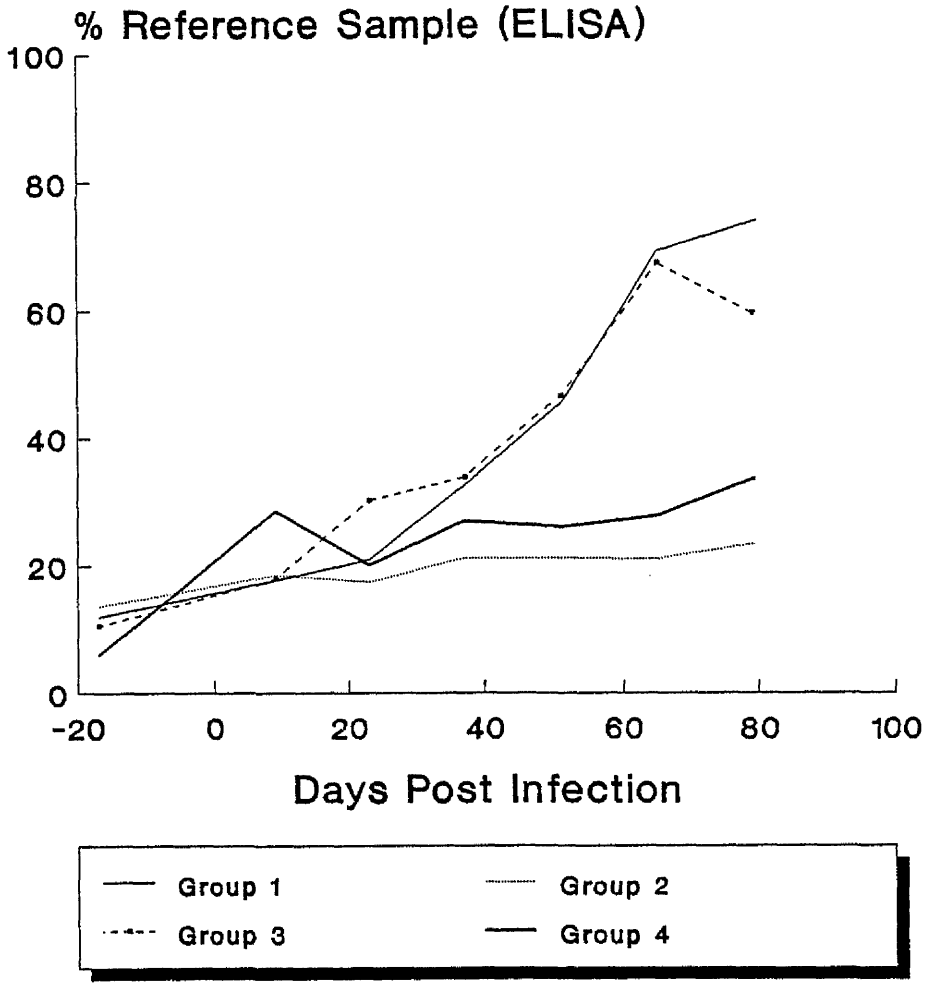
Statistical analysis showed that titres from infected animals were significantly higher than non-infected controls by 37 days PI until the end of Phase I ($P < 0.01$, $P < 0.05$ and $P < 0.001$) in both supplemented and non-supplemented lambs. Comparison of titres of supplemented and non-supplemented, non-infected sub-groups revealed no significant differences during Phase I except for a single occasion 77 days PI.

Antibody response to *C. tetani* vaccination:

Serum samples from lambs, vaccinated according to the schedule described in Chapter 2 were assayed to determine antibody

FIGURE 39

Antibody Response to O. circumcincta Lambs



Phase I

Group 1: Vitamin E Supplemented, Infected Lambs

Group 2: Vitamin E Supplemented, Non-Infected Lambs

Group 3: Non Supplemented, Infected Lambs

Group 4: Non Supplemented, Non-Infected Lambs

response to *C. tetani* vaccination in samples taken two weeks after their second vaccination and compared to pre-vaccination levels. The mean results from this assay are shown in Table 24 below and original data is listed in Table XX, Appendix II. No comparison was possible of pre- and post-vaccination titres in selenium deficient lambs.

Pre-vaccination titres in both supplemented and non-supplemented groups were low at < 1.0 iu/ml but did not differ significantly from each other. By comparison, post-vaccination titres were high but not significantly different from each other, 6.14 and 5.93 iu/ml for supplemented and non-supplemented lambs respectively. Comparison of pre- and post-vaccination titres showed that post-vaccination titres of non-supplemented lambs were significantly higher than their pre-vaccination level ($P < 0.001$). Considerable individual variation occurred in post-vaccination titres of Vitamin E supplemented lambs which did not differ significantly from pre-vaccination values.

TABLE 24: Vaccine Response to *C. tetani*

GROUP	NO ANIMALS	MEAN ANTITOXIN TITRE (± SE) PRE-VACC. (iu/ml)	POST-VACC. (iu/ml)	'p'
Supplem.	16	0.666 (0.158)	6.14 (2.80)	NS
Non-supplem.	36	0.749 (0.168)	5.93 (1.21)	P < 0.01
'p'		NS	NS	

(Student's T-test)

4.4 RESULTS - PHASE II

As discussed in the experimental design section, animals from Phase I were reassigned to groups dependent on their selenium status and treated with selenium and/or Vitamin E, 77 days PI before allocation into the six groups detailed in Table 25 (Table 20 repeated below) and falling into micronutrient status levels defined as selenium and Vitamin E adequate, Groups 1 and 2, selenium deficient, Groups 3A and 4A and Vitamin E deficient, Groups 3B and 4B.

TABLE 25: Summary of Experimental Groups - Phase II

Group 1 (7 lambs)	-	Vitamin E/Se Supplemented Infected
Group 2 (8 lambs)	-	Vitamin E/Se Supplemented Non-Infected
Group 3A (8 lambs)	-	Vitamin E Supplemented **Selenium Deficient** Infected
Group 3B (7 lambs)	-	Selenium Supplemented **Vitamin E Deficient** Infected
Group 4A (8 lambs)	-	Vitamin E Supplemented **Selenium Deficient** Non-Infected
Group 4B (8 lambs)	-	Selenium Supplemented **Vitamin E Deficient** Non-Infected

4.4.1 Biochemistry

GSH-Px:

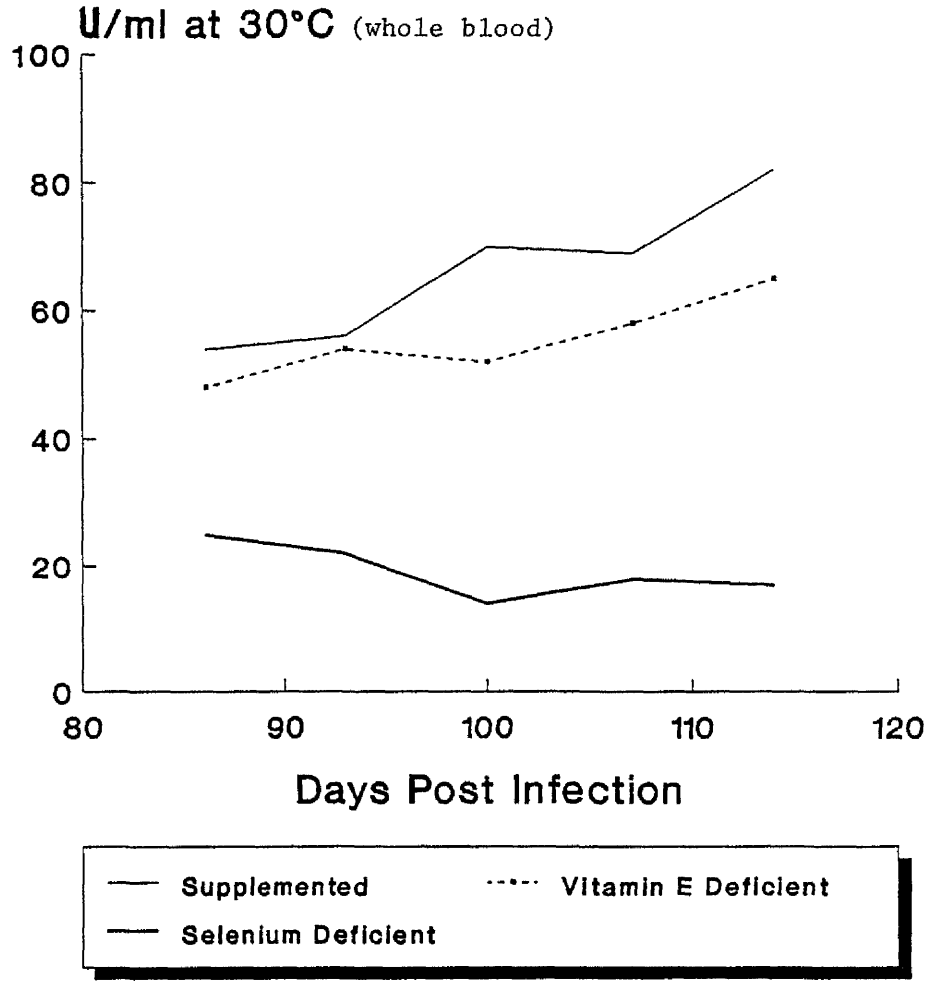
Figure 40 shows mean GSH-Px activity of the three status groups, with individual mean values and standard errors listed in Table XXI, Appendix II. The selenium supplement given to all non-selenium deficient lambs 77 days PI, prevented any further fall in GSH-Px activity of Vitamin E deficient lambs, Groups 3B and 4B, from their Phase I levels. A mean level of 48 U/ml was recorded 86 days PI rising to a peak of 65 U/ml by 114 days PI, levels considerably in excess of the threshold derining deficiency (27 U/ml).

Control lambs, Groups 1 and 2, were neither selenium nor Vitamin E deficient during the greater part of Phase I and Phase II. The mean GSH-Px values of infected and non-infected sub-groups rose from 54 U/ml, 86 days PI to a peak of 82 U/ml 114 days PI. Selenium deficient lambs, ie Groups 3A and 4A, showed mean GSH-Px activities of 25 U/ml at the start of Phase II and continued low dietary selenium intake caused their mean GSH-Px activity to fall further to a minimum of 14 U/ml, 100 days PI.

Statistical analysis revealed no significant difference between infected and non-infected sub-groups of the same micronutrient status other than an isolated incident 114 days PI ($P < 0.05$). GSH-Px activities of Vitamin E deficient lambs were not significantly different from those of supplemented controls for the duration of Phase II but the selenium deficient group showed

FIGURE 40

Mean Glutathione Peroxidase Activity
Lambs



Phase II

activities significantly lower than all others during Phase II, ($P < 0.001$).

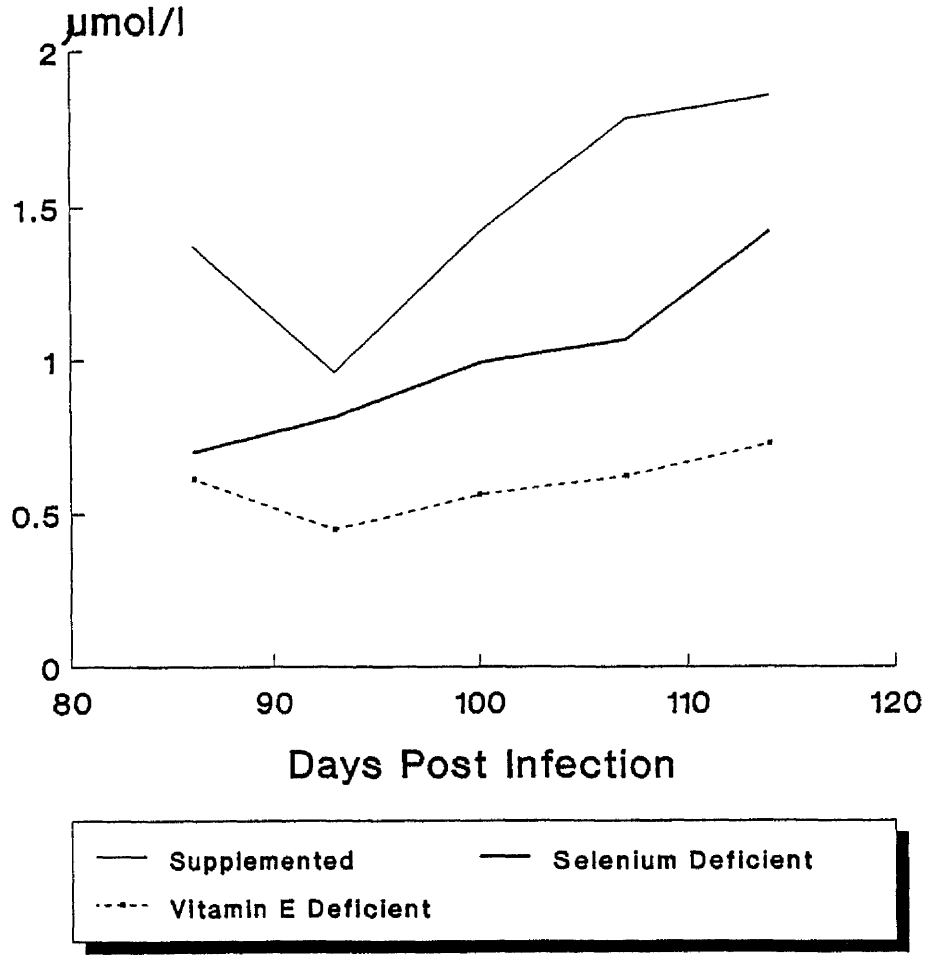
Vitamin E:

The effect of Vitamin E supplementation was revealed in mean Vitamin E concentrations shown in Figure 41 and listed in Table XXII, Appendix II. Adequate control lambs, Groups 1 and 2, always exceeded the upper limit defining deficiency (1.0 µmol/l) in this phase of the experiment and remained in the region 1.37-1.86 µmol/l. Supplementation of selenium deficient lambs, Groups 3A and 4A, at the start of Phase II caused their mean serum Vitamin E level to rise to 0.705 µmol/ml at 86 days PI and exceed 1.0 µmol/l 107 days PI and for the remainder of the experiment. Vitamin E deficient lambs, Groups 3B and 4B, continued to show mean levels below 1.0 µmol/l, which ranged from 0.617 µmol/ml, 86 days PI before falling to a low of 0.449 µmol/l, 93 days PI.

Statistical analysis demonstrated no significant differences in Vitamin E status attributable to *O. circumcincta* infection. Adequate, Group 1 and 2 lambs had significantly higher mean Vitamin E concentrations than either selenium deficient or Vitamin E deficient equivalents ($P < 0.05$), after 77 days PI. Vitamin E deficient lambs, Groups 3B and 4B, had significantly lower Vitamin E concentrations than either selenium deficient or adequate controls from 77 days PI ($P < 0.05$). Vitamin E concentrations rose in supplemented groups after supplementation but concentrations in adequate lambs, Groups 1 and 2, were significantly greater than selenium deficient (Vitamin E supple

FIGURE 41

Mean Vitamin E Concentration Lambs



Phase II

mented) lambs on two occasions during Phase II, 107 and 114 days PI ($P < 0.05$).

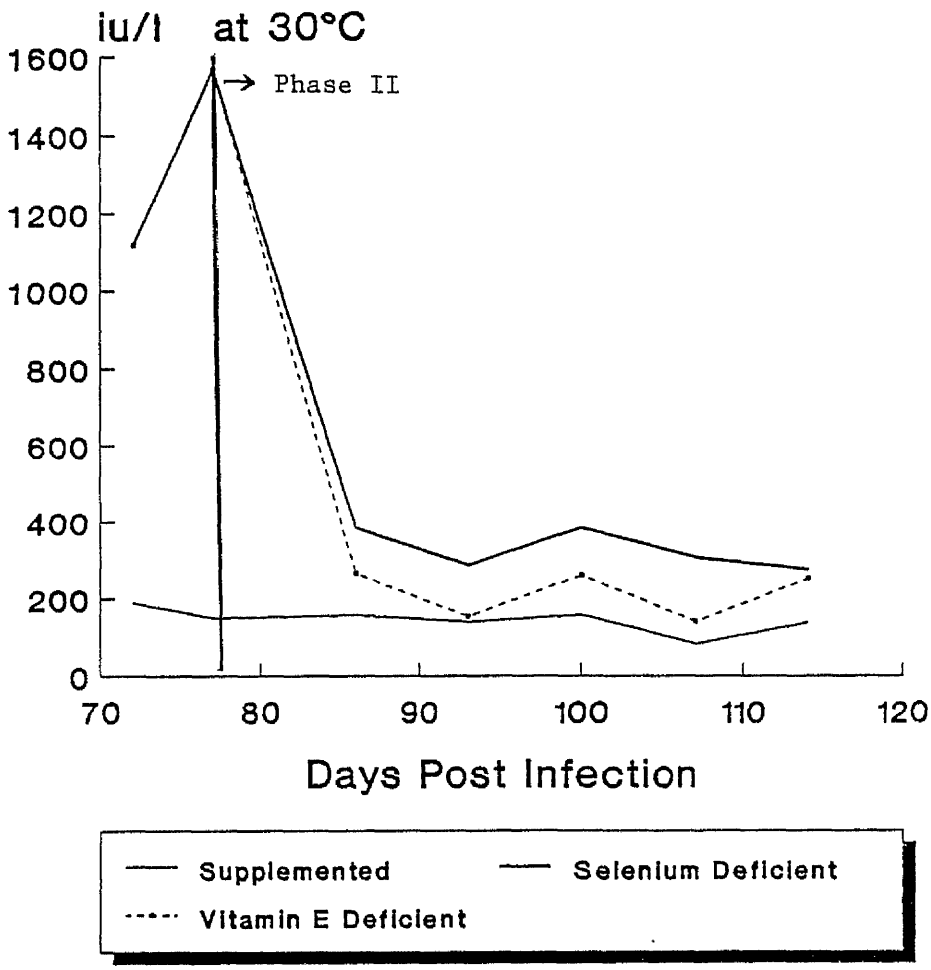
Creatine kinase:

Mean CK activity and standard errors are listed in Table XXIII, Appendix II and illustrated in Figure 42. Lowest activity in Phase II was recorded among supplemented lambs, Groups 1 and 2, which remained greater than normal (ie > 50 iu/l) for the duration of Phase II, in the range 83-158 iu/l. During Phase II, Vitamin E deficient lambs always exceeded 140 iu/l and often exceeded 250 iu/l ranging from 141-266 iu/l, levels considerably lower than Vitamin E deficient lambs during Phase I. Highest levels of CK activity were detected in selenium deficient lambs (range 276-385 iu/l). Statistical analysis showed that *O. circumcincta* infection had no significant effect on CK activity of any status group during Phase II.

Comparison of the three status groups showed that selenium supplementation of Vitamin E deficient lambs from Phase I, significantly lowered CK activity ($P < 0.05$) in Phase II as shown in Figure 42, to values not significantly different from adequate controls, Groups 1 and 2. CK activities of selenium deficient lambs were significantly greater than both Vitamin E deficient and adequate lambs ($P < 0.01$). Analysis of data showed however that CK activity of lambs subsequently assigned selenium deficient, were significantly greater than others during the latter part of Phase I ($P < 0.05$).

FIGURE 42

Mean Creatine Kinase Activity Lambs



Phase II

4.4.2 Production

Mean liveweights of the six groups in Phase II and their standard errors are listed in Table XXIV, Appendix II and shown in Figures 43 and 44 for infected and non-infected sub-groups of the three status groups. No significant difference was detected between infected and non-infected sub-groups of the same micronutrient status during any part of Phase II, despite a clear 2.0 kg mean deficit of infected lambs compared to their non-infected counterparts.

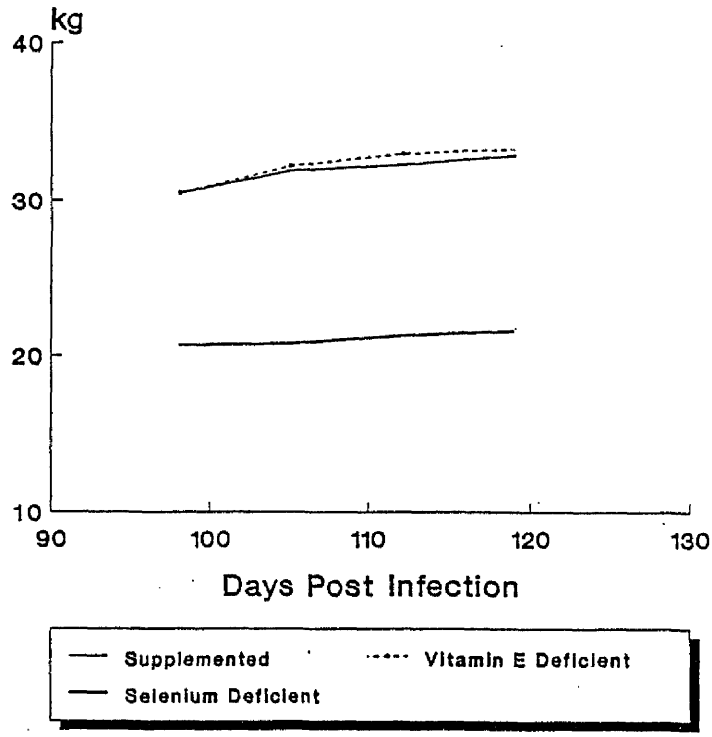
Comparison between status groups showed that selenium deficient lambs were significantly lighter than both Vitamin E deficient and adequate, supplemented lambs ($P < 0.05$) in both infected and non-infected sub-groups. Overall liveweight gain for the duration of Phase II revealed that rate of gain was impaired among infected lambs of all three status groups when compared with non-infected controls. Mean gains of 0.110 vs. 0.152 kg/day, 0.114 vs. 0.131 kg/day and 0.045 vs. 0.117 kg/day were found for infected and non-infected equivalents from the supplemented, Vitamin E deficient and selenium deficient status groups respectively. However statistical analysis of liveweight gain revealed no significant difference attributable to infection in any status groups.

4.4.3 Parasitology

Mean plasma pepsinogen levels and worm egg counts and standard errors of infected and non-infected lambs for the duration of Phase II are listed in Table XXV and XXVI, Appendix II respec-

FIGURE 43

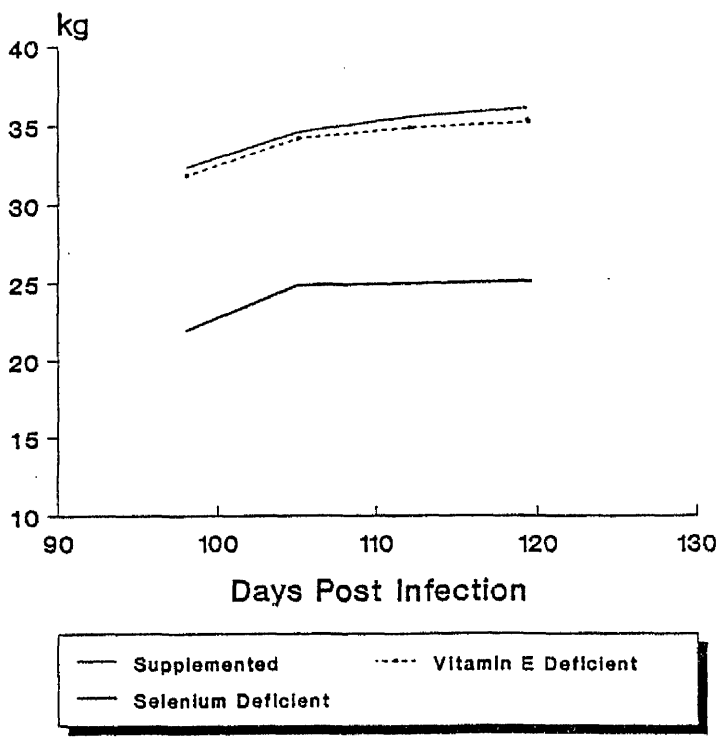
Mean Liveweight
Infected Lambs



Phase II

FIGURE 44

Mean Liveweight
Non Infected Lambs



Phase II

tively. Periodic analysis of faecal and blood samples from non-infected lambs during Phase II did not reveal the presence of strongyle eggs in faeces nor any elevation in plasma pepsinogen associated with infection.

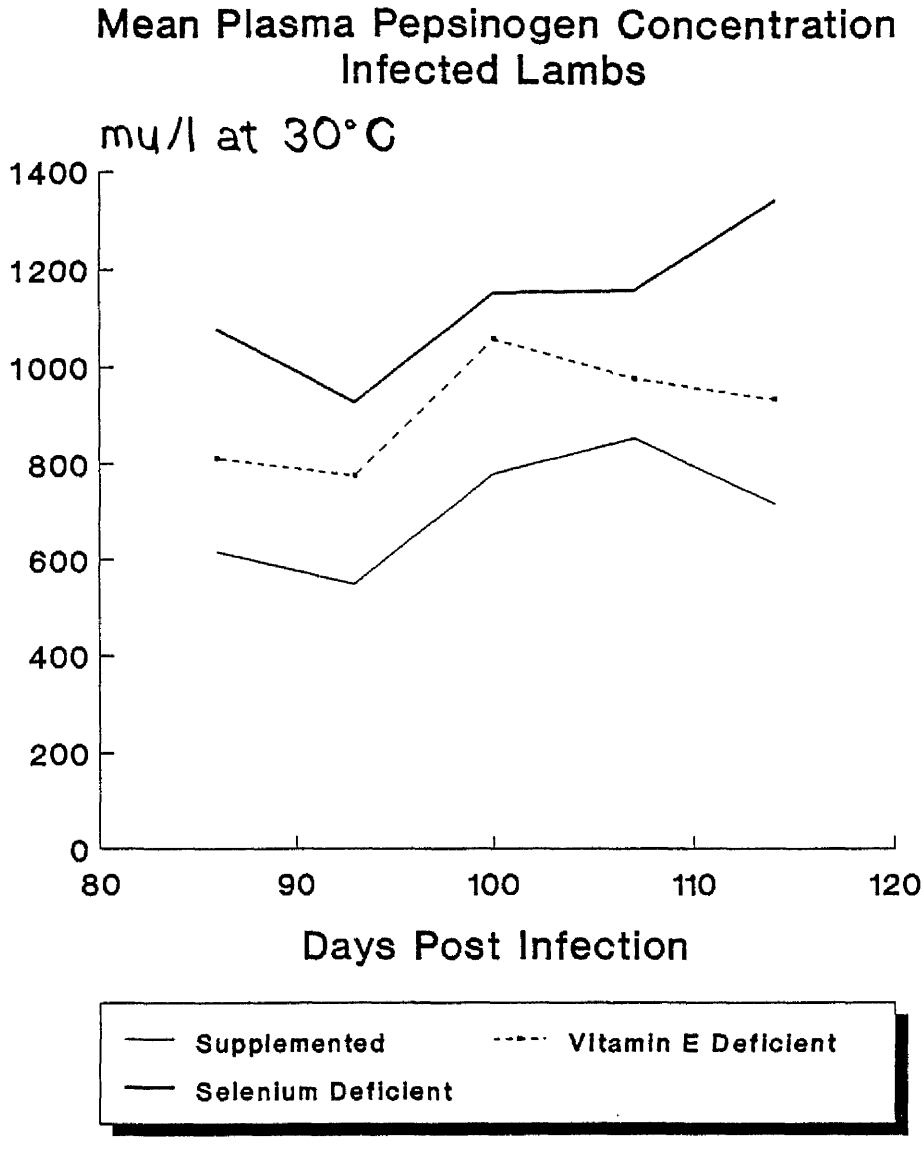
Plasma pepsinogen

Figure 45 shows mean plasma pepsinogen concentrations of infected lambs of the three status groups during Phase II. Group 1 lambs (supplemented, infected) showed mean plasma pepsinogen concentrations in the range 549-854 mu/l, considerably lower than the means ranging from 811-1057 and 928-1158 mu/l for Vitamin E and selenium deficient lambs respectively. Statistical analysis revealed no significant difference between any group during Phase II despite obvious differences evident in Figure 45. Comparison of plasma pepsinogen concentration in Vitamin E deficient lambs between Phases I and II revealed that a slight drop in concentration in Phase II was not significant.

Worm egg counts:

Mean worm egg counts and standard errors listed in Table XXVI, Appendix II, revealed that during Phase II selenium deficient lambs had the highest mean WEC which varied between 600 and 1000 epg and compared unfavourably with the Vitamin E deficient groups, 3B, with a mean count generally between 250 epg and 500 in the range 238-486 epg. By comparison, adequate, Group 1 lambs, had relatively low WEC's, usually < 260 epg, except for a single occurrence 91 days PI. These results are shown in Figure 46.

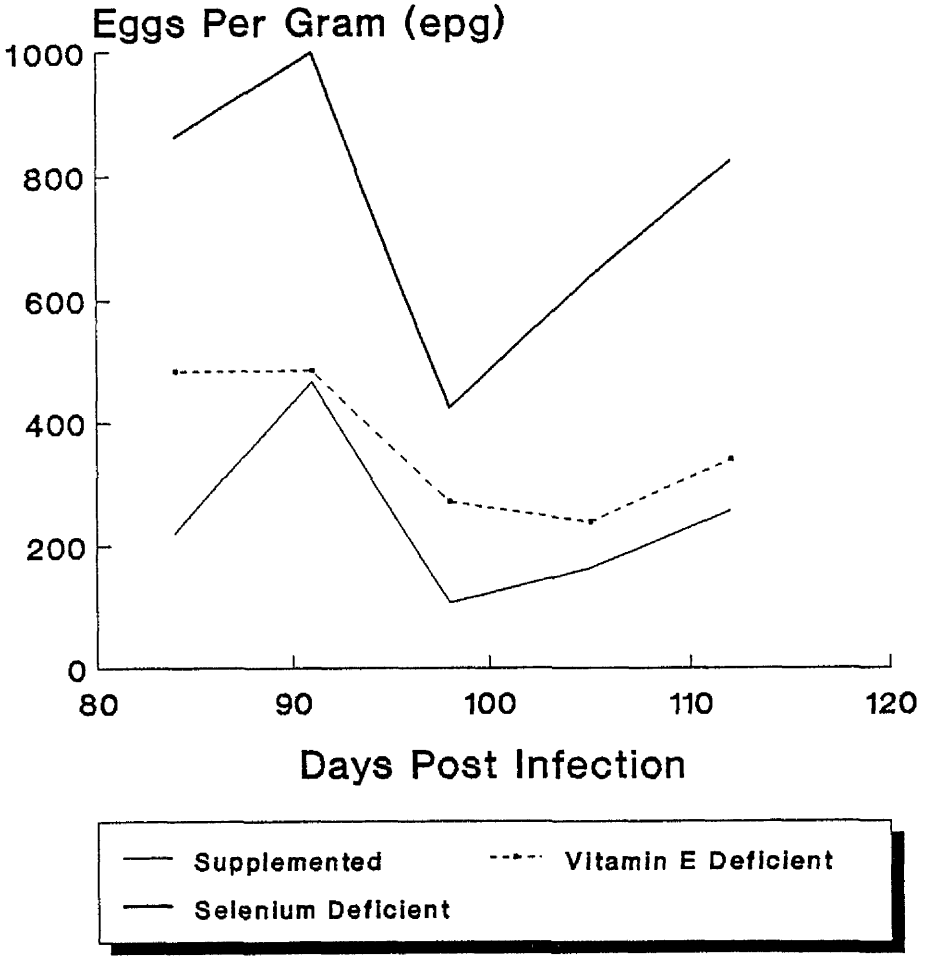
FIGURE 45



Phase II

FIGURE 46

Mean Worm Egg Count
Infected Lambs



Phase II

Statistical analysis revealed that WEC's of selenium deficient lambs were significantly elevated in comparison with both Vitamin E deficient and adequate controls from 98 days PI until the end of the experiment ($P < 0.05$). WEC's of Vitamin E deficient lambs were not significantly different from supplemented controls for the duration of Phase II apart from a single exception 105 days PI.

Total worm count (TWC):

Four lambs from the three infected status groups in Phase II were slaughtered for total worm count determination 18 weeks PI. *Post mortem* analysis was as described in Chapter 2. Statistical analysis revealed no significant difference in abomasal pH (Table 26) between the three groups although pH values of selenium deficient lambs were lower than those of the other groups.

TABLE 26: **Abomasal pH at Slaughter**

GROUP	STATUS	ABOMASAL pH	MEAN (SE)
1	Adequate	4.7 6.1 5.8 5.1	5.43 (0.32)
3A	Selenium Deficient	3.9 4.6 5.2 4.9	4.65 (0.28)
3B	Vitamin E Deficient	4.0 4.7 5.6 5.9	5.05 (0.43)

TWC findings are summarised in Table 27 below. Selenium deficient lambs had the highest mean burden of 10000 which was significantly greater than both Vitamin E deficient and supplemented lambs, 4425 and 1738 respectively ($P < 0.05$). Vitamin E deficient lambs' burdens were also significantly greater than supplemented equivalents ($P < 0.05$).

Table 28 shows mean and individual percentage breakdown of TWC of slaughtered animals. Selenium deficient lambs showed the greatest percentage adults, 65.49, of the three groups and lowest percentage larval stages, 36.51%. Supplemented lambs showed the lowest percentage adults, 51.91%, and correspondingly, the highest percentage of larvae, 48.09%. Vitamin E deficient lambs were intermediate.

TABLE 27: Total Worm Count - Phase II

GROUP	STATUS	MEAN COUNT ADULTS	L5	L4	TOTAL	GROUP MEAN (SE)
1	Supplemented	2350	500	1000	3850	
		450	250	550	1259	1738[a]
		400	250	350	1000	(709)
		600	100	150	850	
3A	Selenium Deficient	3900	850	1650	6400	
		7950	2400	1750	12100	10000[b]
		7100	1700	1900	10700	(1242)
		7450	2000	1350	10800	
3b	Vitamin E Deficient	3700	1500	1050	6250	
		2050	950	1950	4950	4225[c]
		850	350	400	1600	(992)
		3450	950	500	4900	

Values with different superscript letters are significantly different, ($P < 0.05$, Student's T-test)

Statistical analysis showed no significant difference in the number of or percentage of adults, L5's and L4's between any of the groups. Within group comparisons revealed that significanly more adults than L5's and L4's in selenium and Vitamin E deficient groups respectively ($P < 0.001$). Adequate, (Group 1) lambs had significantly more adults than L5's ($P < 0.01$), but the mean percentages of adults and L4's were not significantly different. Comparison of relative proportion of L5's:L4's in all three groups, expressed as a percentage, revealed no significant difference between groups.

TABLE 28: Proportions of Adults and Larvae as percentage of TWC

		PERCENTAGE OF TWC		
GROUP	STATUS	ADULTS	L5	L4
1	Adequate	61.04	12.99	29.97
		36.00	20.00	44.00
		40.00	25.00	35.00
		70.59	17.64	11.76
Mean (SE)		51.91[a]	18.91[b]	29.18[a]
		(8.30)	(2.50)	(6.87)
3A	Selenium Deficient	60.94	13.25	25.78
		65.70	19.83	14.46
		66.36	15.89	17.76
		66.98	18.52	12.50
Mean (SE)		65.49[a]	16.88[b]	17.63[b]
		(1.68)	(1.45)	(2.93)
3B	Vitamin E	59.20	24.00	16.80
		41.41	19.19	39.39
		53.13	21.88	25.00
		70.41	19.39	10.20
Mean (SE)		23.01[a]	21.11[b]	22.85[b]
		(6.11)	(1.14)	(6.29)

Values with different superscripts differed significantly from other values in the same row ($P < 0.01$ or less)

Effect of deficiency on parasite growth:

Fifty female and fifty male adult worms were recovered from the abomasal washing of sacrificed animals and measured to give the results listed in Table 29. Statistical analysis showed that male worm length was significantly lower than female length in all three status groups ($P < 0.01$). Within sex and group comparisons showed no significant difference in lengths of either males or females from any status group. No significant difference between the mean lengths of worms recovered was recorded attributable to micro-nutrient status except that male and female worms recovered from selenium deficient lambs were significantly longer ($P < 0.05$) than those from supplemented adequate controls.

TABLE 29: Mean Worm Lengths

GROUP	STATUS	FEMALE (SE) mm		MALE (SE) mm	
1	Adequate	11.41	(0.200)	7.21	(0.190)
		11.15	(0.170)	7.37	(0.180)
		10.91	(0.210)	7.27	(0.430)
Mean (SE)		11.17[a]	(0.113)	7.29[b]	(0.126)
3A	Selenium Deficient	11.53	(0.170)	7.63	(0.170)
		11.83	(0.180)	7.83	(0.160)
		12.35	(0.740)	8.26	(0.210)
		11.35	(0.180)	7.33	(0.210)
Mean (SE)		11.72[c]	(0.205)	7.77[d]	(0.100)
3B	Vitamin E Deficient	11.23	(0.160)	7.55	(0.210)
		11.60	(0.160)	7.79	(0.190)
		11.34	(0.160)	7.79	(0.190)
Mean (SE)		11.39[a]	(0.090)	7.55[b]	(0.116)

Values with different subscripts differed significantly ($P < 0.05$ or less)

Sex differences:

Table 30 shows mean and individual male:female ratios determined from the three status groups.

TABLE 30: Sex Ratios in TWC

GROUP	STATUS	% FEMALE	% MALE
1	Adequate	61	39
		66	34
		71	29
Mean (SE)		66.0^{a} (2.89)	34.0^{b} (2.89)
3A	Selenium Deficient	65	35
		68	32
		75	25
		70	30
Mean (SE)		39.5^{a} (2.10)	30.5^{b} (2.10)
3B	Vitamin E Deficient	60	40
		71	29
		65	35
Mean (SE)		63.3^{a} (5.50)	34.7^{b} (5.50)

Values with different superscripts differed significantly ($P < 0.05$)

Ratios from all three groups fell in the ranges 60-75 and 25-40% for females and males respectively. Statistical analysis revealed no significant differences between any male:female ratio of three status groups, although all groups had significantly more female than male worms ($P < 0.05$).

Faecal collection:

Three groups of four lambs from the infected sub-group of each micronutrient status level, which had been selected for slaughter

for TWC, were placed in metabolism cages for 2 weeks at the end of Phase II for collection of faeces and subsequent larval culture. Daily faecal output was weighed and WEC determined to calculate potential total egg output. Faecal collection and Baermannisation as described in Chapter 2 allowed larvae to be harvested and counted. Table 31 below, shows possible total number of eggs and recovery expressed as actual larval recovery and as a percentage of potential total.

TABLE 31: Mean Recovery Rates of Larvae

GROUP	STATUS	TOTAL LARVAE EXPECTED (Faecal wt. x epg)	NUMBER COUNTED	% RECOVERY
1	Adequate	5.8×10^6	0.32×10^6	5.42
3A	Selenium Deficient	21.6×10^6	1.67×10^6	7.74
3B	Vitamin E Deficient	14.4×10^6	0.79×10^6	5.51

More larvae were expected from selenium deficient lambs than from either Vitamin E deficient or adequate, supplemented controls and similarly from Vitamin E deficient lambs than adequate controls. Recovery in all cases was relatively low at < 8.0 % although recovery among selenium deficient lambs was slightly higher than that found in other groups. No statistical comparison was possible due to the method of collection.

4.4.4 Haematology

The nature of blood sampling frequency fo haematological examination as discussed in this Chapter meant that no haematological examination of newly formed groups (ie during Phase II) took place during Phase II. Groups 1, 2, 3B and 4B were examined and the results appear in Section 4.3.5.

4.4.5 Immunity

White cell function tests:

Logistical reasons prevented NFT assay of the six groups in Phase II, however, neutrophil function testing was carried out on a single occasion during the faecal collection period among the infected sub-groups of the three micronutrient status groups and gave the results listed in Table 32. KI of selenium deficient lambs, which was consistently less than 30% within the group, was significantly lower than both Vitamin E deficient and adequate lambs ($P < 0.001$). In contrast to Phase I results however, it was found that KI's from adequate and Vitamin E deficient lambs were not significantly different on this single assay date.

TABLE 32: Killing Indices of Lambs, 122 days PI

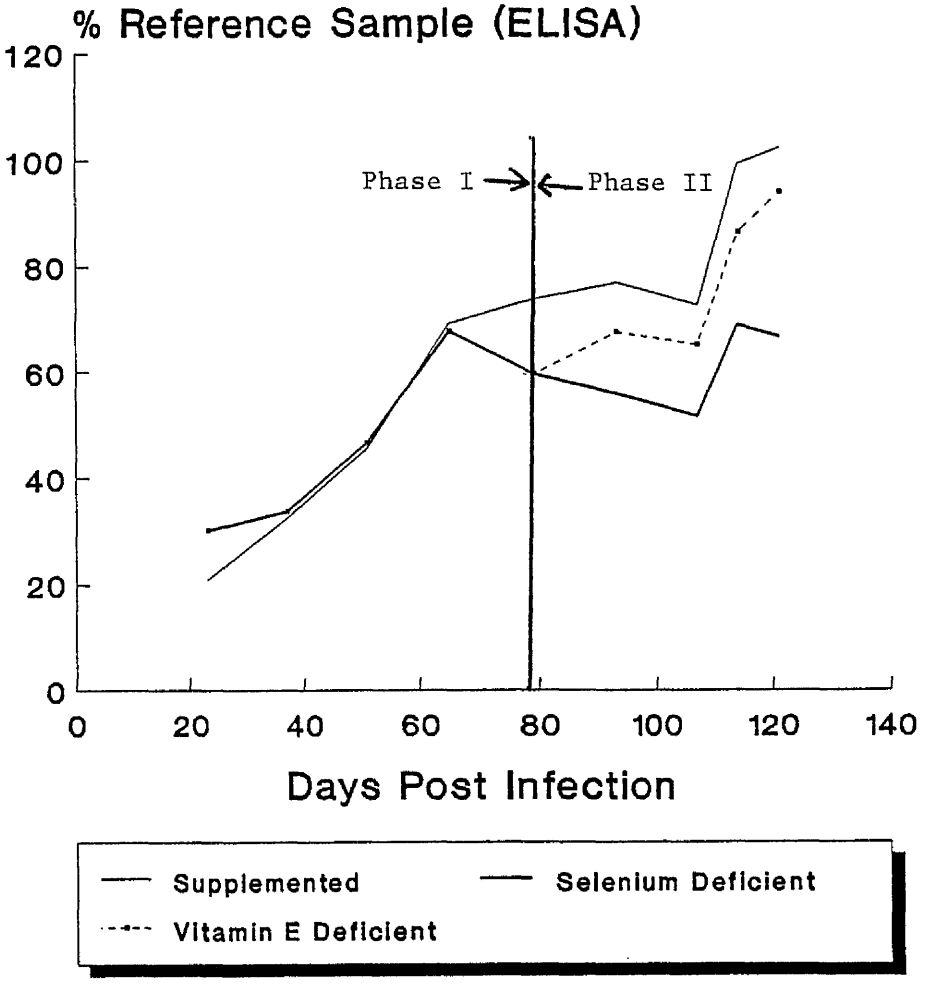
GROUP	STATUS	% KI	MEAN	'P'
1	Adequate	58 49 51	54.0	-
3A	Selenium Deficient	26 29 31 21	26.75	P < 0.001
3B	Vitamin E Deficient	48 54 45 40	46.75 (2.93)	-

Antibody response to *O. circumcincta*:

Figure 47 shows mean antibody titres of infected lambs from Phase I which were split into the three status groups at the start of Phase II, 77 days PI. Non-infected controls are not illustrated since their titres remained < 30% and were not significantly different from those found in Phase I. Mean titres and standard errors for the six groups in Phase II are listed in Table XXVII, Appendix II. Highest titres were noted in the adequate group which rose from 73.9%, 79 days PI to reach a peak of 102.5% by 121 days PI. The value for selenium deficient infected lambs, Group 3A, fell from 59.6% at the end of Phase I to 56.0%, 107 days PI and did not exceed 70% during Phase II. Vitamin E deficient infected lambs, Group 3B occupied an intermediate position with titres which rose after the start of Phase II to 67.5%, 93 days PI and reached a peak of 94.0%, 121 days PI.

FIGURE 47

Antibody Response to O. circumcincta Infected Lambs



Phase II

Statistical analysis revealed that infected lambs had significantly higher titres than non-infected equivalents in all three status groups throughout Phase II, ($P < 0.001$). No significant difference was detected between non-infected sub-groups of any of the three status groups at any time during Phase II. Comparison of infected lambs however showed that titres of selenium deficient lambs were significantly lower than those of adequate lambs for the duration of Phase II ($P < 0.05$). No significant difference was detected between adequate and Vitamin E deficient lambs on any assay date during Phase II nor was any significant difference detected between selenium deficient and Vitamin E deficient lambs.

4.5 DISCUSSION

4.5.1 Vitamin E

The findings of Coop *et al* (1977) and Sykes and Coop (1977) showing *O. circumcincta* mediated poor utilisation of diet possibly through nutrient malabsorption, alteration of digestibility of the induction of inappetance (Coop, 1981) were not supported by results from Phases I and II of the present experiment since no significant lowering of mean serum Vitamin E concentration was noted in either supplemented or non-supplemented lambs due to infection. Similarly, and although no direct comparison was possible, equal Vitamin E status in infected and non-infected sub-groups in the present study contrast with the findings of Weir *et al* (1948), of lowered Vitamin A and C level in serum of *H. contortus* infected sheep when compared to non-infected controls. This phenomenon may be due to the lower rate

of *O. circumcincta* infection used in the present study (6000 per week) in comparison with the larger dose rate used by Coop *et al* (1977), 21000 per week and Sykes and Coop (1977) who used 28000 per week and to the different parasite species used by Weir *et al*, (1948).

Problems of low Vitamin E status, as shown in Figure 27, in supplemented lambs in the pre-infective and early part of Phase I emphasised the low Vitamin E content of the diet. Difficulties in supplementation were overcome by increasing the dietary level of Vitamin E absorbate and by the weekly administration of a gelatin capsule containing more than the required weekly amount of Vitamin E (ARC, 1980). Comparison of the results for other parameters obtained from these groups was still possible since supplementation raised Vitamin E levels in Groups 1 and 2 above their non-supplemented counterparts and significantly so by 37 days PI. Previous studies have reported the beneficial effects of Vitamin E supplementation on disease resistance and immune response to *E. coli* infection in chicks and turkeys (Tengerdy and Nockels, 1975, Nockels, 1979), Chlamydial infection in lambs (Stephens *et al*, 1979, and Nockels, 1979), *E. tenella* infection in chicks (Colgano *et al*, 1984) and in LSI indices to IBR antigen in calves (Cipriano *et al*, 1982 and Reddy *et al*, 1985).

A continued rise in mean serum Vitamin E concentration in supplemented lambs during Phase I confirmed the effectiveness of this mode of supplementation. Mean Vitamin E concentrations in control (Groups 1 and 2) lambs during Phase II (Figure 41 indicated the successful supplementation of those lambs although

similar problems to those found in Phase I occurred in raising Vitamin E concentrations of selenium deficient lambs, Groups 3A and 4A. Accordingly, comparisons between the three status groups in Phase II (adequate, selenium deficient and Vitamin E deficient), were influenced by the fact that Vitamin E deficiency continued in selenium deficient lambs until 100 days PI (Figure 42). The beneficial effects of Vitamin E supplementation noted in Groups 1 and 2 in Phase I were also noted at the start of Phase II and accordingly the trends illustrated in Figure 42 show that supplementation of Groups 3A and 4A raised their mean Vitamin E concentration above those of the Vitamin E deficient group.

4.5.2 GSH-Px

A high correlation between the concentration of GSH-Px and the concentration of selenium in blood has been reported in many species including sheep (Wilson and Judson, 1976, Oh *et al*, 1976, Anderson *et al*, 1978, 1979 and Black *et al*, 1978). Despite the findings of Thompson *et al* (1980) that an elevation in blood selenium levels after selenium supplementation was not immediately matched by a rise in GSH-Px activity, it can be concluded that GSH-Px activity was indicative of selenium status in the present experiment.

Initial high dietary selenium intakes resulted in GSH-Px activities much in excess of the lower normal limit up to the end of Phase I and meant that no lambs were selenium deficient. Falling GSH-Px activity as seen in Figure 38 illustrated the effectiveness of the selenium deficient diet in lowering lamb

selenium levels. By the end of Phase I, GSH-Px activities had fallen close to the lower normal limit. "Changeover" of groups 77 days PI resulted in GSH-Px activities seen in Figure 41 thus in Phase II, Groups 3A and 4A, selenium deficient, continued to fall to low GSH-Px activities. Selenium supplementation of adequate controls, Groups 1 and 2, reversed this decline in GSH-Px activity and Vitamin E deficient lambs, Groups 3B and 4B, showed a similar response to selenium supplementation but remained at a non-significantly lower level than adequate controls. No significant lowering in whole blood GSH-Px activity attributable to *O. circumincta* infection was noted in either Phase I or II, in any status group. These findings also contradict the report of Coop *et al* (1977) of impaired nutrient uptake in *O. circumcincta* infected sheep as discussed previously. This was probably due to the relatively higher rate of infection used by the latter authors, 21000 per week, in comparison with the present study.

4.5.3 Creatine Kinase (CK)

CK activity in the present experiment was used to compare muscle damage among selenium deficient, Vitamin E deficient and supplemented controls since it has been reported to be a sensitive indicator of muscle damage in sheep (Boyd, 1976), cattle (Anderson *et al*, 1976) and other species (After Anderson *et al*, 1976). Mean CK activity in lambs in Phase I of the present experiment confirmed the trends found in Vitamin E concentration and GSH-Px activity where successful Vitamin E supplementation of Groups 1 and 2 alleviated deficiency and, as Figure 29 shows, prevented possible muscle damage in that group. This can be

claimed since CK activity fell with time in supplemented lambs but remained slightly greater than normal (normal < 50 iu/l) although Anderson *et al* (1976) reported activities > 100 iu/l in cattle showing no signs of myodegeneration. Elevation of CK in supplemented lambs in this experiment was probably due to the Vitamin E and selenium deficient nature of the diet fed and the slow response to Vitamin E supplementation in the early part of Phase I. Non-supplemented lambs, Groups 3 and 4, demonstrated variable but consistently elevated CK activities throughout Phase I, reflecting their relatively lower Vitamin E status during that period.

The absence of helminth mediated nutrient malabsorption which resulted in no differences in selenium (Phase II) and Vitamin E (Phases I and II) status in infected and non-infected sub-groups was also expressed indirectly by similar CK activity. Elevated CK activities in both selenium and Vitamin E deficient lambs in the present study confirmed the findings of Anderson *et al*, (1976) that CK activity was a sensitive indicator of sub-clinical, myodegeneration in selenium deficient calves.

After experimental changover 77 days PI, mean CK levels in Vitamin E deficient lambs which had received selenium supplementation fell and remained low for the duration of Phase II. Adequate, selenium and Vitamin E supplemented lambs remained at low levels of CK activity similar to Phase I, however selenium deficient lambs showed less of a reduction in CK activity after Vitamin E supplementation. It was interesting to note that values from selenium deficient (Vitamin E supplemented) lambs

still exceeded those of the other two status groups. Again no infection effects were noted and thus it appeared that selenium supplementation (of Vitamin E deficient lambs) has more effect in preventing muscle damage than Vitamin E supplementation of selenium deficient lambs.

The fall in CK activity in selenium deficient lambs after Vitamin E supplementation supported the findings of Boyd (1976), who stated that CK half-life in serum was decreased after Vitamin E supplementation of WMD affected sheep but cautioned that actual recovery from myodegenerative disease was much slower. The fall in CK activity in both selenium deficient (Vitamin E supplemented) and Vitamin E deficient (selenium supplemented) lambs could be explained by the mode of action of the two micronutrients shown in Figure 4 where peroxide damage was limited by both selenium and Vitamin E. Thus selenium and Vitamin E supplementation at 77 days PI halted many of the destructive oxidative processes possibly operating during Phase I.

4.5.4 Clinical Findings

The death of a lamb in the supplemented group during Phase I was attributable to Vitamin E deficiency since this lamb did not recover from deficiency after supplementation and was euthanased *in extremis*. Gross findings of WMD were recorded similar to those reported by Maas *et al* (1984) in selenium normal, Vitamin E deficient lambs. Al Tekrity and Telfer (1985) described ultrastructural changes in muscles of selenium and Vitamin E deficient sheep and while similar analysis was not carried out in this experiment, histopathological findings revealed lesions consis-

tent with WMD (Underwood, 1981). Nephrosis also evident in this lamb may have been due to a factor other than low Vitamin E status since Kennedy and Rice (1986) concluded that a renal lesion demonstrated in low Vitamin E status calves was due to sodium hydroxide treatment of the grain being fed and not to the deficiency. In the present study nephrosis may well have been related to the presence of large amounts of myoglobin in the bloodstream associated with myodegeneration in that lamb (Gray, personal communication).

Establishing a link between the cause of death and Vitamin E deficiency in non-supplemented lambs was more difficult since varying degrees of Vitamin E induced WMD were found in three of five casualties with typical findings of pale musculature, elevated CK and low serum Vitamin E concentrations. However, detection of clostridial toxins in one lamb and gross findings suggestive of enterotoxaemia in another implied that immunity to these (and possibly other) pathogens was impaired by low Vitamin E status and thus confirmed similar findings in chicks and turkeys infected with *E. coli* (Tengerdy and Nockels, 1975 and Nockels, 1979), *E. tenella* infected chicks (Colgano *et al*, 1984) and response to Chlamydial infection in lambs (Nockels, 1979 and Stephens *et al*, 1979).

Measurement of the vaccinal response to Clostridial vaccination using the *C. tetani* component as a marker revealed no significant difference in post-vaccination titres between any of the groups in this experiment. This may be explained by the Vitamin E deficient state of all lambs (supplemented and non-supplemented)

at the time of vaccination. Alternatively, similar vaccine response may have been an effect comparable with that reported by Larsen *et al* (1988a) where response to *C. tetani* vaccination (as part of a multicomponent vaccine) in Vitamin E deficient-selenium normal 6 month old lambs was similar to that found in selenium deficient-Vitamin E normal and selenium normal-Vitamin E normal controls.

Evidence of suspected immune incompetence was suggested by the fifth casualty in Vitamin E deficient lambs in which pasteurellosis (*P. haemolytica*, Serotype A2) and *M. ovipneumoniae* infections were noted, despite the fact that *P. haemolytica* (A2) was a component of the vaccine administered to all lambs in this experiment.

Subjective assessment of an outbreak of pneumonia implied that non-supplemented lambs suffered more than control lambs although measurement of clinical infection using an objective cough index failed to reveal any difference in mean index score and suggested that both groups responded equally to the pneumonic infective challenge. Alternatively, it was possible that the timing of the assessment was too late and did not detect the earlier subjective differences.

Supplementation with Vitamin E has been implicated in disease resistance, eg increased resistance to bovine mastitis (Smith, 1988), and enhanced ovine immune response to *Salmonella dublin* infection, (Finch and Turner, 1986 and Finch *et al*, 1986) and which ultimately has been shown to lead to decreased mortality to

E. coli infection (Heinzerling *et al*, 1974, Tengerdy and Nockels, 1975) and *E. tenella* infection in poultry (Colnago *et al*, 1984). Thus the low mortality in Vitamin E supplemented groups in the present experiment is supported by those studies although no impaired antibody titre to *C. tetani* vaccination was found in Vitamin E deficient lambs. The increased mortality in the present study, possibly associated with inability of Vitamin E deficient lambs to resist or fully respond to infection also confirmed the above hypothesis.

Statistical assessment of mortality rates showed no significant difference between groups but the differing rates suggested a relationship between Vitamin E deficiency and mortality. A similar ill-thrift syndrome in selenium deficient lambs was reported by Hartley (1967), McDonald (1975) and cited by Underwood (1981), where selenium deficient lambs had lower liveweights and suffered greater mortality than selenium supplemented equivalents. In the present study, however, no direct link proving the existence of a Vitamin E ill-thrift syndrome could be demonstrated, since liveweight was not impaired by Vitamin E deficiency in the same manner.

The influence of selenium status was unclear in the present experiment since no selenium ill-thrift mortality was noted in Phase II, ie the five deaths which occurred during Phase I in Vitamin E deficient lambs in the absence of selenium deficiency. Previous authors had linked low selenium status, poor immune function and increased mortality in sheep (Hartley, 1967), mice

(Spallholtz, 1981) and many other species, all of which were reviewed by Kiremidijian-Schumacher and Stotsky (1987).

4.5.5 Production Effects

A clear difference in mean liveweight was noted during patency and for the duration of Phase I (Figure 30) between infected and non-infected lambs. *O. circumcincta* infection caused approximately a 2.0 kg deficit in both Vitamin E supplemented and non-supplemented infected sub-groups although this difference was not significant. This finding agreed with Coop *et al* (1977), who found that *O. circumcincta* infected sheep suffered a reduction in efficiency of feed utilisation when infected lambs grew at half the rate of pair-fed controls although the 10% infection mediated production impairment in the present experiment was much less. Gibson and Everett (1976) reported that primary *O. circumcincta* infection could cause liveweight loss and this would afford a probable explanation for the weight loss in Group 1 lambs in Phase I from 18 days PI.

Significant differences in liveweight attributable to Vitamin E status were not detected in Phase I despite the liveweight deficit in Vitamin E deficient lambs present in the pre-patent period which was possibly attributable to individual animal variation in weight during random allocation to groups. Changes in rate of weight gain attributable to infection caused a 'crossover' effect 21 days PI as shown in Figure 30 although it was clear after patency that the effect of Vitamin E supplementation on liveweight was minimal since the only liveweight deficit noted occurred in infected sub-groups. Thus supplemented

infected, Group 1 lambs quickly fell to a liveweight similar to non-supplemented infected, Group 3, lambs. Similarly, non-supplemented, non-infected lambs gained weight at a greater rate than their infected controls matching the liveweight of Group 2 (supplemented, non-infected) lambs from 35 days PI until the end of Phase I.

Helminth mediated inappetance as found by Coop *et al* (1977) could not be measured in the present experiment but this phenomenon as a possible explanation for production impairment in infected lambs can be discounted since all concentrates were consumed in Phase I by both infected and non-infected sub-groups. Conversely, the similar concentrate consumption by supplemented and non-supplemented sub-groups in the present study affords a possible explanation for the absence of liveweight difference attributable to Vitamin E status.

Phase II further illustrated trends found in Phase I where liveweights of Vitamin E deficient lambs were similar to supplemented control groups in both infected and non-infected sub-groups. Although the group was absent in Phase I, selenium deficient infected and non-infected lambs showed liveweights during Phase II which were much lower than both other groups. These findings were consistent with previous reports of increased liveweight gain in selenium responsive unthriftiness in the UK, Australasia and North America (Blaxter, 1963, Gabbedy, 1971, Andrews *et al*, 1968, McDonald, 1975, Slen *et al*, 1961 and Oldfield *et al*, 1963). Liveweight differences due to infective status found in Phase I were also present in Vitamin E supplemented and deficient groups

during Phase II and additionally among selenium deficient lambs. While these infection-related differences were non-significant, this trend was consistent with the findings of Coop *et al* (1977), of poor nutrient utilisation in *O. circumcincta* infected sheep.

4.5.6 Parasitology

Elevation of blood pepsinogen concentration associated with mucosal damage and altered membrane permeability to macromolecules was found in *O. circimcuncta* infected lambs in the present experiment and is in agreement with the findings of Holmes and McLean (1971). A similar rise in plasma pepsinogen which occurred in both supplemented and non-supplemented groups may be explained by a similar level of Vitamin E concentration in both groups at the start of infection. The effectiveness of Vitamin E supplementation in restoring mean serum Vitamin E concentration of supplemented lambs to > 1.0 μmol/l could have resulted in an enhanced immune response limiting incoming helminth infection and lowering mean plasma pepsinogen concentration. Justification of this theory may be found in those studies reporting enhanced immune response in Vitamin E supplemented poultry (Tengerdy and Nockels, 1975 and Nockels, 1979) and in Vitamin E supplemented lambs to Chlamydial infection (Stephens *et al*, 1979), although no studies exist examining concurrent Vitamin E deficiency and helminth infection in lambs. No similar immune response enhancement in Vitamin E deficient lambs occurred after selenium supplementation in Phase II probably because they were still Vitamin E deficient at that time.

Individual variation in higher worm egg counts in non-supplemented lambs during Phase I meant that differences were significant on only two occasions. After changeover 77 days PI, Vitamin E deficiency effects remained in infected lambs, ie greater mean plasma pepsinogen concentrations and WEC than controls (Group 1), for the duration of Phase II. Infected selenium deficient lambs proved to have the highest mean plasma pepsinogen concentration and WEC's of any of the three groups and it was not surprising that TWC reflected plasma pepsinogen and WEC data, ie selenium deficient lambs had the highest TWC at slaughter, Vitamin E deficient lambs were intermediate and the lowest burdens occurred in adequate controls. The proportion of adults: larvae suggested that a degree of 'self cure' (Stewart, 1953) was evident in control lambs since almost 50% of their mean burden was larval, and considerably greater than both remaining infected groups. However, this suggestion was partly negated by the absence of stunting in male and female worms in any group which Urquhart *et al* (1986) described as manifestations of the 'self cure' reaction.

Differences were found in WEC whereby adequate, Group 1, lambs had the lowest counts, Vitamin E deficient lambs intermediate and selenium deficient lambs the highest values. These effects were probably related to the worm burden present in each animal since no significant difference in male:female ratio was found at necropsy and consequently did not indicate any effect of selenium or Vitamin E deficiency on fecundity.

Comparison of these findings was difficult due to the absence or similar studies examining concurrent selenium-Vitamin E deficiency and helminth infection as suggested by Suttle and Jones (1989). However two studies have examined mixed *O. circumcincta/T. colubriformis* infection (consisting of 5000 of each) in lambs supplemented with selenium (McDonald *et al*, 1989) and *H. contortus* infection in weaner Merino sheep fed a low selenium diet (Jelinek *et al*, 1988).

Findings in the present experiment disagreed with those of McDonald *et al* (1989) who reported no difference in mean WEC, plasma pepsinogen or TWC at slaughter (28 days PI) attributable to selenium supplementation although it was noted that non-supplemented lambs were selenium deficient and WMD was observed in that group. A possible explanation of this discrepancy may lie in the absence of anticipated selenium-mediated enhanced immune response to pathogenic infection in the 1989 study expected from their previous studies (Paynter *et al*, 1979 and McDonald, 1983) which suggested that immune response to bacterial infection in selenium deficient lambs would increase after supplementation. However the relatively short duration of infection in their 1989 experiment (28 days) in comparison to the present study is a more convincing explanation. Jelinek *et al* (1988) also reported no significant difference in TWC or inflammatory response in selenium supplemented lambs when compared to selenium deficient equivalents, findings which also disagree with the results reported in this experiment. A possible explanation of the differences is that the latter authors used a single low dose (5000 larvae) of a different parasite (*H. contortus*) in

immune sheep, all of which contrast with the experimental parameters employed in Experiment 2.

It was not possible to determine a specific factor responsible for increased worm burden, increased pathogenicity and increased helminth establishment in selenium and Vitamin E deficient lambs in this experiment. The possible influence of a low protein intake on these effects as suggested by Abbott *et al*, (1985a,b, 1986a,b, 1988) in studies examining ovine haemonchosis, was discounted since production rates were not affected by selenium or Vitamin E deficiency. Yong *et al* (1985) and Hucker and Yong (1986) suggested that cell mediated immune reactions in copper deficient sheep infected with *Trichostrongylus* spp, as measured by lymphocyte transformation tests, may be impaired and could be important in resistance to infection. Although no similar measurements were made in Experiment 2, NFT tests did reveal a cellular immune defect in selenium and Vitamin E deficient lambs which could have affected the immune response in *O. circumcincta* infection.

4.5.7 Haematology

No haematological examination of samples from selenium deficient lambs was made for logistical reasons and analysis of results was therefore confined to Vitamin E sufficient and deficient Groups 1/2 and 3(3b)/4(4b) respectively. Absence of any other than isolated significant difference in PCV count attributable to micronutrient status during Phases I and II suggested that Vitamin E deficiency in the present experiment did not cause anaemia in Vitamin E deficient lambs. Additionally, the fact

that no reduction in PCV was detected in infected lambs in comparison to non-infected equivalents contrasted with the findings of Horak and Clark (1964) who reported a marked decrease in PCV level in sheep infected with *O. circumcincta*. This can be explained by the nature of the infective regime which in the present experiment was a low trickle infection designed to mimic field infection and which contrasted markedly with the regime of 240000-618000 L3 *O. circumcincta* given in large doses ranging from 70000-312000 administered by Horak and Clark (1964).

Neutrophilia occasionally associated with WMD (Suttle and Linlater, 1983) was not noted in differential white cell counts of Vitamin E deficient lambs in the present experiment. Eosinophilia characteristic of helminth infections (Andrews, 1962 (after Millar, 1984), Gallacher, 1963 (after Watson, 1986), Huxtable and Rothwell, 1975 and Tizard, 1986) was however detected in *O. circumcincta* infected lambs in the present experiment.

Statistical analysis showed a greater eosinophilia in non-supplemented lambs than in supplemented counterparts on six out of 17 occasions, which may be explained by an increased pathogenicity of *O. circumcincta* infection and a greater parasite establishment in Vitamin E deficient lambs than in sufficient equivalents, a claim supported by higher plasma pepsinogen concentration, WEC and TWC found in these animals. However, a similar duration of eosinophilia in both supplemented and non-supplemented lambs indicated that another possible explanation could be offered. TWC results demonstrated no stunting of male or female adult

worms in either adequate or Vitamin E deficient lambs, Groups 1 and 3B respectively and suggests that 'self cure' (Stewart 1953) may have been absent in infected lambs in the present experiment. This can be claimed since it appeared that even normal (Vitamin E supplemented) Group 1 lambs did not completely expel incoming larval challenge as WEC and plasma pepsinogen concentration remained elevated during the infective phase and mostly adult worms were recovered in all lambs at necropsy 16 weeks after infection.

Comparison of differential white cell counts of the present experiment was not possible since previous studies have examined other haematological components such as impaired haemoglobin concentration in Vitamin E deficient cattle (Reddy *et al*, 1987) and erythrocyte haemolysis in Vitamin E deficient sheep (Suttle and Jones, 1989) and calves (Siddons and Mills, 1981) rather than relative white cell proportions as recorded in the present study.

4.5.8 Non-specific Immunity

Previous studies have shown the beneficial effects of supplementation with Vitamin E on immune function (Nockels, 1979 and Tengerdy *et al*, 1981, 1984) and have reported the detrimental effects of low Vitamin E status of deficiency (Sheffy and Williams, 1980). No studies exist, however, examining the effectiveness of polymorphonuclear leucocytes (in this case, neutrophils) harvested from Vitamin E deficient sheep in killing ingested yeast cells. A sustained significantly lower KI in neutrophils from Vitamin E deficient lambs in the present study suggested such an impairment.

Impairment in candidacidal activity has been reported in neutrophils from selenium deficient cattle, sheep and rats (Boyne and Arthur, 1979, 1981), copper deficient sheep and cattle (Boyne and Arthur, 1981, 1986, Jones and Suttle, 1981), and cobalt deficient sheep and cattle (Wright *et al*, 1982, Fisher and MacPherson, 1986 and MacPherson *et al*, 1987a). The magnitude of the impairment in KI of Vitamin E deficient sheep in the present experiment (17%) was less than that reported by previous authors: 50% in selenium deficient cattle (Boyne and Arthur, 1979), a 33% reduction in copper deficient sheep (Jones and Suttle, 1981) and a reduction of up to 50% in cobalt deficient sheep (Fisher and MacPherson, 1986).

Jones and Suttle (1981) attributed impairment of candidacidal activity in copper deficient sheep to reduced superoxide dismutase (SOD) activity in leucocytes which caused increased superoxide release and damage to phagocytes thus impairing function. The same authors found lower superoxide activity in leucocytes from copper deficient calves. Alternatively, Aziz and Klesius (1986) reported lower leukotriene B4 activity in neutrophils from selenium deficient goats causing a corresponding reduction in chemotaxis. Boyne and Arthur (1985a,b) suggested that impairment in candidacidal activity in neutrophils from selenium deficient animals stemmed from GSH-Px mediated inhibition of the HMPS pathway and reduced superoxide production.

Attempts to measure superoxide activity in NBT reduction assay (Figure 38) in the present experiment revealed only isolated significant differences (due either to Vitamin E or infective

status) which did not explain consistent significant impairment of candidacidal activity in Vitamin E deficient lambs. Variation in dOD in NBT assay could be explained in terms of the limited effectiveness of *C. albicans* as a stimulant in place of Zymosan (*Sarcomycetes cervisae*, Sigma) as used by Nagahata *et al* (1986). The hypothesis forwarded by Boyne and Arthur (1985a) to explain impaired candidacidal activity in neutrophils from selenium deficient cattle suggested that low GSH-Px activities resulted ultimately in impaired functioning but lambs assayed for NBT activity in the present experiment were never selenium deficient (in fact GSH-Px activity greatly exceeded the lower normal limit in most cases) and thus this assertion could not be challenged.

Assessment of KI's of selenium deficient, Vitamin E deficient and adequate infected sub-groups on a single occasion during the faecal collection period of Phase II, revealed around 50% impairment in candidacidal activity in neutrophils from selenium deficient lambs in comparison to controls, similar to that reported by Boyne and Arthur (1981), and markedly greater than the level of impairment found in Vitamin E deficient lambs during Phases I and II. Thus from these findings it appears that selenium deficiency has a greater effect in reducing non-specific immune function than Vitamin E deficiency.

4.5.9 Immune Response to *O. circumcincta* Infection

A specific serological response to infection was measured in infected lambs (Figure 39) during Phase I since titres detected among infected groups were significantly higher than non-infected equivalents. It was interesting to note that Vitamin E supple-

mented adequate lambs had mean titres which were significantly greater than non-supplemented controls on only one occasion at the end of Phase I, probably due to the fact that Vitamin E supplementation was ineffective in the early part of Phase I. Therefore, at that time, all lambs were Vitamin E deficient, with a consequent impairment of immune function which would however have been restored following effective Vitamin E supplementation. This tenet is confirmed by previous reports of similar immune enhancement following Vitamin E supplementation in *E. coli* infected chicks (Tengerdy and Nockels, 1975 and Nockels, 1979), in lambs infected with Chlamydia (Nockels, 1979 and Stephens *et al*, 1979) and in other studies cited in Chapter 1.

In Phase II the serological response to *O. circumcincta* (Figure 47) showed trends similar to those in Phase I whereby selenium/ Vitamin E supplemented adequate controls had the highest titre, Vitamin E deficient lambs had slightly lower titres but the lowest titres occurred in selenium deficient lambs. The high titre detected in selenium/Vitamin E supplemented lambs is supported by previous findings showing enhanced antibody titre to *S. dublin* immunisation in selenium supplemented lambs (Finch and Turner, 1986 and Turner *et al*, 1985, 1986). The same authors reported beneficial effects of selenium on lymphocyte transformation tests (Finch and Turner, 1986). Similar confirmatory findings were recently reported by Norwegian workers (Larsen *et al*, 1988b) who recorded enhanced mitogen responses after low levels but decreased mitogen responses at higher levels of selenium supplementation, although those authors reported no enhanced mitogen responses with Vitamin E supplementation alone.

Selenium deficient lambs in the present experiment showed comparatively low titres to *O. circumcincta*, findings which agreed with reports of lowered serological response in selenium deficient lambs (Finch and Turner, 1986, Turner *et al*, 1985 and 1986), in humoral immune responses of selenium deficient chicks (Marsh *et al*, 1981) and in the inflammatory responses of selenium deficient rodents (Parnham *et al*, 1983). Recently similar findings were confirmed in sheep by Larsen *et al* (1988a).

Enhanced antibody titres in control lambs (supplemented with Vitamin E in Phase I) in comparison with other groups in the present experiment confirmed previous similar reports of enhanced immune response to *E. coli* infection after Vitamin E supplementation (Tengerdy and Nockels, 1975, Tengerdy and Brown, 1977, Nockels, 1979, Tengerdy *et al*, 1981, 1984, Nockels, 1983) and to oil adjuvant vaccination (Franchini, 1989) in poultry, enhanced immune response in selenium supplemented mice (Spallholz *et al*, 1973) and in mitogen responses to PHA and PWM in selenium supplemented lambs (Turner *et al*, 1985). Similarly, the effects of Vitamin E deficiency in comparatively reducing the immune response as measured by NFT and *O. circumcincta* serology in the present study have been previously reported by Sheffy and Williams (1979), as lowered *in vitro* T cell responses in Vitamin E deficient dogs and by Bendich *et al* (1984) in lowered mitogen responses in Vitamin E deficient guinea pigs. Antibody titres found in Phase II of the present experiment indicated that Vitamin E supplementation of selenium deficient lambs did not enhance their *O. circumcincta* titre to levels similar to those achieved by selenium supplementation of Vitamin E deficient and

adequate control lambs. A possible explanation lies in the nature of the connection between selenium and Vitamin E in the immune response whereby the lowest response occurred in lambs which remained selenium deficient in Phase II of the present experiment and which did not respond to vitamin E supplementation as well as selenium supplementation of Vitamin E deficient lambs. Justification of this theory is difficult to pinpoint due to the diverse biological effects of selenium in the immune response which may, according to Kiremidijian-Schumacher and Stotsky (1987), be the result of a number of different mechanisms. Those authors speculated however that a direct link existed between impaired intracellular GSH-Px activity (and thus selenium rather than Vitamin E deficiency), resulting in impaired immune function and bactericidal activity. This explanation is supported in the present experiment by a significantly greater impairment of candidacidal activity in selenium deficient lambs when compared to Vitamin E deficient controls. From these findings it is possible to claim that selenium supplementation was relatively more beneficial in terms of restoring the immune response than Vitamin E supplementation and that supplementation with both micronutrients may be additive in their effects.

This theory is confirmed by the studies cited previously where little or no Vitamin E mediated immune enhancement was noted. For example, Larsen *et al* (1988b) revealed antibody titres in Vitamin E deficient (selenium supplemented) ewes to tetanus toxiod, after vaccination with Ovivac (Hoechst Animal Health) were similar to those of selenium/Vitamin E adequate ewes. The same authors also reported similar *C. tetani* titres in selenium

deficient (Vitamin E supplemented) ewes compared to supplemented controls and only ewes which were deficient in both selenium and Vitamin E had a significant impairment in their vaccine response. Vitamin E deficient lambs which became selenium deficient during Phases I and II of the present study showed the lowest antibody titre to *O. circumcincta* and the greatest impairment of candidacidal activity in NFT. Vitamin E deficiency appeared to be of lesser importance therefore in the immune response as measured by antibody titres to *O. circumcincta*, and which were higher than those of selenium deficient lambs and by lower candidacidal impairment than in selenium deficient counterparts and a vaccine response to *C. tetani* equivalent to supplemented controls.

It is possible that helminth and immunologic effects mediated by Vitamin E deficiency as noted were in fact attributable to selenium status rather than selenium deficiency, ie lambs with higher blood GSH-Px activity and thus higher selenium status, responded better in immunological terms than those of lower, if not necessarily deficient, selenium status as measured by GSH-Px activity. This is supported by the work of Turner *et al* (1986) who suggested that increasing selenium supplementation increased immune response up to and not beyond a theoretical threshold and Anderson *et al* (1986), who investigated the response to IBR vaccination in calves and found that neither Vitamin E deficiency nor supplementation affected the measured immune response.

CHAPTER 5

EFFECTS OF SUPPLEMENTATION AND ANTHELMINTIC TREATMENT ON MICRONUTRIENT DEFICIENT LAMBS

5.1 INTRODUCTION

The addition of micronutrients to anthelmintics is a "relatively recent phenomenon" (Suttle *et al*, 1990) and while the effectiveness of this practice has received only limited attention, Field *et al* (1988) reported that anthelmintics were convenient vehicles with which to supplement the diet with cobalt and selenium (and possibly other micronutrients) since the selenium and cobalt status of lambs declined as they became dependent on herbage, containing nematode larvae, for their nutrients. Recently, a product has become available which combines vitamin supplementation with anthelmintic treatment (Valbazin HiDose Plus, Smith-Kline Animal Health) thus emphasising the need to investigate the effectiveness of micronutrient supplemented anthelmintics. The experiment described below was designed to assess the effectiveness of an anthelmintic containing trace element supplements (Panacur SC, Hoechst Animal Health) in removing *O. circumcincta* infection and remission of experimentally induced cobalt and selenium deficiencies arising from Experiments 1 and 2.

5.2 MATERIALS AND METHODS

Lambs surviving Experiments 1 and 2 were treated according to the manufacturers recommendation with a 5 ml dose of anthelmintic supplemented with selenium and cobalt (SC-anthelmintic) containing 25 mg fenbendazole, 0.4 mg selenium and 0.95 mg cobalt per ml (Panacur SC, Hoechst Animal Health) after 16 weeks of infection and monitored by measuring elimination of burden, response to biochemical parameters and production effects. Experimental treat-

ments in the recovery phase were the same as those used in relevant experiments in Chapters 3 and 4 and relisted here (Table 33). Lambs from Experiment 1 were treated according to the schedule described in Table 34 and were fed the cobalt deficient diet described in Chapter 3.

TABLE 33: Summary of Experimental Groups Experiment 1 (recovery phase)

Group 1	- From cobalt deficient ewes No cobalt supplement Infected with *O. circumcincta*	DEFICIENT
Group 2	- From cobalt deficient ewes No cobalt supplement Non-infected	
Group 3	- From cobalt adequate ewes No cobalt supplement Infected with *O. circumcincta*	DEPLETED
Group 4	- From cobalt adequate ewes No cobalt supplement Non-infected	
Group 5	- From cobalt adequate ewes Cobalt supplemented Infected with *O. circumcincta*	SUFFICIENT
Group 6	- From cobalt adequate ewes Cobalt supplemented Non-infected	

TABLE 34: Schedule of Recovery Treatment of Lambs in Experiment 1

Date of Treatment	:	21 October 1987 (119 days PI)
Treatment	:	5 ml Panacur SC
Additional Supplement	:	7 mg Co (as sulphate) per head 0, 7, 14 and 28 days PT
Parameters Measured (frequency)	:	Vitamin B_{12} — 0, 7, 14, and 28 days PT
	:	GSH-Px — 0, 7, 14, and 28 days PT
	:	Plasma pepsinogen — 0, 7, and 14 days PT
	:	WEC — 0, 7, and 14 days PT
	:	Liveweight 0, 14 and 28 days PT

TABLE 35: Summary of Experimental Groups - Experiment 2 (recovery phase)

Group 1	:	Vitamin E/Se Supplemented Infected
Group 2	:	Vitamin E/Se Supplemented Non-Infected
Group 3A	:	Vitamin E Supplemented **Selenium Deficient** Infected
Group 3B	:	Selenium Supplemented **Vitamin E Deficient** Infected
Group 4A	:	Vitamin E Supplemented **Selenium Deficient** Non-Infected
Group 4B	:	Selenium Supplemented **Vitamin E Deficient** Non-Infected

The experimental design used in Phase II of Experiment 2 (Table 35) was retained during the recovery phase where lambs were treated according to the schedule listed in Table 36, but were still fed the selenium/Vitamin E deficient ration described in Chapter 4.

TABLE 36: Schedule of Recovery Treatment of Lambs in Experiment 2

Date of Treatment	:	21 November 1988 (119 days PI)
Treatment	:	5 ml Panacur SC
Additional Supplement	:	7 mg Co (as sulphate)/head (0 days PT) 150 iu Vitamin E/head 0, 7, 14 and 28 days PT
Parameters Measured	:	Vitamin B_{12}
	:	GSH-Px
	:	Vitamin E
	:	Liveweight
		(0, 7, 14 and 28 days PT)
	:	Plasma pepsinogen
	:	WEC
		(0 and 7 days PT)

5.3 RESULTS - EXPERIMENT 1

No statistical comparison was possible of any parameter on any occasion with surviving lambs from the deficient group since the high mortality rate experienced left only a single animal in each of the infected and non-infected sub-groups and prevented statistical comparison.

5.3.1 Clinical Findings

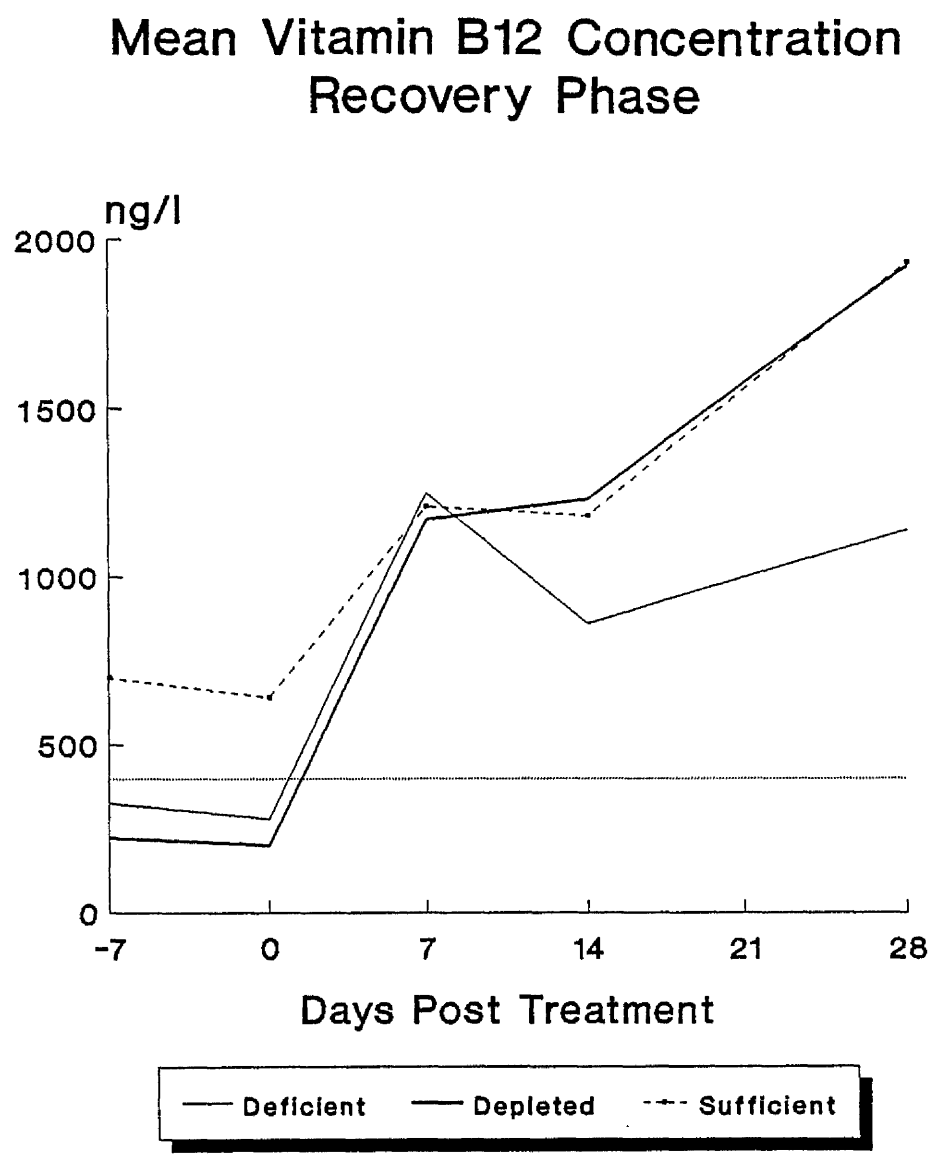
A single casualty was found in the recovery phase, one of the two remaining lambs in the cobalt deficient group which died 133 days PI (= 14 days post-treatment, PT) and showed signs of scour immediately before death but had only non-specific findings post mortem.

5.3.2 Serum Vitamin B_{12}

Pre-treatment levels and the Vitamin B_{12} concentration found on the day of treatment confirmed the previous experience of these lambs whereby Vitamin B_{12} concentrations of depleted lambs were less than 400 ng/l (< 200 ng/l in some cases) on those dates ranging from 50-270 ng/l and compared unfavourably with levels in cobalt sufficient controls (Groups 5 and 6), > 400 ng/l (range 365-925 ng/l). After treatment 119 days PI, mean Vitamin B_{12} concentration of all lambs greatly exceeded 400 ng/l and by 7 days PT exceeded 1000 ng/l even in surviving cobalt deficient lambs. Mean Vitamin B_{12} concentrations remained > 1000 ng/l in depleted and sufficient lambs for the remainder of the recovery phase. Modification of the technique used to assess Vitamin B_{12} concentration revealed that mean values which were previously at the upper limit of detection and classifed as > 1250 ng/l, were elevated to 1930 ng/l (range 950-2500 ng/l) and 1920 ng/l (range 980-2500) in sufficient lambs and depleted counterparts respectively 28 days (PT).

Statistical analysis revealed only an isolated infection effect among cobalt sufficient lambs on the day of treatment (day 0) and

FIGURE 48



Experiment 1

since no other significant difference between infected and non-infected sub-groups of the same cobalt status was detected, mean values of both groups have been used to draw Figure 48. Mean post-treatment Vitamin B_{12} concentrations and standard errors for Experiment 1 are recorded in Table I, Appendix III. Comparison between groups revealed no significant differences attributable to previous micronutrient status since all their Vitamin B_{12} levels were similar in the recovery phase.

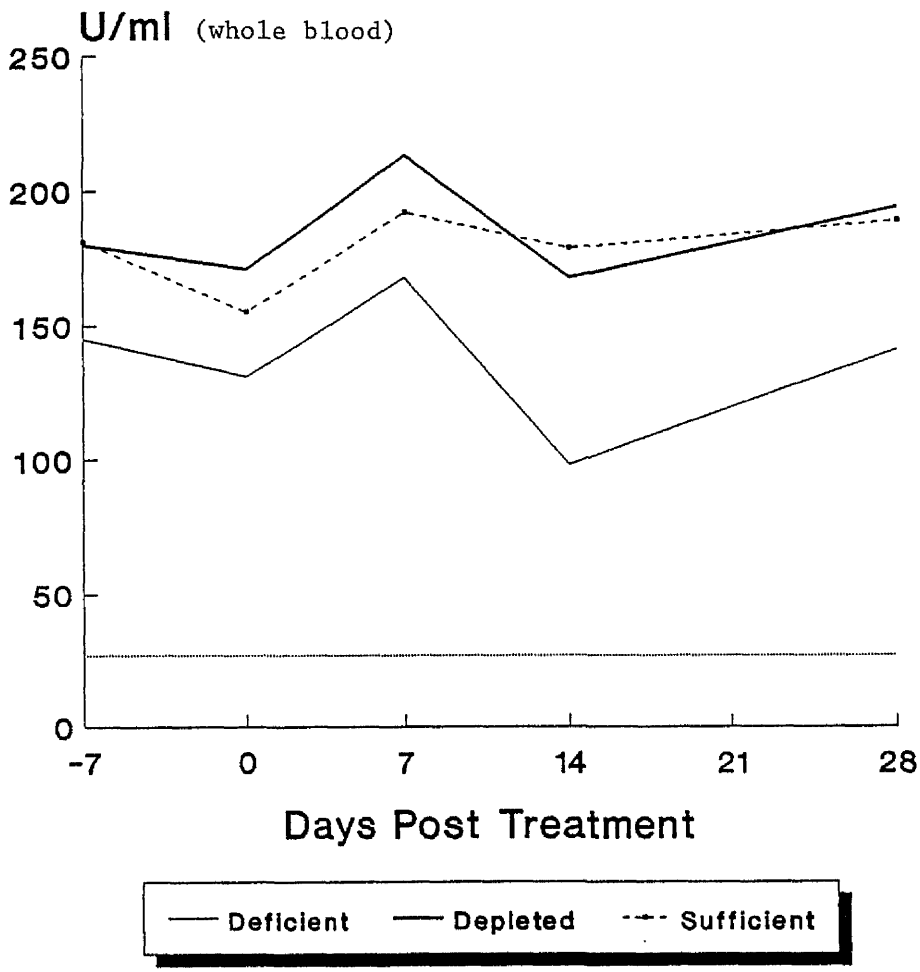
5.3.3 GSH-Px

Pre-treatment GSH-Px activities in all groups revealed no significant differences between infected and non-infected sub-groups of the same cobalt status as all values were high, usually > 100 U/ml and certainly well above the lower normal limit, 27 U/ml. By the day of treatment, the GSH-Px activities of all groups had fallen slightly but still remained very much in excess of 100 U/ml (range 129-177 U/ml). Mean activities were elevated by 7 days PT by between 22 and 61 U/ml above their previous level but thereafter activities in all groups fell slightly.

Statistical analysis revealed no significant differences between infected and non-infected sub-groups of the same cobalt status and thus Figure 49 was constructed from status group means. Comparison of depleted and sufficient groups revealed no significant difference between activities on any occasion during the recovery phase. Mean GSH-Px activities and standard errors for the recovery phase of Experiment 1 are listed in Table II, Appendix III.

FIGURE 49

Mean GSH-Px Activity
Recovery Phase



Experiment 1

5.3.4 Parasitology

Mean pre-treatment (day 0) plasma pepsinogen concentration shown in Table 37 revealed trends which continued from Experiment 1. Infected depleted lambs showed a mean concentration of 2253 mu/l (range 870-4410), significantly higher ($P < 0.05$) than supplemented infected controls which had a mean concentration of 937 mu/l (range 320/1830), both of which were significantly higher than their respective non-infected controls ($P < 0.01$). No significant difference was detected between non-infected status sub-groups. Treatment with SC-anthelmintic resulted in levels of infected lambs still being significantly elevated in both infected groups in comparison with non-infected controls by 7 days PT, ($P < 0.01$) with ranges of 470-3500 mu/l for infected and 270-470 mu/l for non-infected lambs and a significant difference was detected between sufficient and depleted infected sub-groups at that time ($P < 0.01$). By 14 days PT, however, no significant differences were detected between previously infected and their non-infected sub-groups and similarly, no significant difference was detected between sufficient infected and depleted infected lambs, with values ranging from 310-740 mu/l.

Analysis of pre- and post-treatment WEC data (Table 38) showed pre-treatment infection levels which continued from Experiment 1, ie WEC's of depleted lambs were significantly greater than sufficient counterparts ($P < 0.01$). WEC's fell significantly and very sharply by 7 days after treatment ($P < 0.01$) when sufficient lambs, whose values ranged from 0-18 epg, had significantly lower mean counts ($P < 0.01$) than infected depleted controls and both of which were extremely low in comparison with pre-treatment

levels, ie 5.6 versus 309 epg for sufficient lambs and 23.5 versus 3219 epg for depleted counterparts. By 14 days PT mean counts had fallen to 2.6 for sufficient lambs (range 0-5 epg) and 15.6 (range 0-44 epg) for depleted counterparts but the significant difference between those two groups remained ($P < 0.01$).

TABLE 37: Mean Pre- and Post-Treatment Plasma Pepsinogen Concentration - Experiment 1

	PLASMA PEPSINOGEN CONCENTRATION ± SE (mu/l)		
GROUP	PRE TREATMENT	7 DAYS PT	14 DAYS PT
1	960 (-)	1200 (-)	740 (-)
2	320 (-)	320 (-)	310 (-)
3	2253^{c} (382)	1963^{c} (309)	444^{a} (27)
4	423^{a} (34)	401^{a} (27)	468^{a} (48)
5	937^{b} (139)	1186^{b} (215)	446^{a} (66)
6	335^{a} (30)	353^{a} (33)	504^{a} (44)

Values with differing superscripts differed significantly ($P < 0.01$)

TABLE 38: Pre- and Post-Treatment Mean WEC (±SE) - Experiment 1

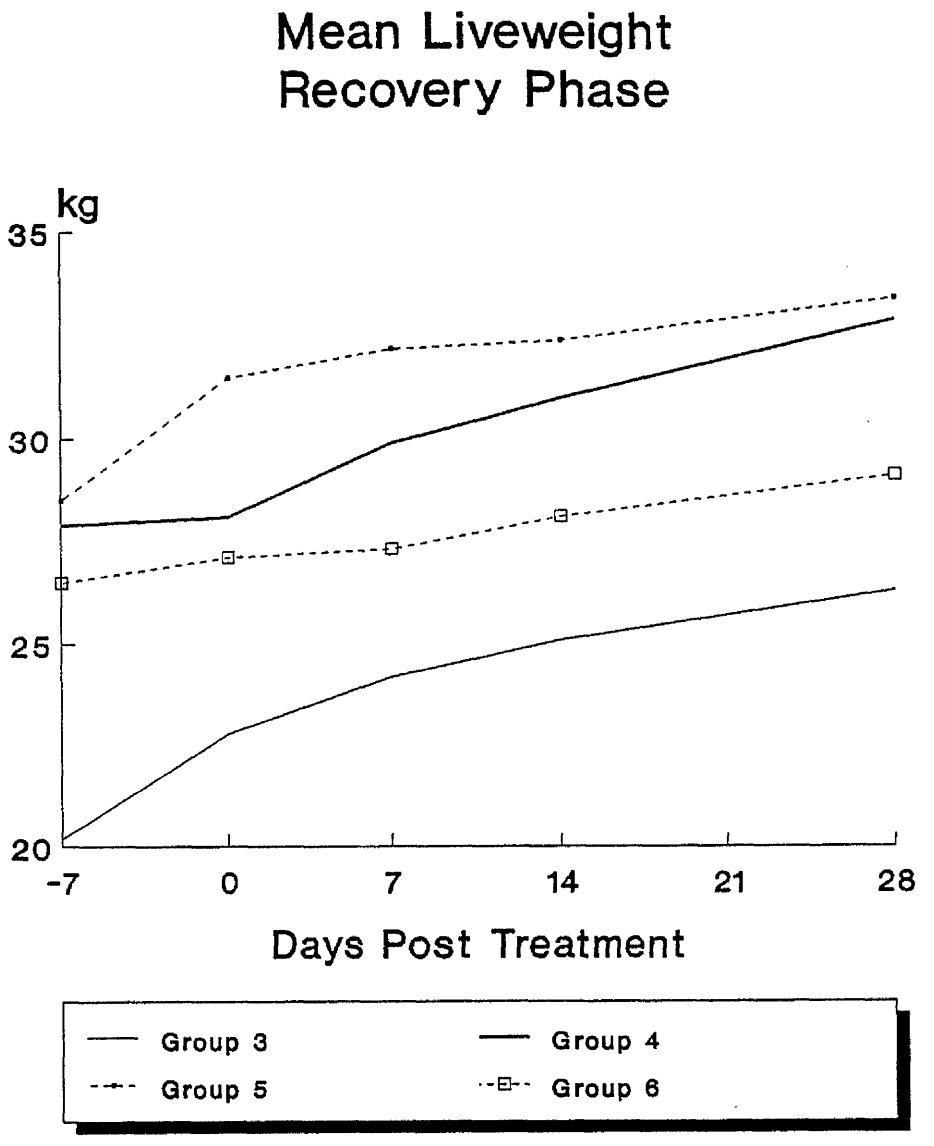
GROUP	WORM EGG COUNT (epg) PRE TREATMENT	7 DAYS PT	14 DAYS PT
1	550.0 (-)	62.0 (-)	32.0 (-)
3	3219[a] (771.4)	23.5[d] (9.4)	15.6[d] (5.4)
5	309[b] (61.2)	5.6[c] (2.4)	2.6[c] (0.8)

Values with differing superscripts differed significantly ($P < 0.01$)

5.3.5 Liveweight

Figure 50 shows the mean liveweights of lambs recorded during the recovery period of Experiment 1. No consistent significant difference attributable to infection was detected in any group before treatment but during the recovery phase, a few significant differences were noted as shown in Table III, Appendix III. On the day of treatment, mean liveweight of Groups 3 lambs (22.8 kg) was significantly lower than those of their non-infected counterparts, Group 4 ($P < 0.05$) and than supplemented infected lambs, Group 5 ($P < 0.01$) at 28.1 and 31.5 kg respectively. Conversely, the weights of Group 5 lambs were significantly higher ($P < 0.05$) than their non-infected controls. Weight gain in all groups was regular and previously depleted infected lambs (Group 3) appeared to gain weight at the same rate as their non-infected controls since the initial significant difference was maintained on all sampling dates ($P < 0.05$). Group 3 lambs gained weight at a rate

FIGURE 50



Experiment 1

Group 3: Depleted, Infected Lambs

Group 4: Depleted, Non-Infected Lambs

Group 5: Sufficient, Infected Lambs

Group 6: Sufficient, Non-Infected Lambs

comparable with sufficient (previously) infected equivalents since the significant difference between those groups remained on all other sampling dates as did weight differences between Groups 5 and 6 ($P < 0.05$). No significant difference was detected between Groups 4 and 6, non-infected depleted and sufficient sub-groups. Group 3 and 4 lambs gained an average of 3.5 and 4.8 kg respectively over the 28 day monitoring period in comparison to 1.9 and 2.0 kg among sufficient equivalents, Groups 5 and 6, although significant differences between those groups still remained.

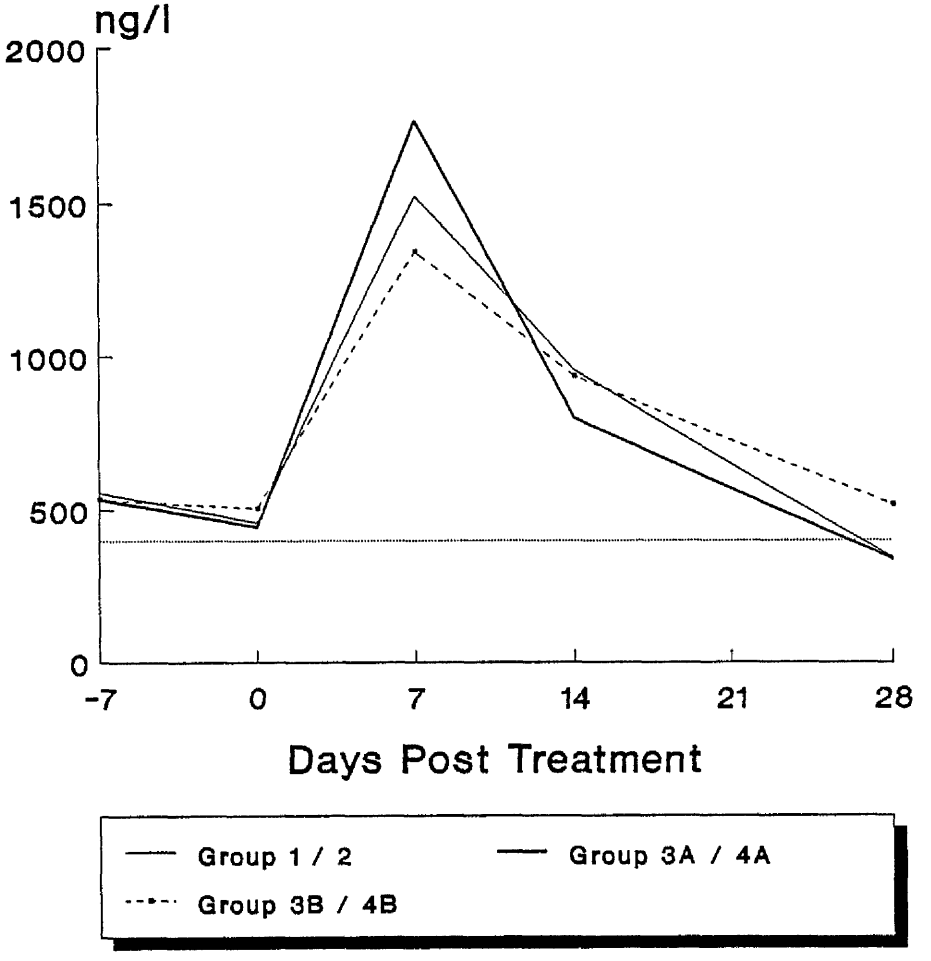
5.4 RESULTS - EXPERIMENT 2

5.4.1 Vitamin B_{12}

Pre-treatment Vitamin B_{12} concentrations of lambs surviving beyond the end of Phase II, Experiment 2 are listed in Table IV, Appendix III and show that no significant difference attributable to infection or micronutrient status existed in Experiment 2 where the Vitamin B_{12} concentrations of all lambs were > 400 ng/l (range 470-646). On the day of treatment, an isolated infection effect occurred when selenium deficient infected lambs had Vitamin B_{12} concentrations (347 ng/l) significantly lower than their non-infected controls, 544 ng/l ($P < 0.05$). The absence of infection effects on other monitoring dates meant that the data used to construct Figure 51 comprised means of infected and non-infected sub-groups of the same micronutrient status. Comparison between micronutrient status groups revealed no significant differences in Vitamin B_{12} concentrations in any group on any sampling date during the recovery phase.

FIGURE 51

Mean Vitamin B12 Concentration Recovery Phase



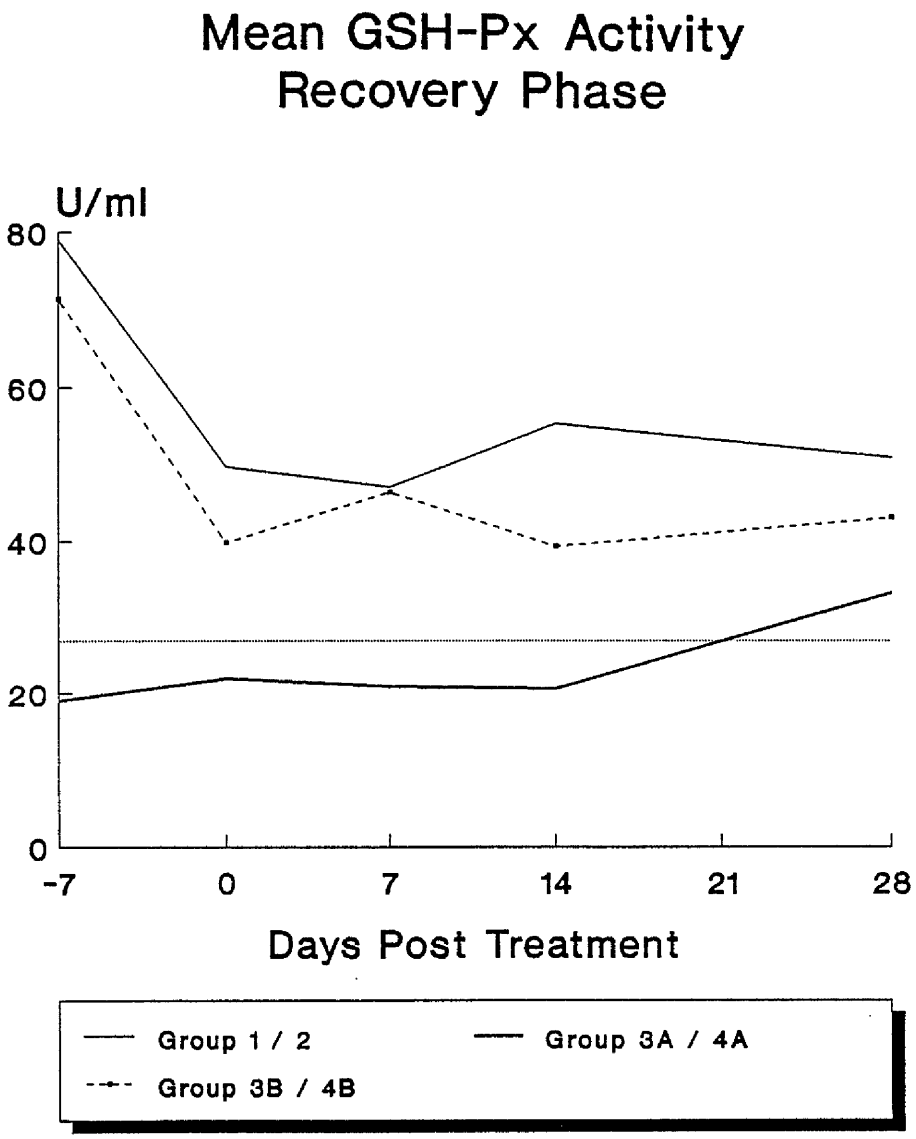
Experiment 2

Mean post-treatment Vitamin B_{12} levels around 530 ng/l (range 470-464 ng/l) were significantly elevated in comparison to pre-treatment values by 7 days PT with mean values in the range 1156-2283 ng/l ($P < 0.01$). Thereafter values fell by 14 days PT to levels in the range 494-1107 ng/l but still in excess of the 400 ng/l threshold. By 28 days PT however, levels in all groups, except 4B, had fallen to less than 400 ng/l ranging from 299-388 ng/l, indicating marginal cobalt deficiency.

5.4.2 GSH-Px

Mean pre-treatment GSH-Px activity (Table V, Appendix III) indicated that the GSH-Px activities of selenium deficient lambs at the end of Phase II were significantly lower than those of the other two groups ($P < 0.01$). Absences of significant difference in GSH-Px activity between infected and non-infected sub-groups of the same micronutrient status allowed construction of Figure 52 representing mean values from both micronutrient status sub-groups. Selenium deficient lambs (Groups 3A and 4B) were significantly lower than remaining groups ($P < 0.01$) for the whole of the recovery phase and although Figure 53 shows mean GSH-Px activity rising with time, activity of selenium deficient lambs only exceeded the lower normal threshold, 27 U/ml, on the last sampling date, 28 days PT with a mean value of 33.3 U/ml. No significant difference was detected between adequate lambs, Groups 1 and 2, and selenium supplemented, Vitamin E deficient equivalents, Groups 3B and 4B, on any sampling date and all had mean values always greater than 27 U/ml (range 34.9 - 58.5).

FIGURE 52



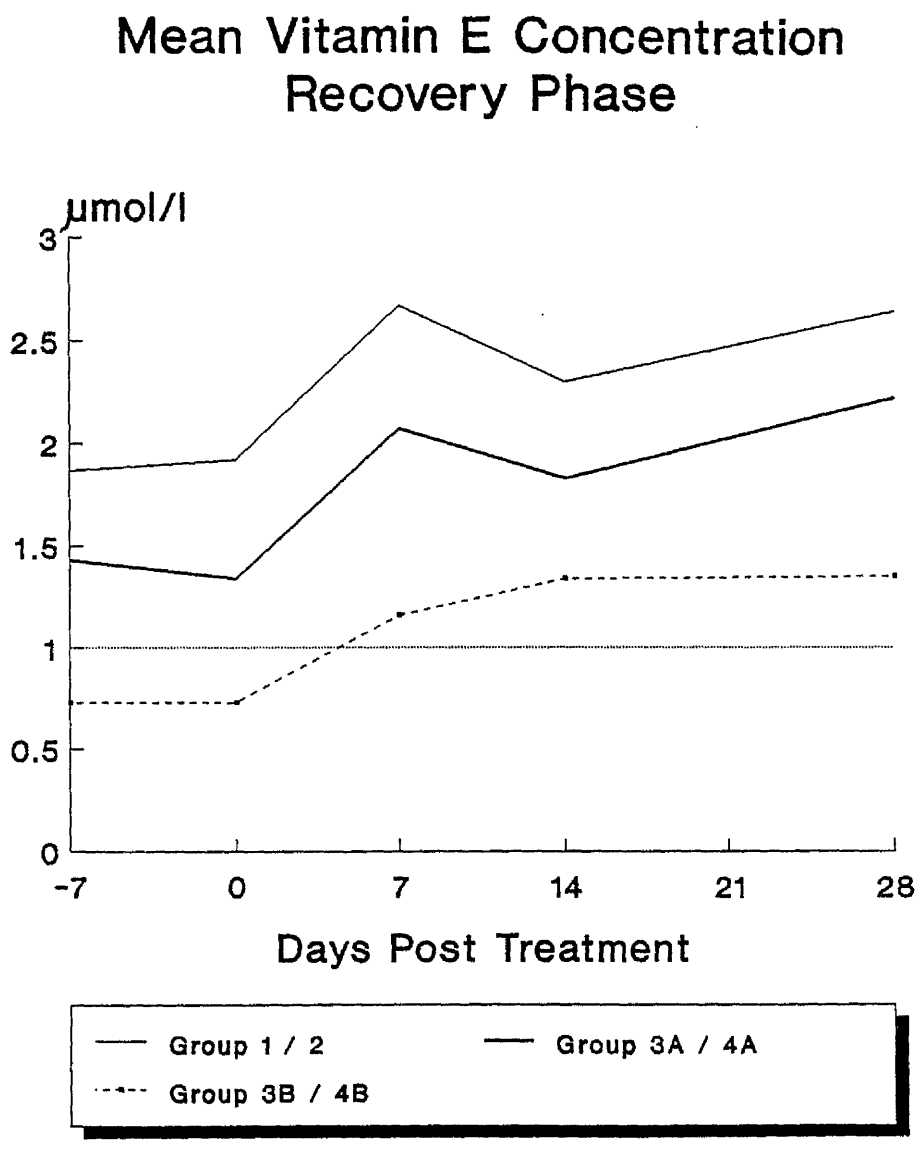
Experiment 2

5.4.3 Vitamin E

Mean Vitamin E concentration before treatment (Table VI, Appendix III) confirmed trends found in Phase II, Experiment 2 that Vitamin E deficient lambs had significantly lower ($P < 0.05$) concentrations than selenium deficient and adequate controls. Lambs which had been supplemented with Vitamin E during Phase II, Groups 1, 2, 3A and 4A, continued to show concentrations > 1.0 µmol/l during the recovery phase. Groups 3A and 4A had concentrations in the range 1.17-2.33 µmol/l which were slightly lower than adequate controls (range 1.77-2.94 µmol/l) a difference which was maintained during the recovery phase.

The serum Vitamin E concentration of Vitamin E deficient lambs increased as the recovery phase progressed but it was not until 14 PT that both infected and non-infected sub-groups exceeded 1.0 µmol/l and their initial lower starting concentration was reflective of the lower level reached by that group by the end of the recovery phase. Mean Vitamin E concentration of lambs in the recovery phase of Experiment 2 are shown in Figure 53 which was constructed from means of infected and non-infected sub-groups of the same micronutrient status since statistical analysis revealed no significant difference attributable to infection in any status group, on any sampling date. Statistical comparison between micronutrient status groups showed that Vitamin E concentrations in lambs which had previously been defined as deficient were significantly lower ($P < 0.05$) than remaining groups for the duration of the recovery phase. No significant difference in Vitamin E status was detected between selenium deficient and adequate controls despite their different initial levels.

FIGURE 53



Experiment 2

5.4.4 Parasitology

Plasma pepsinogen concentrations found in the recovery phase of Experiment 2 reflected the different levels found among groups at the end of Phase II whereby all infected lambs had significantly elevated levels in comparison to non-infected controls ($P < 0.05$) and the effect of SC-anthelmintic treatment affected plasma pepsinogen concentrations such that the lowest levels were detected in adequate lambs, highest values were in selenium deficient lambs and Vitamin E deficient lambs were intermediate. By 7 days PT however, plasma pepsinogen of infected lambs had fallen to levels not significantly different from either non-infected controls or each other as shown in Table 39.

TABLE 39: Mean Pre- and Post-Treatment Plasma Pepsinogen Concentration - Experiment 2

	PLASMA PEPSINOGEN CONCENTRATION ±SE (mu/l)	
GROUP	PRE TREATMENT	7 DAYS PT
1	487[b] (149)	393[a] (61)
2	256[a] (27)	353[a] (27)
3A	773[b] (271)	393[a] (45)
3B	560[b] (31)	377[a] (39)
4A	396[a] (34)	447[a] (45)
4B	363[a] (47)	410[a] (53)

Values with differing superscripts differed significantly ($P < 0.05$)

Similar trends were found in WEC data. Mean pre-treatment WEC's are listed in Table 40 and confirmed trends found at the end of Phase II, similar to those previously described for plasma pepsinogen concentration whereby selenium deficient lambs had the highest count ($P < 0.05$), adequate lambs the lowest and Vitamin E deficient lambs were intermediate. However no eggs were detected in faeces of previously infected lambs by 7 days PT in any micronutrient status group.

TABLE 40: Pre- and Post-Treatment WEC's (±SE) - Experiment 2

GROUP	WORM EGG COUNT ±SE (epg)	
	PRE TREATMENT	7 DAYS PT
1	257[a] (69)	0 (0)
2	0 (0)	0 (0)
3A	621[b] (141)	0 (0)
3B	336[a] (111)	0 (0)
4A	0 (0)	0 (0)
4B	0 (0)	0 (0)

Values with differing superscripts differed significantly ($P < 0.05$)

5.4.5 Liveweight

Table VII, Appendix III shows mean pre- and post-treatment liveweights of lambs in Experiment 2 during the recovery period.

Pre-treatment liveweight levels illustrated trends similar to those found at the end of Phase II where Vitamin E deficient lambs had matched their adequate controls but selenium deficient lambs were significantly lighter than both of those status groups ($P < 0.05$) and infected lambs were lighter than their non-infected counterparts although not significantly so. After slaughtered lambs were removed from infected groups, mean liveweight on the day of treatment (day 0), showed no significant difference between any group attributable to either selenium or Vitamin E supplementation or to infection. These findings were repeated on all other sampling dates post-treatment. Figure 54, which shows mean liveweight of infected and non-infected subgroups of the same micronutrient status, showed that weight gain was similar in all non-infected groups, 4.4-4.9 kg, over the treatment period. Infected selenium and Vitamin E deficient lambs, Groups 3A and 3B, gained slightly more weight, 5.3 kg over the recovery phase, than their non-infected controls whereas adequate lambs, Group 1, gained slightly less, 3.5 kg, over the same period. Differences in liveweight gain were not significant.

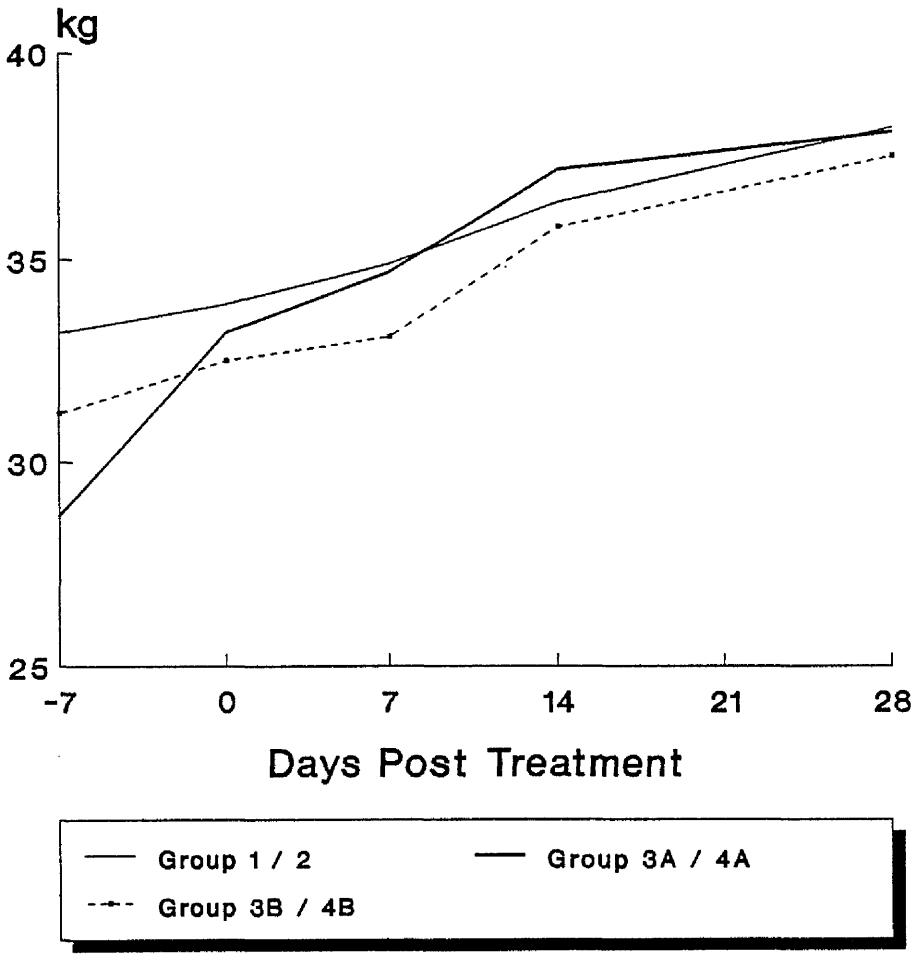
5.5 DISCUSSION - EXPERIMENT 1

5.5.1 Vitamin B_{12}

Anthelmintic treatment and supplementation with cobalt increased and maintained serum Vitamin B_{12} concentrations in all groups greatly beyond the 400 ng/l threshold for the entire 28 day period. The different treatment regimes in the present study caused longer lasting effects than the transient rise in serum

FIGURE 54

Mean Liveweight Recovery Phase



Experiment 2

Vitamin B_{12} after SC-anthelmintic treatment previously reported (MacPherson *et al*, 1987b and Field *et al*, 1988). Explanation of the different findings comes from the additional cobalt dose given to all lambs in Experiment 1 of the present study which was thought necessary for the remission of chronic, severe cobalt deficiency induced during the experimental period. Maintained elevation of serum Vitamin B_{12} concentration in the present study is however in agreement with similar findings in cattle dosed weekly with cobalt as reported by MacPherson (1973) and confirmed recently in sheep by MacPherson *et al* (1987b) using SC-anthelmintic with additional cobalt supplements in lambs and also in ewes (MacPherson, 1989).

5.5.2 GSH-Px

Previous studies have reported that SC-anthelmintic treatment raised GSH-Px activity and that treated sheep showed a lasting response, > 3 months (MacPherson *et al*, 1987b, Bremner *et al*, 1988, Field *et al*, 1988 and Suttle *et al*, 1990). Findings in the present study (Experiment 1) confirmed the findings of those authors since GSH-Px activities of lambs in this experiment also increased even though the nature of their diet meant that their GSH-Px activities were very high during Experiment 1 (ie > 100 U/ml) and remained so after treatment thus possibly masking longevity effects after SC-anthelmintic treatment report by previous authors.

5.5.3 Liveweight

A rapid return of appetite following treatment with anthelmintic and oral cobalt dosing of deficient lambs in this experiment is in agreement with MacPherson (1973) of a similar rapid effect in deficient cattle after cobalt dosing. Therefore it was not surprising that lambs previously defined as depleted (and subsequently becoming cobalt deficient) in Experiment 1 showed recovery of appetite noted subjectively and thereafter gained weight more rapidly throughout the recovery phase than sufficient controls although not significantly so. The findings in the present study confirmed the report of Bremner *et al* (1988) that SC-anthelmintic treatment prevented continued weight loss and appetite suppression noted in untreated animals in their experiments.

5.5.4 Parasitology

It was evident from results that SC-anthelmintic treatment was not 100% effective in removal of parasite burden as measured by the continued presence of nematode eggs in the faeces of previously infected lambs. Biochemistry, however, had revealed that pathological damage was limited and that plasma pepsinogen concentrations had returned to normal. The findings in the present study disagree with the report of Ross (1975) of 100% effectiveness of fenbendazole in removing a mixed nematode burden in lambs but is explained by the very short duration of infection allowed in that experiment (28 days) in comparison with the longstanding adult burden which was allowed to establish in the present experiment. Previous studies reporting 99.0-99.5%

effectiveness of fenbendazole in removing *O. circumcincta* infection in sheep (Wilkins, 1975) were confirmed by findings in the present study. Similarly removal of less than 100% of burden has been reported in *O. ostertagi* infected cattle treated with fenbendazole (Lancaster and Hong, 1977).

5.6 DISCUSSION - EXPERIMENT 2

5.6.1 Vitamin B_{12}

Regular supplementation with cobalt during Phase II of Experiment 2 resulted in adequate Vitamin B_{12} levels before treatment. However dosing with SC-anthelmintic (Panacur-SC, Hoechst Animal Health) significantly raised Vitamin B_{12} levels to > 1000 ng/l by 7 days PT, which were maintained until 14 days PT. This agrees with the findings of Field *et al* (1988) that treatment with SC-anthelmintic (Ovithelmin-SC, Jansen Animal Health) raised serum Vitamin B_{12} above 1000 ng/l for up to 7 days although those authors noted a rapid fall in serum Vitamin B_{12} thereafter. Field *et al* (1988) also reported that serum Vitamin B_{12} levels were lower than their initial levels by 28 days after treatment and these findings are confirmed in the present study where Vitamin B_{12} levels fell below 400 ng/l by 28 days PT. Bremner *et al* (1988) also noted that Vitamin B_{12} concentrations fell by 10 days after treatment. MacPherson *et al* (1987b) administered SC-anthelmintic monthly to show maintained cumulative beneficial effects in serum Vitamin B_{12} and later reported (MacPherson, 1989) that three-weekly treatment with SC-anthelmintic (Panacur SC, Hoechst Animal Health) was beneficial but suggested that a rate yet to be defined, but in the range 21-100 mg cobalt per

treatment, would increase the duration and effectiveness of this method of cobalt supplementation.

5.6.2 GSH-Px

Lambs which were previously selenium deficient in Experiment 2 showed elevation in mean GSH-Px activity after SC-anthelmintic treatment but it was not until 28 days PT that their levels exceeded 27 U/ml and thus did not regain adequate selenium status until that time. These findings confirmed the report of Bremner *et al* (1988) that GSH-Px activities of previously selenium deficient lambs rose from 9.5 U/ml pre-treatment to 40 U/ml 28 days after treatment. The findings in the present study also confirm the earlier report of MacPherson *et al* (1987b) of pre-treatment GSH-Px activities being boosted from 13.3 to 44.4 U/ml and from 16.6 to 26.1 U/ml, 28 days after SC-treatment at two separate field sites. GSH-Px activities in other (selenium normal) groups in Experiment 2 were adequate before treatment and dosing with Panacur-SC simply increased their mean activities by the end of the recovery phase and confirmed earlier similar findings of MacPherson *et al* (1987b) in SC-anthelmintic treated selenium adequate sheep.

5.6.3 Vitamin E

The findings of the present study illustrated that oral Vitamin E supplementation did not raise the Vitamin E status of previously deficient animals to adequate levels immediately and disagrees with the report of Doncon and Steel (1988) that oral drenching with Vitamin E, stated by them to be the preferred

route of supplementation to avoid damage to carcass quality associated with Vitamin E injection, raised alpha-tocopherol levels by 4 hours post-treatment. Explanation of those differences comes from the much larger Vitamin E dose rates used by those authors (1000 or 2000 iu oral dose) and that in the present study (150 iu on 4 occasions). However it was clear from findings in the present study that difficulties in administering Vitamin E supplements via the alimentary tract that investigation of effectiveness of Vitamin E supplementation of anthelmintics requires further study.

5.6.4 Parasitology

Results in Experiment 2 indicated that anthelmintic treatment was completely effective in removing parasitic burden in lambs and agrees with the findings of Ross (1975) of 100% effectiveness of fenbendazole in removing nematodes in lambs. Effectiveness of anthelmintic therapy in Experiment 2 contrasts with the residual infection (as measured by WEC) noted in Experiment 1. This was probably related to metabolism impairment associated with cobalt deficiency and which resulted in impaired utilisation of anthelmintic in those lambs in comparison to selenium and Vitamin E deficient counterparts which suffered neither metabolism impairment nor poor effectivenss of anthelmintic treatment. No direct assessment of this effect was made in the present study although this may require further study.

5.6.5 Liveweight

The large change between mean pre-treatment liveweight of selenium deficient, previously infected lambs, Group 3A, and their mean liveweight on the day of treatment reflected the removal of slaughtered lambs from that group. No significant different in growth rates was detected between previously infected lambs and their non-infected counterparts during the recovery period and confirmed findings in Phase II. Improvement in liveweight in lambs which were previously Vitamin E and selenium deficient in comparison with adequate controls, indicated a slight growth response attributable either to Vitamin E or selenium supplementation during the recovery phase which indirectly confirmed the findings of MacPherson *et al* (1987b) that SC-anthelmintic treatment did not cause a growth response in selenium adequate sheep, ie where none was expected and whereby adequate lambs in the present study (Groups 1 and 2) grew at a lower rate than remaining groups.

CHAPTER 6

GENERAL DISCUSSION

6.1 DEFINING COBALT DEFICIENCY

Results from Experiment 1 of the present study illustrated the widely held contention that both serum Vitamin B_{12} and MMA concentrations are reasonably reliable indicators of cobalt status in ruminant animals. It was not possible to assess which was the more sensitive indicator of cobalt status as this was outwith the remit of Experiment 1, and all serum samples were not assayed for MMA concentration on each sampling occasion. However Experiment 1 did illustrate that normal limits defining serum Vitamin B_{12} concentration may not be appropriate markers for young lambs prior to development of rumen microflora capable of *in situ* Vitamin B_{12} synthesis. This was exemplified by the fact that sufficient lambs had serum Vitamin B_{12} concentrations considerably below the 400 ng/l threshold defining deficiency in the early part of that experiment. However normal MMA concentrations in that group, at that time suggests that this may prove to be the more reliable indicator. This is in broad agreement with the views of McMurray *et al* (1985) that MMA is an active marker of cobalt status rather than the passive form of serum Vitamin B_{12}.

An accurate assessment of cobalt status could be obtained by taking liver biopsy samples and determining liver cobalt or Vitamin B_{12} content. However the comparative expense and distress caused by this procedure may not be merited in a commercial flock when low or marginal serum Vitamin B_{12} or cobalt status could be corrected simply and cheaply by dosing with a cobalt sulphate solution or another suitable method of supplementation.

6.2 DEFINING MUSCLE DAMAGE IN VESD SYNDROME

Experiment 2 in the present study showed that supplementation with a single selenium dose (as sodium selenite) was effective in maintaining GSH-Px levels greater than the threshold defining deficiency during phases I and II. However, the slow response to Vitamin E supplementation during those periods showed that feed incorporated alpha-tocopheryl acetate absorbate was not immediately effective in curing deficiency and that elevation of serum Vitamin E concentration by intramuscular injection with Vitamin E was only transient.

Biochemical assessment of CK activity proved to be a useful measure of muscle damage in the present study. However the presence of white muscle disease in a casualty lamb with normal selenium status indicated that the diagnosis of WMD requires a biochemical "package", which should consist of serum Vitamin E concentration, CK activity, and whole blood GSH-Px.

The supplementation of Vitamin E deficient lambs with selenium and vice versa at the start of Phase II, rapidly reduced muscle damage as measured by CK in both groups and indicated that recovery from muscle damage was more rapid following selenium rather than Vitamin E supplementation.

6.3 MORTALITY, DISEASE RESISTANCE AND MICRONUTRIENT STATUS

Comparison of Experiments 1 and 2 revealed that higher mortality rates were experienced in most micronutrient deficient groups than

in their supplemented controls. Adequate cobalt status of the pregnant and or lactating ewe was the most important factor in, and was required for, resistance to common pathogens such as *P. haemolytica* in their lambs. The mortality rate (88%) in lambs from ewes deficient in cobalt during pregnancy and lactation was higher than in either their less severely cobalt deficient (ie, depleted) counterparts (19%) in Experiment 1, or in Vitamin E deficient lambs (22%) in Experiment 2. These losses compared unfavourably with no mortality in cobalt sufficient lambs in Experiment 1 and a single anomalous casualty among Vitamin E supplemented lambs in Experiment 2.

No casualties occurred in selenium deficient lambs in Experiment 2, but as deficiency lasted 5 weeks only, no direct comparison was possible. *O. circumcincta* infection was not thought to have played a significant role in the mortality rates experienced since, although characteristic signs of infection were found post mortem, mortality rates were equally high among noninfected subgroups of the same micronutrient status and although specific deficiency disease syndromes such as:

a) White liver disease/hepatic encephalopathy; or

b) Vitamin E deficiency/white muscle disease

were found, it was possible that different processes were operating in either micronutrient deficiency.

Casualties in Experiment 2 were related both to the Vitamin E deficiency syndrome and probably also to direct immune effects associated with Vitamin E deficiency. The former can be claimed since varying degrees of myopathy associated with Vitamin E

deficiency was found in all groups in that experiment. However confirmed and suspected vaccination failure to both pneumonic and enteric infection justify the latter contention. In Experiment 2, WMD associated with Vitamin E deficiency confirms firstly the involvement of Vitamin E in preventing myopathy as cited by Underwood (1981) and secondly, decreased immunity to, for example pneumonia, suggests immune defects in response to infection in Vitamin E deficient animals similar to those previously discussed (Tengerdy and Nockels, 1975, Nockels, 1979, Tengerdy *et al*, 1981, 1984).

Cobalt deficient lambs showed specific hepatic effects and also succumbed in much greater numbers to pneumonia, possibly due to cobalt deficiency-mediated inappetance inducing a secondary protein deficiency in those lambs. However, regardless of the mechanisms operating, it was clear that the net effect in both experiments was a poorer resistance to infection. For example, the serotypes of *P. haemolytica* recovered post-mortem (A2 and A6) from pneumonic lambs formed components of the vaccine which they had been given and similar effects were probably responsible for vaccination failures noted in lambs in both Experiments 1 and 2, which revealed signs consistent with enterotoxaemia.

6.4 PRODUCTION DIFFERENCES

Production differences were noted in both Experiments 1 and 2 but comparison between the two must be limited since they appear to have been caused by different factors. Inappetance and concentrate refusal caused by cobalt deficiency and noted in Experi-

ment 1, resulted in reduction in weight gain and eventual weight loss in cobalt deficient lambs and subsequently in depleted counterparts after exhaustion of cobalt reserves. The severity of this effect on both infected and non-infected lambs of the same cobalt status sub-group however, probably masked any possible weight loss attributable to *O. circumcincta* infection similar to that reported by Coop *et al* (1977), which may otherwise have been evident in Experiment 1. This contrasts with Experiment 2 in which no liveweight effect attributable to Vitamin E deficiency occurred. However, a liveweight deficit was evident in all infected lambs compared to their non-infected counterparts in Experiment 2 but was not statistically significant. Liveweight deficit or loss due to *O. circumcincta* infection in Experiment 2 agreed with the previous studies of Coop *et al* (1977). However the absence of significance could be due to the slightly lower infection rate used in Experiment 2 in comparison with the latter authors.

Liveweight differences were noted in selenium deficient, infected and non-infected lambs in Phase II, in comparison with remaining lambs in Experiment 2. Liveweight impairment in a selenium ill-thrift syndrome similar to that described by Hartley (1967) was thus confirmed in the present study. As with other groups in Experiment 2, non-significant liveweight deficit due to infection noted in selenium deficient lambs was also probably due to the lower infection rate used in this study in coparison with others.

A production difference noted between lambs of sufficient groups in Experiments 1 and 2, which gained weight at the desired rate,

showed that Vitamin E supplemented lambs in Experiment 2 had higher liveweights, and probably reflected their higher initial starting weights. Comparison between lambs in deficient groups in Experiments 1 and 2 is limited by the protracted nature of the deficiency in Experiment 1, by virtue of the cobalt deficient state of their dams, but which nonetheless demonstrated that cobalt deficiency resulted in lower liveweights in affected lambs than in selenium and Vitamin E deficient equivalents and which emphasised the production limiting effect of cobalt deficiency cited by Underwood (1977, 1981). Selenium deficient lambs showed a lesser degree of growth impairment, confirming the selenium deficiency ill-thrift syndrome reported by Hartley (1967). Vitamin E deficiency did not however cause any production impairment.

6.5 PARASITOLOGY AND DEFICIENCY

In both Experiments 1 and 2, higher plasma pepsinogen levels were found in infected lambs than in their non-infected equivalents and deficient infected lambs showed considerably higher plasma pepsinogen concentrations than supplemented controls. WEC's in deficient lambs in both experiments were also higher than those of their supplemented controls and similar trends were revealed after slaughter for TWC. Comparison between Experiments 1 and 2 showed no difference in time to patency in either experiment but pathogenic infection as measured by plasma pepsinogen concentration was greater in cobalt deficient lambs. Cobalt depleted lambs also suffered a more pathogenic infection after depletion of cobalt reserves compared with cobalt sufficient lambs in Experiment 1.

Selenium deficient lambs had the highest plasma pepsinogen concentrations in Experiment 2 but these were still approximately equivalent to those of cobalt sufficient lambs in Experiment 1. Vitamin E deficient lambs had intermediate concentrations and adequate infected lambs in Experiment 2 had the lowest levels but were still significantly elevated in comparison with non-infected counterparts. Worm egg counts showed identical trends to plasma pepsinogen concentration and since similar proportions of female: male worms were recovered at necropsy, fecundity effects probably mirror establishment trends and thus TWC. TWC's in the worst affected group, cobalt depleted infected lambs in Experiment 1, were much greater than that found in the least affected group, adequate infected lambs in Experiment 2, and intermediate burdens were recovered in the remaining groups in Experiments 1 and 2.

A self-cure reaction to *O. circumcincta* infection was absent in both Experiments 1 and 2 since plasma pepsinogen and haematology indicated continuing establishment of incoming larvae in all infected groups in both experiments with consequent maturity, the presence of worm eggs in faeces and the recovery of parasites at necropsy. Additionally only minimal evidence of stunting of male worms recovered from sufficient lambs in Experiments 1 and 2 and the relative abundance of adult parasites compared to larval stages in all TWC's confirmed incomplete immunity. This result contrasts with the report of Smith *et al* (1985a) who reported that 10 month old parasite naive lambs mounted a full immune response as measured by local IgA production, stunting of worms, and absence of developing larvae (due to a greater proportion of arrested fourth stage larvae). Such immunity was absent at TWC-

necropsy in 7-8 month old lambs in Experiments 1 and 2 of the present study and thus confirms their lack of immunity.

Comparison between Experiments 1 and 2 again demonstrated the relatively greater effect of cobalt deficiency on resistance to pathogenic *O. circumcincta* infection. Specific and non-specific immune effects are discussed later in this chapter.

6.6 HAEMATOLOGY

In contrast to the findings of helminth mediated anaemia by Horak and Clark (1964) and Holmes and MacLean (1971), no anaemia due to *O. circumcincta* infection was noted in either Experiment 1 or 2 of the present study and was probably due to the low trickle nature of the infective regime. While anaemia was completely absent in Experiment 2, a relative normocytic, normochromic anaemia similar to that reported by Gawthorne *et al* (1966) in cobalt deficient sheep, was observed in all cobalt deficient, depleted and even in a number of sufficient lambs in Experiment 1, which may have masked helminth mediated anaemia. However the total absence of a helminth induced anaemia in Experiment 2 suggests that the result from Experiment 1 is genuine, since infective regimes in both experiments were identical.

Persistant eosinophilia in deficient infected sub-groups in both experiments was largely indicative of continued establishment of ingested helminth larvae, confirmed by continued elevation of plasma pepsinogen levels in infected lambs, which as discussed above, suggested lack of complete immunity in lambs in the present

study. During the infective phase of Experiment 1 however, eosinophilia in the sufficient sub-groups diminished in comparison with deficient counterparts by returning to normal limits by the end of experimentation. Percentages of lymphocytes and other white cells percentages, which may have been lowered due to eosinophil proliferation returned however to normal limits. The continued presence of worm eggs in faeces and maintained elevation of plasma pepsinogen concentration discounted "immunity" in those lambs.

6.7 NON-SPECIFIC IMMUNITY

Neutrophil function as measured by candidacidal assay revealed impairment in all deficient lambs in comparison to their sufficient controls, suggestive of direct immune effects due to selenium and Vitamin E deficiency. However, it remained unclear whether the impairment noted in cobalt deficient lambs was due to a functional immune role of cobalt or to a secondary induced protein intake deficit. No infection effects were noted in any status group in either experiment. The KI of cobalt sufficient lambs in Experiment 1 was, however, lower than that of Vitamin E selenium supplemented (Group 1) counterparts in Experiment 2, the former being of similar magnitude to Vitamin E deficient lambs in Experiment 2. Previous studies (Fisher and MacPherson, 1986, Fisher, 1988) have tended to record higher KI's for cobalt sufficient sheep.

Impaired candidacidal activity in micronutrient deficient lambs confirmed previous findings due to cobalt deficiency (Fisher and

MacPherson, 1986, Fisher, 1988) and selenium deficiency (Boyne and Arthur, 1979, 1981). However the findings in the present study that similar limited impairment occurred in Vitamin E deficient lambs is possibly the first reported incidence of such a phenomenon.

Comparison of deficient lambs revealed that the impairment in KI in cobalt deficient/ depleted lambs in Experiment 1 was much greater than that experienced in Vitamin E deficient equivalents in Experiment 2. Impairment of KI similar to that previously reported (Boyne and Arthur, 1979, 1981) was detected on a single occasion in Phase 2, Experiment 2, in selenium deficient lambs. The net effect of these non-specific immune function analyses is to suggest that cobalt deficient and depleted lambs again suffered greater immune impairment than either selenium or Vitamin E deficient lambs. However firm conclusions regarding this contention must be limited by the different nature of the deficiencies used in Experiments 1 and 2, ie the deficiency in lambs in Experiment 1, by virtue of the cobalt deficient nature of their dams, was protracted in comparison with Vitamin E or selenium deficient equivalents in Experiment 2.

Sufficient controls showed a lower KI in cobalt sufficient lambs (Experiment 1) than in Vitamin E/selenium sufficient equivalents in Experiment 2. Attempts to further investigate neutrophil phagocytosis using NBT reduction further provided only inconclusive results in both Experiments 1 and 2.

6.8 SPECIFIC IMMUNITY

Experiments 1 and 2 showed contrasting results in serological response to *O. circumcincta* as measured by ELISA, where a clear difference between infected and non-infected sub-groups of the same micro-nutrient status was found in Experiment 2 but the difference was poorly demonstrated in Experiment 1.

Titres in Experiment 1 were much lower than those detected in all groups in Experiment 2 but the former showed an interesting trend in the pre-infective phase and the early part of the experiment, where the antibody response, presumed to be maternally derived, diminished prior to experimental *O. circumcinta* infection before rising thereafter. Such an effect, however, was not noted in Experiment 2 since these lambs were older at the start of infection and consequently the level of maternally derived antibody may have diminished.

TWC's in Experiments 1 and 2 showed a similar inverse relationship to ELISA titres to *O. circumcincta* L_3 antigen as measured in the present study where lambs with the lowest burden had the highest titre and vice versa. This suggests that the serological response may be a measure of immunity rather than of pathogenic infection. Although the specificity of this test is relative since apparent titres were detected in non-infected lambs, regression analysis of final serological titres to *O. circumcincta* with log TWC revealed a significant correlation (R^2 = 48.8 % ($P < 0.05$)). While inferences from this test must be limited, it tends to confirm previous suggestions in the present study of greater

impairment of the immune response in cobalt deficient and depleted lambs. Lambs in Experiment 2 showed a greater response to *O. circumcinta* infection as measured by ELISA, relatively low TWC's and a higher level of resistance to other pathogens as measured by lower mortality.

The role of antibodies and the serological response to *O. circumcincta* infection in the present study is difficult to appraise since most previous studies have concentrated on local responses at the site of infection. For example, Sinski (1975) and Sinski and Bezubik (1980) concluded from their studies that while a serological humoral response to *O. circumcincta* and its excretory-secretor antigens was detectable, the levels measured did not reflect immunity. Recently, however, Klesius (1988) speculated that the role of antibody in *O. ostertagi* infected calves, which could also be applicable to *O. circumcincta* infected sheep, may be affected by a number of factors including antigen-sharing, but nonetheless could be summarised thus:

1. The antibody response (in calves) to *O. ostertagi* was weak (Keus *et al*, 1981, Klesius *et al*, 1986).
2. Increased titres to *O. ostertagi* antigen corresponded to resumption of larval development or to increased parasite challenge (Kloosterman *et al*, 1984, 1985, Enterocasso, 1986).
3. Serum IgG IgM and IgA antibody response to *O. ostertagi* was detectable with anamnestic IgG responses (Klesius *et al*, 1986).
4. Animals with higher antibody titres had fewer, shorter worms with fewer ova per female and a greater incidence of smaller vulval flap (Kloosterman *et al*, 1984, 1985).

Klesius (1988) concluded that higher antibody titres suggested increased immunity to reinfection which is broadly confirmed by the findings in Experiments 1 and 2 of the present study where highest titre against *O. circumcincta* infection were found in lambs with lowest worm burdens at slaughter.

Measurement of the serological response to specific vaccination showed interesting results in the present study when considered with deaths due to enterotoxaemia and pneumonia, apparently due to vaccination failures in Experiments 1 and 2, and the pneumonia being associated with serotypes of *P. haemolytica* which were also vaccine components. No difference in pre-vaccination titre was noted between any status group of either experiment although in Experiment 2 the titres were much lower than those found in Experiment 1.

Such a result was expected in Experiment 2, since no nutritional stress was placed on pregnant ewes. However, despite the nutritional stress in Experiment 1, in which one-third of ewes were in a cobalt deficient state for the whole of their gestation period, this did not affect passive transfer of antibodies to the lamb and contrasts with previous findings of Fisher (1988). Pre-vaccination levels in Experiment 1 were much higher than those measured in lambs in Experiment 2, possibly because of lower residual maternal antibody in the 1-2 month older lambs of Experiment 2.

Post vaccination titres in both status groups in Phase I of Experiment 2 showed considerable elevation from their pre-vaccination level in most cases. However large variations in individual

titres of supplemented lambs prevented this from being a significant difference. Post vaccination titres in Experiment 1 were somewhat lower than those found in Experiment 2, and further proof of the relatively low immune response of cobalt deficient lambs in the various assays and the comparatively greater influence of cobalt deficiency on immune status.

6.9 THE EFFICACY OF SC-ANTHELMINTIC (Panacur-SC)

Results in Experiment 1 revealed that simultaneous oral supplementation of all lambs with cobalt sulphate and SC-anthelmintic (Panacur SC, Hoechst Animal Health) raised and maintained mean serum Vitamin B_{12} levels of deficient, depleted and sufficient lambs near to the upper limit of detection for the entire treatment period and caused the reduced rate of liveweight gain previously noted in deficient lambs to return to levels similar to those of sufficient controls. The remission of cobalt deficiency and return of appetite in Experiment 1 after SC-anthelmintic treatment confirm previous findings in other similar studies (MacPherson *et al*, 1987b and MacPherson, 1988).

A slightly different treatment regime used in Experiment 2, in which SC-anthelmintic was used on its own, produced results contrasting with Experiment 1 since serum Vitamin B_{12} levels increased only transiently before returning to initial levels by 28 days post-treatment. The present study confirmed previously reported similar transient rises in serum Vitamin B_{12} concentrations after SC-anthelmintic treatment (Bremner *et al*, 1988).

The reverse situation was noted in GSH-Px activity where consistently high levels during the course of Experiment 1 were only marginally raised after SC-treatment and contrasted with Experiment 2 where minimal elevation of a much lower GSH-Px activity was noted. This probably resulted from the selenium deficient nature of the diet used in Experiment 2 and thus accounts for the relative delay in the rise of GSH-Px levels of selenium deficient lambs.

Examination of helminth effects in the recovery phases of Experiments 1 and 2 showed a rapid return to normal plasma pepsinogen values of infected lambs after SC-anthelmintic treatment. Worm egg counts however contrasted between the two experiments where complete removal of burdens within 7 days was noted in Experiment 2 but the low residual presence of worm eggs was noted in faeces of remaining previously infected lambs in Experiment 1. This may have been due to impaired metabolism of the anthelmintic in cobalt deficient lambs associated with helminth-mediated abomasal damage and impaired abomasal function (Coop *et al*, 1977, Holmes and MacLean, 1971,) which would have been greater in Experiment 1 by virtue of the much larger burden found in those lambs in comparison with lambs in Experiment 2.

Production effects in the recovery phase of Experiment 1 were associated with a return of appetite, similar to that quoted by MacPherson *et al* (1973), in depleted lambs and consequent liveweight gain and were probably not comparable with differences noted in Experiment 2 where no production limiting deficiency occurred. This tenet is confirmed when analysing the level of

liveweight in Experiments 1 and 2, ie liveweight of all lambs in the recovery phase of Experiment 2 was much greater than their equivalents in Experiment 1.

It was interesting to observe, however, that while no helminth mediated production deficit was noted in Experiment 1 but occurred in Experiment 2, little or no compensatory growth associated with improved nutrient utilisation after the removal of infection was noted in either experiment. However supplementation with relevant micronutrients in Experiments 1 and 2 resulted in compensatory growth and suggests that the mooted 'ill-thrift' syndromes clearly demonstrated in cobalt deficiency in Experiment 1 and suspected in Vitamin E and selenium deficiencies in Experiment 2 was confirmed during the recovery phase.

6.10 FUTURE WORK

In Experiment 1 a production effect associated with inappetance and resultant higher mortality suggested that it would be important to assess the role of protein intake in the response to infection, parasitic or otherwise, using either pair fed controls for cobalt deficient lambs or by metabolic studies measuring protein intake of infected cobalt deficient lambs using fistulated animals to determine, for example, possible change in rumen microflora caused by cobalt deficiency or by assessing the quality and quantity of protein passing through into the abomasum of deficient lambs.

This would permit the resolution of the relative importance of primary and secondary protein deficiency on impairment of immune functions. Additionally, it would be feasible to justify claims of increasing pathogenic infection in depleted lambs in Experiment 1 after exhaustion of cobalt reserves by sacrificing similar lambs sequentially to monitor the establishment of infection. Measurement of true cobalt status via liver biopsy sampling could provide additional information in the experiments described above.

O. circumcincta larvae used in the present study were cultured from faeces of a donor lamb which had normal micronutrient status. Repeated passage of infection in deficient lambs in the field may ultimately result in the production of 'deficient' infective larvae which may have different pathogenic effects from their normal counterparts. This could be tested by passing larvae repeatedly through micronutrient deficient sheep until the parasites themselves were deficient and then using these to infect helminth-naive sheep and for comparison with infection with normal parasites. Alternatively *in vitro* culture of parasites in cobalt deficient media may be used to provide 'deficient' larvae for suitable comparison.

Finally, Experiment 2 used a change-over design to compare infection in selenium deficient, Vitamin E deficient and normal lambs, the results of which suggested that a three-way comparison, with a longer duration of selenium deficiency, could assess whether Vitamin E deficiency or selenium deficiency was the greater obstacle to functional resistance to disease.

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APPENDIX I

TABLE I: Dietary Constituent Analysis - Experiment 1, Timothy Hay

PARAMETER MEASURED	UNITS	CONTENTS					MEAN (Standard Error)
Cobalt	mg/kg	0.01	0.12	0.01	0.13	0.08	0.07 (0.006)
Dry Matter	g/kg	861.00	863.00	863.00	841.00	778.00	841.00 (16.30)
Crude Protein	g/kg	58.00	60.00	60.00	59.00	58.00	59.00 (0.45)
Organic Matter	g/kg	946.00	946.00	946.00	946.00	946.00	946.00 (0.00)
Rumen Degradable Protein	g/kg	35.00	36.00	36.00	36.00	35.00	35.60 (0.24)
Undegradable Protein	g/kg	23.00	24.00	24.00	23.00	23.00	23.40 (0.24)
Calcium	g/kg	2.90	3.60	3.60	3.40	3.20	3.34 (0.13)
Phosphorous	g/kg	1.10	1.50	1.50	1.30	2.00	1.48 (0.15)
Magnesium	g/kg	0.90	1.10	1.10	0.90	0.80	0.96 (0.06)
Potassium	g/kg	10.90	10.70	10.70	10.90	8.50	10.34 (0.46)
Nitrogen	g/kg	9.30	9.60	9.60	9.20	9.30	9.40 (0.08)
Sodium	g/kg	1.01	0.95	0.95	0.98	1.01	0.98 (0.01)
Copper	mg/kg	3.25	2.34	4.13	2.57	3.14	3.09 (0.31)

All values are expressed on a dry matter (DM) basis

TABLE I (Cont): Dietary Constituent Analysis - Experiment 1, Flaked Maize

PARAMETER MEASURED	UNITS	CONTENTS					MEAN (Standard Error)
Cobalt	mg/kg	0.01	0.01	0.01	0.01	0.015	0.01 (0.001)
Dry Matter	g/kg	863.00	862.00	863.00	862.00	863.00	862.50 (0.26)
Crude Protein	g/kg	89.00	86.00	89.00	86.00	89.00	87.80 (0.73)
Organic Matter	g/kg	992.00	991.00	992.00	991.00	992.00	991.60 (0.24)
Rumen Degradable Protein	g/kg	54.00	60.00	55.00	62.00	56.00	57.40 (1.54)
Undegradable Protein	g/kg	35.00	26.00	34.00	24.00	23.00	28.40 (2.54)
Calcium	g/kg	10.90	0.20	0.20	0.20	10.90	4.48 (2.62)
Phosphorous	g/kg	4.90	2.20	1.50	2.20	4.90	3.14 (0.73)
Magnesium	g/kg	1.80	0.80	0.60	0.80	1.80	1.16 (0.26)
Potassium	g/kg	8.49	2.35	2.10	2.35	8.49	4.76 (1.53)
Nitrogen	g/kg	14.20	13.70	14.20	14.20	13.70	14.00 (0.12)
Sodium	g/kg	0.10	0.06	0.05	0.06	0.10	0.07 (0.01)
Copper	mg/kg	2.14	1.44	1.26	1.04	1.21	1.42 (0.19)

All values are expressed on a dry matter (DM) basis

TABLE I (Cont): Dietary Constituent Analysis - Experiment 1, Prairie Meal (maize gluten 60%)

PARAMETER MEASURED	UNITS	CONTENTS					MEAN (Standard Error)
Cobalt	mg/kg	0.01	0.01	0.07	0.12	0.11	0.06 (0.0015)
Dry Matter	g/kg	891.00	891.00	892.00	892.00	890.00	891.20 (0.37)
Crude Protein	g/kg	695.00	695.00	666.00	666.00	675.00	679.40 (6.58)
Organic Matter	g/kg	918.00	918.00	949.00	949.00	928.00	932.40 (7.02)
Rumen Degradable Protein	g/kg	385.00	398.00	401.00	390.00	385.00	391.80 (3.31)
Undegradable Protein	g/kg	310.00	297.00	265.00	276.00	290.00	287.60 (7.88)
Calcium	g/kg	0.20	0.20	0.20	0.20	0.20	0.20 (0.00)
Phosphorous	g/kg	1.50	6.00	6.20	6.20	5.80	5.14 (0.91)
Magnesium	g/kg	0.60	0.30	0.30	0.30	0.40	0.38 (0.06)
Potassium	g/kg	2.11	0.90	0.66	0.66	1.24	1.11 (0.27)
Nitrogen	g/kg	111.20	111.20	106.60	106.60	110.00	109.10 (1.05)
Sodium	g/kg	0.10	0.10	0.08	0.08	0.10	0.09 (0.005)
Copper	mg/kg	1.24	0.72	1.04	0.50	0.72	0.84 (0.13)

All values are expressed on a dry matter (DM) basis

TABLE II: Mean Serum Vitamin B_{12} Concentration ±SE (ng/L)

DAYS POST INF[N]	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
STATUS	DEFICIENT		DEPLETED		SUFFICIENT	
-28	68[b](9.69)	55[c](5.00)	272[a](46.80)	294[a](64.20)	202[a](35.70)	142[a](23.40)
-20	138[a](49.40)	204[a](106.50)	284[a](58.10)	286[a](51.20)	320[a](119.70)	318[a](87.50)
- 8	118[a](33.20)	132[a](28.90)	223[a](57.90)	248[a](42.20)	216[a](46.50)	262[a](68.60)
2	141[a](68.80)	123[a](23.50)	170[a](26.00)	203[a](42.00)	109[a](12.60)	152[a](29.10)
7	120[a](18.90)	141[a](20.90)	186[a](24.70)	206[a](32.30)	267[c](30.20)	476[c](114.80)
14	93[a](6.19)	135[a](28.80)	275[a](18.10)	335[a](22.30)	452[c](63.20)	747[c](125.60)
23	104[a](9.96)	121[a](16.30)	125[a](12.60)	160[a](21.40)	741[c](118.90)	839[c](81.30)
28	108[a](16.20)	86[a](11.10)	114[a](11.40)	139[a](19.60)	841[c](132.20)	1001[c](91.30)
35	91[a](12.98)	79[a](9.88)	180[a](34.50)	149[a](18.40)	785[c](107.60)	788[c](84.30)
42	123[a](36.50)	83[a](11.40)	143[a](17.70)	134[a](17.10)	768[c](107.10)	730[c](87.40)
49	76[a](7.47)	83[a](7.38)	215[a](53.20)	183[a](24.50)	684[c](114.80)	950[c](95.10)
56	95[a](7.64)	95[a](10.90)	147[a](11.90)	160[a](16.60)	540[c](70.90)	683[c](72.60)
63	120[a](0.00)	113[a](17.90)	285[a](77.20)	166[a](14.40)	561[c](81.80)	681[c](80.40)
70	118[a](2.25)	102[a](12.60)	137[a](13.40)	181[a](24.40)	463[c](62.80)	618[c](72.40)
77	110[a](15.00)	84[a](22.70)	118[a](21.50)	147[a](28.70)	507[c](95.40)	719[c](95.10)
84	65 (-)	93[a](18.30)	136[a](24.70)	126[a](11.10)	684[c](106.20)	937[c](115.00)
91	70 (-)	88[a](28.30)	133[a](30.70)	134[a](19.00)	531[c](87.70)	779[c](87.50)
98	150 (-)	88[a](2.50)	140[a](28.00)	216[b](57.50)	431[c](66.70)	647[c](71.80)
105	90 (-)	63[a](7.50)	106[a](16.30)	134[a](25.30)	485[c](62.60)	657[c](67.60)
111	475 (-)	180 (-)	171[a](46.00)	279[a](76.40)	585[c](80.80)	810[c](84.60)

Values with different superscripts were significantly different from others in the same row ($P < 0.05$ or less)

TABLE III: Mean Serum MMA Concentration ±SE (μMol/l)

DAYS POST INF^N	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
STATUS	DEFICIENT		DEPLETED		SUFFICIENT	
-20	18.55^b(6.15)	9.84^b(3.04)	1.599^a(0.76)	2.09^a(0.90)	2.78^a(0.74)	2.92^a(1.13)
-8	33.11^b(7.60)	62.20^b(36.60)	3.92^a (1.15)	6.99^a(3.25)	5.53^a(1.81)	4.92^a(1.20)
0	91.01^b(20.13)	83.00^b(26.70)	6.16^a (1.78)	14.74^a(7.72)	17.09^a(5.66)	10.19^a(3.50)
7	91.10^b(17.60)	78.30^b(27.80)	12.08^a (3.02)	14.86^a(6.73)	9.22^a(2.42)	15.19^a(2.89)
35	138.80^b(58.20)	168.20^b(50.30)	20.35^c (6.04)	54.30^c(29.40)	6.93^a(0.92)	3.55^a(0.63)
70	171.30^b(78.60)	311.10^b(70.30)	93.20^c (25.90)	111.60^c(48.40)	6.34^a(1.54)	6.45^a(1.60)
105	308.10 (-)	189.50^b(54.30)	133.30^c (26.80)	107.20^c(21.20)	6.05^a(0.93)	3.13^a(0.30)

Values with different superscripts were significantly different from others in the same row ($P < 0.01$)

TABLE IV: Mean GSH-Px Activity ±SE (U/ml cells at 30°C)

DAYS POST INF^N	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
STATUS	DEFICIENT		DEPLETED		SUFFICIENT	
-8	132.1 (11.55)	147.7 (8.19)	164.6 (18.10)	180.5 (21.41)	160.6 (20.01)	156.0 (8.14)
35	140.4 (7.63)	150.9 (14.54)	180.2 (6.06)	178.7 (9.40)	193.1 (14.37)	179.0 (10.00)
76	110.8 (12.32)	127.4 (18.06)	141.5 (9.04)	161.2 (21.13)	133.3 (7.21)	142.9 (11.68)

TABLE V: Mean Serum Vitamin E Concentration ±SE (μmol/l)

DAYS POST INF^N	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
STATUS	DEFICIENT		DEPLETED		SUFFICIENT	
	1.42 (0.29)	1.35 (0.11)	1.03 (0.13)	1.51 (0.27)	1.36 (0.20)	1.16 (0.15)
	1.19 (-)	1.49 (-)	1.35 (0.27)	1.83 (0.21)	2.17 (1.27)	3.25 (1.40)
	1.25 (-)	1.11 (-)	1.79 (0.72)	1.90 (0.46)	1.47 (0.72)	2.36 (0.61)

TABLE VI: Mean Serum Albumin Concentration ±SE (g/L)

DAYS POST INF^N	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
STATUS	DEFICIENT		DEPLETED		SUFFICIENT	
-8	33.4 (1.01)	35.1 (0.85)	36.3 (1.40)	35.7 (0.73)	35.1 (0.49)	34.4 (0.47)
2	35.7 (0.44)	35.1 (1.10)	36.3 (0.79)	34.8 (0.97)	36.0 (1.03)	34.9 (0.46)
7	35.2 (1.09)	34.3 (0.60)	35.4 (0.94)	35.0 (0.73)	34.7 (0.56)	33.7 (0.89)
13	33.6 (1.24)	33.7 (0.86)	34.9 (0.69)	34.2 (0.63)	33.9 (0.73)	33.7 (0.43)
21	32.6 (1.62)	32.7 (0.77)	31.5 (0.83)	33.5 (0.83)	32.4 (0.46)	31.6 (0.41)
28	32.0 (1.92)	34.2 (0.61)	33.5 (0.55)	33.1 (0.82)	33.0 (0.89)	31.9 (0.54)
42	37.3 (1.95)	37.9 (1.12)	37.2 (0.86)	37.2 (1.36)	36.0 (0.73)	36.2 (0.77)
56	33.6 (1.07)	33.4 (1.10)	33.4 (1.01)	32.7 (0.47)	32.1 (1.20)	29.5 (0.78)
70	35.8 (1.10)	34.8 (0.89)	33.1 (0.50)	33.5 (0.70)	34.0 (0.60)	33.4 (0.74)
84	38.2 (-)	33.6 (1.49)	32.9 (0.80)	27.1 (1.93)	33.6 (0.58)	30.7 (1.33)
98	35.1 (-)	35.6 (0.35)	32.3 (0.86)	32.7 (0.65)	33.6 (0.72)	33.6 (1.99)
111	28.5 (-)	28.8 (-)	30.7 (1.05)	32.2 (0.78)	32.9 (0.76)	32.6 (1.24)
119	27.4 (-)	31.9 (-)	27.9 (1.09)	31.3 (0.91)	32.7 (0.48)	31.5 (1.16)

TABLE VII: Mean Serum Total Protein Concentration ±SE (g/L)

DAYS POST INF[N]	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
STATUS	DEFICIENT		DEPLETED		SUFFICIENT	
-8	67.9 (3.43)	69.8 (2.83)	67.5 (2.97)	63.3 (2.18)	65.8 (1.82)	64.9 (2.25)
2	72.2 (0.86)	71.4 (3.04)	67.3 (1.83)	67.1 (1.74)	73.0 (2.05)	67.5 (1.58)
7	68.8 (1.57)	65.3 (1.35)	66.9 (2.39)	64.2 (1.39)	66.4 (1.33)	63.5 (1.89)
13	62.6 (1.55)	59.9 (1.59)	61.4 (1.28)	59.8 (1.15)	62.5 (1.93)	60.2 (1.46)
21	67.0 (1.95)	67.1 (1.70)	65.6 (2.81)	64.2 (1.49)	65.1 (1.02)	63.8 (1.16)
28	62.0 (1.90)	59.9 (1.15)	61.3 (1.21)	60.3 (1.17)	60.8 (1.06)	58.5 (1.06)
42	68.1 (3.25)	65.5 (1.31)	67.3 (1.78)	64.5 (1.94)	66.0 (1.72)	65.7 (2.12)
56	63.6 (0.34)	62.1 (1.33)	63.3 (1.08)	59.8 (2.09)	60.0 (1.08)	57.6 (1.56)
70	63.5 (0.25)	63.5 (2.45)	62.9 (1.42)	61.6 (1.07)	63.3 (0.81)	64.9 (0.92)
84	84.3 (-)	61.5 (3.65)	60.5 (1.69)	52.8 (2.20)	64.9 (0.72)	59.8 (1.47)
98	63.8 (-)	69.2 (3.25)	62.3 (1.70)	61.8 (0.73)	64.2 (0.76)	65.8 (2.55)
111	53.1 (-)	65.3 (-)	65.0 (3.63)	65.3 (2.42)	65.8 (1.38)	65.1 (1.48)
119	54.3 (-)	74.4 (-)	60.8 (4.83)	63.6 (2.10)	64.0 (0.87)	64.4 (1.01)

TABLE VIII: Summary of Post-Mortem Findings from Casualties in Experiment 1

LAMB NO (Tube No)	GROUP	STATUS	TIME OF DEATH (Days PI)	REMARKS
44 (9)	1	Deficient infected	6/8/87 (-18)	Ruminal acidosis (pH = 5.0) Liver Cu = 110 ppm; Liver Co = 0.04 ppm Histopathology also revealed muscular dystrophy, centrilobular fatty degeneration in hepatocytes and a degree of proximal renal tubular degeneration. No significant neuropathological changes.
80 (7)	1	Deficient infected	27/7/87 (33)	Uraemic carcass, local area of enteritis, hepatic degeneration and sub-capsular haemorrhage in renal cortex and areas suggestive of nephrosis. Liver Co = 0.01 mg/kg (very low).
96 (2)	1	Deficient infected	6/7/89 (12)	Acidosis (pH = 4.8), abomasitis plus rupture and and enteritis due to overeating. Liver Co = 0.01 mg/kg.
46 (3)	1	Deficient infected	23/7/87 (29)	Apical pneumonia plus pale kidneys and liver. P. haemolytica (A6) recovered from lung and kidney. Liver Co = 0.01 ppm.
93 (5)	1	Deficient infected	7/8/87 (44)	Lung abscess, pneumonia and septicaemia due to P. haemolytica (A6). Lamb was cachexic and carcass was poor and oedematous. TWC = 6250 Liver Cu = 272 ppm (normal); Liver Co = 0.03 ppm (low) **Histophathology:** Pneumonic pasteurellosis. Centrilobular necrosis evident in liver plus parasitic abomasitis. **Neuropathology:** Spongy change possibily indicative of regional preferrential susceptibility to cytotoxic process.
78 (6)	1	Deficient infected	18/8/87 (55)	Very poor condition (wt = 26 lbs) and oedematous carcass. Degenerative fatty change in liver and pale kidneys. Parasitic abomasitis although normal pH (3.5). TWC = 10650 GSH-PX = 189 umol/L B_{12} = 110 ng/l Liver Co = 0.01 ppm; Liver Cu = 55 ppm **Histopathology:** Widespread fatty degeneration of hepatocytes with proliferative changes in small bile ducts. Disruption of villious architecture with eosinophil infiltration in abomasum. **Neuropathology:** Some vacuolation in cerebellar white matter - not significant.
95 (4)	1	Deficient infected	31/8/87 (68)	Euthanased. Poor oedematous carcass with local apical pneumonia or collapse. Severe fatty degeneration of liver parenchyma and soft fatty kidneys. TWC = 14299 **Neuropathology** Some non-significant vacuolation of cerebral and cerebellar white matter. **Histopathology:** Eosinophil infiltration of abomasal epithelium and mucosa. Severe extensive nephrosis of the proximal tubules. Severe extensive centrilobular fatty degeneration of hepatocytes. Liver Co = 0.01 ppm; Liver Cu = 37ppm

LAMB NO (Tube No)	GROUP	STATUS	TIME OF DEATH (Days PI)	REMARKS
68 (1)	1	Deficient infected	11/10/87 (109)	Euthanased in extremis. Poor oedematous carcass, enlarged adrenals and pale liver and kidney. TWC = 3385 Liver Cu = 240 ppm; Liver Co = 0.02 ppm **Neuropathology:** Pathological vacuolation whose type and distribution resemble those arising from hyper-ammonaemia characteristic of hepatic encephalopathy in sheep.
52 (14)	2	Deficient non-infected	13/9/87 (81)	Died via a haemhorrage into the abdominal cavity via a number of tears in the liver capsule. Liver Co = 95 ppm; Liver Co = 0.01 ppm.
81 (16)	2	Deficient non-infected	15/9/87 (83)	Fatty liver and pale fatty kidneys. Enlargement of the adrenals and small areas of pneumonia or collapse anteriorly. Elevated SGOT indicated hepatic dysfunction. Liver Co = 0.01 ppm; Liver Cu = 18 ppm Non-significant neuropathological change.
84 (13)	2	Deficient non-infected	15/9/87 (83)	Died suddenly in good condition revealing PM changes changes consistent with enterotoxaemia. C perfringens isolated from small intestine.
69 (10)	2	Deficient non-infected	15/9/87 (83)	Lamb was extremely poor, emaciated and oedamatous. Collapse was noted in apical lobe of lungs and kidneys appeared pale and fatty. Liver Cu = 164 ppm; Liver Co = 0.01 ppm. **Neuropathology:** Non-significant vacuolation of white matter of the occipital region.
94 (12)	2	Deficient non-infected	30/9/87 (97)	Pneumonia and septicaemia from P. haemolytica (A6). Liver Co = 0.02 ppm; Liver Cu = 210 ppm.
90 (17)	2	Deficient non-infected	7/10/87 (104)	Euthanased in extremis. Cachexic oedematous carcass. Localised anterior pneumonia. Congestion of the liver and soft bronze coloured kidneys. Liver Cu = 156 ppm; Liver Co = 0.01 ppm. **Neuropathology:** Spongy transformation found in white matter in brain stem and spinal cord suggests possible genuine cytotoxic process.
79 (15)	1	Deficient non-infected	8/10/87 (105)	Sacrificed in extremis. Had a flabby heart, bronze liver and pale kidneys. Liver Cu = 93 ppm; Liver Co = 0.04 ppm.
59 (42)	5	Depleted infected	2/9/87 (70)	Acidosis (Rumen pH = 5.2), TWC = 18251, WEC = 600 epg. Liver Cu = 58 ppm; Liver Co = 0.01 ppm. **Histopathology:** Moderate centrilobular fatty degeneration of hepatocytes, proximal tubular nephrosis of the kidney and eosinophil infiltration of abomasal epithelium and sub-mucosa.
60 (53)	5	Depleted infected	20/9/87 (87)	Euthanased in extremis. Hypostatic congestion of lungs and widespread fatty dengeration of liver tissue. Fatty kidneys with subcapsular haemorrhage. Poor, oedematous carcass with impaction of the omasum. Liver Cu = 121 ppm; Liver Co = 0.02 ppm. **Neuropathology:** Vacuolation of the neuropil and cerebellar white matter showed mild spongy change associated with vacuolation.

LAMB NO (Tube No)	GROUP	STATUS	TIME OF DEATH (Days PI)	REMARKS
73 (60)	6	Depleted non-infected	31/8/87 (68)	Acidosis (Rumen pH = 4.0). Abomasal congestion. Pulmonary congestion. Liver Co = 0.02 ppm; Liver Cu = 111 ppm. **Histopathology:** Sub capsular haemorrhages in the renal cortex and congestion of liver tissue.
45 (58)	6	Depleted non-infected	24/9/87 (92)	Grossly emaciated, pyloris impacted with wool balls. Organised pneumonia affecting right apical and cardiac lobes from which P. haemolytica (A6) was isolated. Liver Cu = 53 ppm; Liver Co = 0.01 ppm. **Neuropathology:** Non-significant vacuolation in frontal and parietal cortices. Vacuolation also found in grey matter of corpus striatum and cerebellar cortex.

TABLE IX: Mean Liveweight ±SE (kg)

DAYS POST INF[N]	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
STATUS	DEFICIENT		DEPLETED		SUFFICIENT	
-20	10.7[a](1.04)	10.5[a](0.87)	12.4[a](1.41)	12.8[a](1.14)	11.7[a](0.94)	12.6[a](0.90)
-8	11.6[a](1.02)	11.0[a](0.98)	13.2[a](1.17)	14.4[a](1.13)	13.5[a](1.00)	13.1[a](0.78)
7	12.9[b](1.23)	13.6[b](1.22)	16.4[a](1.12)	17.5[a](1.29)	17.0[a](0.91)	16.3[a](0.99)
23	14.0[b](2.00)	15.9[b](1.75)	19.0[a](1.30)	20.4[a](1.55)	20.1[a](0.92)	19.4[a](1.12)
35	14.0[b](1.93)	16.7[b](2.07)	20.5[a](1.34)	21.9[a](1.64)	22.1[a](1.07)	21.0[a](1.18)
49	16.0[b](2.84)	17.8[b](1.98)	20.9[a](1.22)	21.2[a](1.51)	23.0[a](1.13)	21.4[a](1.10)
61	18.0[b](7.50)	17.5[b](1.95)	20.9[a](1.38)	21.4[a](1.88)	24.3[c](1.35)	23.3[c](1.13)
75	17.5[b](7.50)	18.5[b](3.21)	22.4[a](1.23)	23.8[a](2.12)	25.8[c](1.47)	24.4[c](1.11)
90	24.0 (-)	20.2[b](4.51)	21.7[a](1.16)	24.2[a](2.29)	26.2[c](1.32)	24.1[c](1.22)
103	21.0 (-)	22.8[b](5.75)	20.2[a](1.17)	25.2[a](2.51)	27.5[c](1.49)	25.4[c](1.36)
117	21.0 (-)	26.5 (-)	20.2[a](1.11)	25.8[a](2.48)	28.0[a](1.65)	26.5[a](1.15)

Values with different superscripts were significantly different from others in the same row ($P < 0.05$ or less)

TABLE X: Mean Worm Egg Count ±SE (epg)

DAYS POST INF[N]	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
STATUS	INFECTED	NON-INFECTED	INFECTED	NON-INFECTED	INFECTED	NON-INFECTED
7	0 (0)	- (-)	0 (0)	- (-)	0 (0)	- (-)
14	0 (0)	- (-)	0 (0)	- (-)	0 (0)	- (-)
23	545[a](301.5)	- (-)	310[a](118.5)	- (-)	301[a](93.7)	- (-)
35	256[a](122.9)	- (-)	261[a](73.6)	- (-)	360[a](116.2)	- (-)
42	2250[a](1087.0)	0 (0)	1345[a](566.0)	0 (0)	304[a](128.0)	0 (0)
49	2350[a](1000.0)	0 (0)	1438[a](626.3)	0 (0)	361[a](94.9)	0 (0)
56	2797[a](756.1)	- (-)	486[a](136.1)	- (-)	317[a](126.8)	- (-)
63	1550[a](1250.0)	- (-)	881[a](195.2)	- (-)	232[b](88.1)	- (-)
70	275[a](175.0)	- (-)	1481[d](691.5)	- (-)	289[b](87.1)	- (-)
77	1200[a](1100.0)	- (-)	644[a](192.4)	- (-)	301[b](92.5)	- (-)
84	250 (-)	- (-)	1425[a](424.2)	- (-)	518[b](118.4)	- (-)
91	1100 (-)	- (-)	1779[a](504.6)	- (-)	618[b](258.9)	- (-)
98	2900 (-)	- (-)	2200[a](550.8)	- (-)	683[b](202.1)	- (-)
105	2850 (-)	- (-)	1869[a](545.9)	- (-)	218[b](46.4)	- (-)
112	500 (-)	0 (0)	2128[a](391.3)	0 (0)	250[b](64.3)	0 (0)
117	550 (-)	- (-)	3219[a](770.7)	- (-)	309[a](61.2)	- (-)

Values with different superscripts were significantly different from others in the same row ($P < 0.05$ or less)

TABLE X: Mean Worm Egg Count ±SE (epg)

DAYS POST INF	GROUP 1		GROUP 2		GROUP 3		GROUP 4		GROUP 5		GROUP 6	
STATUS	INFECTED		NON-INFECTED		INFECTED		NON-INFECTED		INFECTED		NON-INFECTED	
7	0	(0)	-	(-)	0	(0)	-	(-)	0	(0)	-	(-)
14	0	(0)	-	(-)	0	(0)	-	(-)	0	(0)	-	(-)
23	545[a]	(301.5)	-	(-)	310[a]	(118.5)	-	(-)	301[a]	(93.7)	-	(-)
35	256[a]	(122.9)	-	(-)	261[a]	(73.6)	-	(-)	360[a]	(116.2)	-	(-)
42	2250[a]	(1087.0)	0	(0)	1345[a]	(566.0)	0	(0)	304[a]	(128.0)	0	(0)
49	2350[a]	(1000.0)	0	(0)	1438[a]	(626.3)	0	(0)	361[a]	(94.9)	0	(0)
56	10267[a]	(756.1)	-	(-)	486[a]	(136.1)	-	(-)	317[a]	(126.8)	-	(-)
63	1550[a]	(1250.0)	-	(-)	881[a]	(195.2)	-	(-)	232[b]	(88.1)	-	(-)
70	275[a]	(175.0)	-	(-)	1481[d]	(691.5)	-	(-)	289[b]	(87.1)	-	(-)
77	1200[a]	(1100.0)	-	(-)	644[a]	(192.4)	-	(-)	301[b]	(92.5)	-	(-)
84	250	(-)	-	(-)	1425[a]	(424.2)	-	(-)	518[b]	(118.4)	-	(-)
91	1100	(-)	-	(-)	1779[a]	(504.6)	-	(-)	618[b]	(258.9)	-	(-)
98	2900	(-)	-	(-)	2200[a]	(550.8)	-	(-)	683[b]	(202.1)	-	(-)
105	2850	(-)	-	(-)	1869[a]	(545.9)	-	(-)	218[b]	(46.4)	-	(-)
112	500	(-)	0	(0)	2128[a]	(391.3)	0	(0)	250[b]	(64.3)	0	(0)
117	550	(-)	-	(-)	3219[a]	(770.7)	-	(-)	309[a]	(61.2)	-	(-)

Values with different superscripts were significantly different from others in the same row ($P < 0.05$ or less)

TABLE XI: Mean Plasma Pepsinogen Concentration ±SE (mU/l at 37°C)

DAYS POST INF[N]	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
STATUS	DEFICIENT		DEPLETED		SUFFICIENT	
-8	518[a](31.6)	596[a](48.6)	543[a](32.5)	489[a](35.4)	526[a](34.9)	493[a](21.3)
2	499[a](20.0)	560[a](85.7)	461[a](23.9)	442[a](25.0)	475[a](35.2)	457[a](18.8)
7	381[a](30.5)	394[a](29.6)	474[a](28.6)	443[a](17.1)	423[a](40.9)	466[a](19.2)
14	368[a](35.5)	358[a](19.6)	414[a](33.6)	376[a](32.8)	447[a](45.1)	371[a](24.4)
21	1082[b](240.3)	424[a](35.9)	990[b](119.8)	476[a](29.8)	901[b](87.5)	427[a](31.6)
28	1548[c](351.8)	- (-)	1180[a](136.7)	- (-)	933[a](99.9)	- (-)
35	1610[c](351.1)	- (-)	1093[a](153.3)	- (-)	1187[a](125.6)	- (-)
42	1974[c](430.3)	- (-)	1002[a](144.2)	- (-)	965[a](110.0)	- (-)
49	1550[c](279.7)	- (-)	708[a](105.2)	- (-)	725[a](112.1)	- (-)
56	2043[c](492.0)	- (-)	1135[a](164.0)	- (-)	907[a](186.4)	- (-)
63	1645[c](325.0)	- (-)	916[a](94.9)	- (-)	766[a](101.4)	- (-)
70	1535[c](715.0)	390[a](3.99)	1245[b](146.3)	423[a](33.7)	798[b](120.6)	363[a] (30.6)
77	1065[c](97.5)	- (-)	1807[c](333.6)	- (-)	763[a](127.4)	- (-)
84	1690 (-)	- (-)	1916[c](350.7)	- (-)	842[a](127.4)	- (-)
91	2060 (-)	- (-)	1759[c](237.0)	- (-)	1009[a](165.6)	- (-)
98	2330 (-)	- (-)	1724[c](270.4)	- (-)	851[a](116.6)	- (-)
105	3450 (-)	- (-)	1838[c](289.3)	- (-)	899[a](131.0)	- (-)
112	960 (-)	- (-)	2253[a](381.7)	- (-)	937[a](138.7)	- (-)

Values with different superscripts were significantly greater than others in the same row ($P < 0.01$)

TABLE XII: Mean Packed Cell Volume ±SE (%)

DAYS POST INF[N]	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
STATUS	DEFICIENT		DEPLETED		SUFFICIENT	
14	25.8[a](1.03)	30.3[b](1.44)	- (-)	- (-)	30.3[b](1.21)	30.8[b](1.11)
21	29.3[a](1.38)	31.3[a](1.03)	- (-)	- (-)	30.5[a](0.91)	29.0[a](0.82)
28	26.0[a](2.06)	29.0[a](0.71)	- (-)	- (-)	25.0[a](2.68)	28.8[a](0.85)
35	24.0[a](1.58)	28.3[a](0.48)	28.8[b](0.85)	31.3[b](0.95)	28.3[b](1.11)	29.5[b](0.96)
46	26.8[a](0.25)	26.7[a](1.20)	- (-)	- (-)	27.8[a](1.03)	28.5[a](0.65)
51	28.3[a](1.55)	28.0[a](0.29)	26.5[a](0.65)	30.8[a](2.25)	30.3[a](1.11)	29.5[a](1.49)
58	26.7[a](2.91)	27.3[a](1.44)	- (-)	- (-)	37.3[a](0.65)	28.5[a](0.50)
65	25.8[a](1.03)	24.0[a](0.48)	26.5[a](0.65)	28.5[a](1.04)	27.5[a](0.29)	29.5[b](0.87)
72	26.3[a](1.03)	26.5[a](0.87)	- (-)	- (-)	28.0[b](0.25)	28.0[b](1.29)
79	24.8[a](0.63)	26.0[a](0.58)	26.5[a](0.65)	28.3[a](0.63)	27.3[a](1.25)	26.0[a](0.86)
85	24.5[a](2.53)	24.3[a](1.25)	- (-)	- (-)	25.0[a](0.50)	25.5[a](0.87)
93	26.3[a](0.85)	24.5[a](1.85)	25.5[a](0.65)	23.8[a](0.63)	27.5[a](0.87)	23.5[a](0.65)
100	24.0[a](0.91)	23.5[a](1.66)	- (-)	- (-)	26.5[a](0.87)	26.0[a](0.71)
107	- (-)	- (-)	25.8[b](0.95)	21.0[a] (1.19)	23.5[b](1.25)	25.0[b](1.73)
113	- (-)	- (-)	23.8[a](2.17)	23.8[a](1.55)	25.5[a](0.65)	26.7[a](1.20)

Values with different superscripts were significantly different from others in the same row ($P < 0.05$)

TABLE XIII: Mean Differential White Cell Count ±SE (%)
Group 1 (Deficient Infected Lambs)

DAYS POST INF[N]	NEUTROPHILS	LYMPHOCYTES	MONOCYTES	EOSINOPHILS	BASOPHILS
14	22.50 (1.85)	54.25 (2.87)	13.75 (1.55)	7.50 (3.72)	0 (0.00)
21	23.50 (2.25)	38.50 (3.40)	11.50 (2.02)	21.75[b] (4.01)	0 (0.00)
28	28.25 (3.12)	44.25 (3.22)	10.00 (3.54)	17.00[b] (3.72)	0 (0.00)
35	29.75 (4.97)	43.75 (4.17)	6.00 (2.48)	20.50[bc] (1.32)	0 (0.00)
46	30.50 (2.22)	36.50 (4.65)	6.50 (2.50)	25.25[b] (2.69)	1.25 (0.00)
51	28.75 (1.38)	36.50 (2.53)	9.75 (1.49)	22.50[bc] (1.85)	1.50 (0.50)
58	22.25 (1.03)	39.50 (4.05)	10.25 (1.70)	26.25[b] (2.95)	1.75 (0.70)
65	24.75 (2.56)	30.50 (4.29)	9.00 (1.35)	32.75[bd] (1.65)	3.50 (0.60)
72	26.25 (2.78)	34.75 (4.87)	9.25 (1.89)	28.00[bd] (3.03)	1.75 (1.00)
79	24.25 (2.02)	27.75 (3.90)	10.75 (1.25)	31.25[bd] (1.80)	1.00 (1.00)
85	22.50 (2.53)	39.75 (3.04)	8.50 (0.86)	27.50[bd] (2.53)	0.25 (0.25)
93	26.00 (2.35)	36.50 (3.75)	8.75 (0.95)	30.25[be] (1.65)	1.00 (1.00)
100	22.25 (1.03)	32.25 (2.29)	9.00 (1.35)	32.75[be] (1.38)	0.75 (0.00)

[b] = Significantly different from non-infected equivalent in Table XIV ($P < 0.01$)
[c] = Significantly different from equivalent values among depleted lambs, Table XV ($P < 0.01$)
[d] = Significantly different from equivalent values among depleted and sufficient lambs, Tables XV and XVII respectively ($P < 0.01$)
[e] = Significantly different equivalent values among sufficient lambs, Table XVII ($P < 0.01$)

TABLE XIV: Mean Differential White Cell Count ± SE (%)
Group 2 (Deficient Non-infected Lambs)

DAYS POST INF^N	NEUTROPHILS	LYMPHOCYTES	MONOCYTES	EOSINOPHILS	BASOPHILS
14	25.50 (1.04)	62.25 (2.14)	12.25 (1.49)	0.50 (0.29)	0 (0.00)
21	29.25 (1.25)	46.50 (1.76)	16.25 (2.02)	8.00 (1.08)	0 (0.00)
28	29.67 (4.26)	55.67 (6.17)	10.67 (1.20)	4.00 (2.00)	0 (0.00)
35	29.50 (1.04)	53.75 (1.75)	11.50 (0.95)	5.25 (0.63)	0 (0.00)
46	30.00 (0.82)	52.25 (3.15)	9.00 (0.87)	8.50 (1.25)	0.25 (1.20)
51	26.25 (2.50)	59.00 (2.86)	7.25 (1.11)	7.50 (1.19)	0 (0.00)
58	26.00 (2.68)	57.25 (2.93)	8.50 (1.32)	8.25 (1.18)	0 (0.00)
65	23.50 (0.86)	63.25 (3.33)	10.25 (1.70)	2.75 (1.35)	0 (0.00)
72	26.00 (2.12)	53.50 (2.75)	12.75 (2.06)	7.75 (1.55)	0 (0.00)
79	27.00 (2.36)	52.25 (0.95)	8.50 (1.32)	7.25 (1.11)	0 (0.00)
85	26.25 (1.93)	55.00 (2.12)	9.75 (1.38)	8.75 (1.11)	0 (0.00)
93	26.75 (2.36)	55.75 (2.63)	8.00 (1.47)	9.00 (1.08)	0.50 (0.25)
100	24.75 (2.29)	57.50 (1.55)	8.75 (0.85)	8.50 (1.32)	0 (0.00)

TABLE XV: Mean Differential White Cell Count ±SE (%)
Group 3 (Depleted Infected Lambs)

DAYS POST INF[N]	NEUTROPHILS	LYMPHOCYTES	MONOCYTES	EOSINOPHILS	BASOPHILS
35	28.25 (1.75)	32.00 (0.816)	8.50 (1.32)	30.00 (1.41)	0 (0.00)
51	23.25 (2.25)	33.50 (3.66)	10.00 (1.58)	33.00 (1.29)	0 (0.00)
65	26.25 (2.95)	40.50 (5.84)	10.25 (1.70)	25.00 (1.91)	0.50 (0.50)
79	27.25 (6.26)	40.50 (7.56)	9.00 (1.29)	22.50 (2.22)	0.75 (0.25)
93	24.50 (2.02)	33.25 (4.61)	9.75 (1.49)	34.50[b] (0.957)	0.50 (0.28)
107	26.50 (1.32)	29.25 (3.09)	9.75 (1.55)	32.75[b] (0.48)	1.75 (1.00)
113	24.25 (1.25)	37.00 (1.08)	7.00 (1.22)	31.75[b] (0.85)	0 (0.00)

[b] = significantly greater than sufficient equivalents, Table XVII ($P < 0.05$)

TABLE XVI: Mean Differential White Cell Count ±SE (%)
Group 4 (Depleted Non-infected Lambs)

DAYS POST INF[N]	NEUTROPHILS	LYMPHOCYTES	MONOCYTES	EOSINOPHILS	BASOPHILS
35	31.00 (1.00)	53.50 (2.06)	12.50 (2.10)	3.25 (1.31)	0 (0.00)
51	29.00 (1.29)	52.00 (2.27)	9.00 (1.08)	9.75 (1.65)	0.25 (0.25)
65	30.00 (1.08)	53.75 (4.17)	11.00 (1.78)	5.25 (1.55)	0 (0.00)
79	30.25 (0.854)	51.50 (1.44)	10.00 (1.78)	8.25 (1.11)	0 (0.00)
93	30.50 (1.71)	54.50 (2.12)	8.50 (1.65)	6.50 (0.65)	0.50 (0.20)
107	32.00 (0.408)	50.50 (1.94)	7.25 (1.11)	11.00 (0.816)	0.25 (0.75)
113	31.50 (0.500)	52.25 (1.97)	8.50 (1.32)	7.50 (1.19)	0.25 (0.20)

TABLE XVII: Mean Differential White Cell Count ±SE (%)
Group 5 (Sufficient Infected Lambs)

DAYS POST INF[N]	NEUTROPHILS	LYMPHOCYTES	MONOCYTES	EOSINOPHILS	BASOPHILS
14	24.25 (1.65)	59.75 (2.14)	12.50 (1.32)	5.25 (2.29)	0.00 (0.00)
21	28.75 (2.29)	30.00 (2.38)	7.50 (2.96)	34.50[b] (0.886)	0.00 (0.00)
28	31.75 (1.18)	34.00 (1.47)	7.75 (2.02)	26.50[b] (2.10)	0.00 (0.00)
35	28.25 (1.44)	31.50 (2.50)	10.00 (1.41)	29.75[b] (3.57)	0.50 (0.25)
46	24.75 (2.29)	40.50 (3.07)	7.50 (1.94)	32.00[b] (1.41)	0.75 (0.48)
51	26.00 (1.87)	36.25 (2.14)	6.00 (2.04)	31.75[b] (1.55)	0.00 (0.00)
58	23.50 (1.85)	39.25 (1.89)	7.75 (0.63)	29.50[b] (0.65)	0.25 (0.25)
65	22.00 (0.93)	42.50 (2.22)	9.75 (1.70)	25.00[b] (0.58)	0.75 (0.75)
72	24.25 (1.65)	42.25 (2.59)	9.75 (0.63)	21.25[b] (1.25)	0.50 (0.28)
79	29.50 (2.25)	40.75 (3.33)	9.00 (0.71)	19.75[b] (1.31)	1.00 (0.00)
85	23.00 (1.58)	55.25 (2.36)	6.00 (0.816)	15.25[b] (2.56)	0.50 (0.50)
93	22.00 (1.08)	55.50 (2.84)	8.50 (0.50)	14.00[b] (2.55)	0.00 (0.00)
100	29.25 (1.93)	53.75 (4.23)	7.25 (1.38)	9.75 (3.12)	0.00 (0.00)
107	23.50 (2.53)	60.25 (3.47)	6.75 (1.49)	9.50 (3.10)	0.00 (0.00)
113	25.00 (2.08)	56.50 (2.40)	9.50 (1.32)	9.00 (1.47)	0.00 (0.00)

[b] = significant difference from non-infected equivalents in Table XVIII

TABLE XVIII: Mean Differential White Cell Count ±SE (%)
Group 6 (Sufficient Non-infected Lambs)

DAYS POST INF[N]	NEUTROPHILS		LYMPHOCYTES		MONOCYTES		EOSINOPHILS		BASOPHILS	
14	26.25	(1.55)	61.75	(2.63)	9.75	(1.93)	0.25	(0.25)	0	(0.00)
21	28.25	(1.80)	52.50	(2.63)	12.75	(1.89)	6.75	(1.97)	0.25	(0.25)
28	29.25	(2.06)	57.00	(3.24)	10.50	(1.71)	3.75	(1.18)	0	(0.00)
35	31.00	(1.00)	53.00	(1.53)	9.67	(0.33)	7.33	(1.20)	0	(0.00)
46	28.75	(1.11)	57.00	(3.24)	10.00	(1.41)	4.25	(1.80)	0.25	(0.25)
51	28.00	(1.47)	53.25	(3.09)	8.00	(0.00)	10.75	(2.29)	0	(0.00)
58	32.00	(1.78)	53.50	(2.10)	8.50	(1.32)	7.00	(1.78)	0	(0.00)
65	26.00	(2.35)	60.50	(1.71)	8.75	(1.31)	4.75	(2.29)	0	(0.00)
72	30.50	(0.96)	55.00	(2.12)	10.50	(0.87)	3.75	(0.63)	0	(0.00)
79	25.50	(1.71)	58.50	(1.94)	8.25	(0.63)	7.75	(0.85)	0.25	(0.25)
85	27.50	(2.99)	54.75	(3.01)	10.25	(1.18)	7.50	(1.19)	0	(0.00)
93	27.00	(1.73)	55.50	(2.22)	9.75	(0.89)	7.75	(1.49)	0	(0.00)
100	28.50	(1.89)	55.75	(2.17)	8.00	(1.22)	8.25	(1.11)	0.25	(0.25)
107	24.75	(0.95)	59.00	(1.78)	9.25	(1.38)	7.00	(0.707)	0	(0.00)
113	26.00	(1.11)	53.00	(1.71)	6.00	(1.18)	6.75	(0.707)	0.25	(0.25)

TABLE XIX: Mean dOD in NBT Assay ±SE

DAYS POST INF[N]	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
STATUS	DEFICIENT		DEPLETED		SUFFICIENT	
8	0.209[a](0.054)	0.311[a](0.051)	- (-)	- (-)	0.313[a](0.042)	0.304[a](0.044)
21	0.096[a](0.044)	0.134[a](0.031)	- (-)	- (-)	0.324[a](0.075)	0.193[a](0.027)
35	0.202[a](0.089)	0.178[a](0.035)	0.242[a](0.066)	0.239[a](0.019)	0.239[a](0.036)	0.220[a](0.053)
65	0.205[a](0.100)	0.136[a](0.043)	0.262[a](0.067)	0.258[a](0.059)	0.331[b](0.052)	0.239[b](0.027)
79	0.010[a](0.000)	0.038[a](0.017)	0.124[a](0.030)	0.146[a](0.071)	0.094[b](0.028)	0.083[b](0.036)
93	0.150[a](-)	0.040[a](0.603)	0.067[a](0.016)	0.125[a](0.017)	0.106[a](0.025)	0.102[a](0.054)
107	0.045[a](-)	0.090[a](-)	0.112[a](0.067)	0.145[a](0.023)	0.144[a](0.035)	0.102[a](0.060)

Values with different superscripts differed significantly from others in the same row ($P < 0.05$)

TABLE XX: Mean ELISA Titre to *O. circumcincta* ±SE (% reference sample)

DAYS POST INF[N]	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
STATUS	DEFICIENT		DEPLETED		SUFFICIENT	
-8	30.4[a](9.33)	29.0[a](6.90)	20.1[a](6.97)	9.8[a](3.52)	32.3[a](10.54)	7.5[a](3.99)
7	16.0[a](9.59)	16.01[a](5.14)	19.9[a](6.69)	8.5[a](6.15)	21.5[a](7.50)	5.3[a](1.41)
13	12.2[a](2.91)	9.5[a](6.00)	18.4[a](4.57)	1.2[a](1.20)	15.1[a](5.41)	7.8[a](2.14)
21	5.2[a](0.69)	12.5[a](3.48)	19.8[a](7.04)	3.1[a](1.62)	12.2[a](3.67)	5.5[a](2.82)
35	8.8[a](2.76)	5.5[a](1.07)	22.4[a](7.82)	3.7[a](1.85)	13.2[a](2.69)	21.5[a](10.25)
49	43.6[a](6.65)	43.0[a](4.28)	48.1[a](4.07)	47.8[a](13.55)	45.2[a](2.82)	46.3[a](2.73)
63	99.5[b](26.65)	39.6[a](2.38)	51.8[b](2.69)	37.8[a](0.00)	49.5[a](3.30)	54.1[a](9.47)
77	91.6[b](7.50)	53.5[a](10.12)	51.4[b](2.89)	45.5[a](2.82)	52.4[a](2.24)	54.0[a](10.02)
91	63.7 (-)	38.6[a](5.03)	45.6[b](2.79)	37.6[a](2.64)	45.2[b](2.33)	39.7[a](5.82)
98	83.8 (-)	50.6[a](4.35)	51.7[b](3.80)	26.5[a](5.40)	57.3[b](3.85)	48.2[a](3.46)
119	54.4 (-)	51.7 (-)	53.7[b](8.56)	32.5[a](6.54)	62.2[b](4.39)	39.4[a](0.73)

Values with different superscripts differed significantly from others in the same row ($P < 0.05$)

TABLE XXI: Pre- and Post-Clostridial Vaccination Titres for Individual Animals in Experiment 1

Group 1 - Deficient

TUBE NO	ANIMAL NO	PRE-VACCINATION	POST-VACCINATION
1	Y68	1.10	12.17*
2	Y96	-	-
3	Y46	-	-
4	Y95	-	-
5	Y93	-	-
6	Y78	-	-
7	Y80	-	-
8	Y56	2.90	2.34
9	Y44	-	-
10	Y69	2.26	3.29
11	Y74	1.25	1.80
12	Y94	0.83	0.216
13	Y84	1.24	1.99
14	Y52	1.66	2.14
15	Y79	1.09	0.601
16	Y81	3.99	0.91
17	Y80	1.29	19.66*

Group 2 - Depleted

TUBE NO	ANIMAL NO	PRE-VACCINATION	POST-VACCINATION
40	Y82	0.32	1.84
41	Y64	0.98	1.51
42	Y59	0.42	3.05
43	Y100	-	0.91
44	Y91	1.89	0.61
45	Y58	0.88	1.46
46	Y75	1.84	2.25
47	Y97	0.45	1.19
48	Y43	2.69	1.06
49	Y47	3.17	0.98
50	Y87	0.25	0.96
51	Y83	-	2.74
52	Y65	0.79	3.33
53	Y60	-	-
54	Y71	2.54	1.44
55	Y76	4.55	1.58
56	Y92	1.25	3.35
57	Y63	1.76	1.27
58	Y45	1.55	4.97
59	Y51	1.27	1.85
60	Y73	-	-

Group 3 - Sufficient

TUBE NO	ANIMAL NO	PRE-VACCINATION	POST-VACCINATION
18	Y88	1.07	4.23
19	Y42	2.76	4.52
20	Y54	0.33	10.82
21	Y66	0.53	4.67
22	Y99	0.86	0.261
23	Y53	1.25	6.60
24	Y101	1.27	0.22
25	Y50	1.96	1.92
26	Y57	0.524	1.01
27	Y98	0.92	0.201
28	Y48	3.23	4.43
29	Y89	1.09	0.261
30	Y41	4.19	3.25
31	Y55	0.43	3.575
32	Y67	0.52	5.98
33	Y62	1.50	3.70
34	Y85	1.34	7.73
35	Y70	0.57	2.33
36	Y61	0.383	-
37	Y72	1.24	5.69
38	Y49	-	-
39	Y77	6.69	1.30

* = Result rejected due to unfair bias on mean

- = No sample

APPENDIX II

TABLE I: Dietary Constituent Analysis - Experiment 2, Barley

PARAMETER MEASURED	UNITS	CONTENTS			MEAN (Standard Error)	
Selenium	mg/kg	0.026	0.019	0.011	0.0187	(0.008)
Vitamin E	iu/kg	2.93	4.50	6.11	4.50	(0.84)
Dry Matter	g/kg	860.00	872.00	865.00	865.70	(3.48)
Crude Protein	g/kg	113.00	121.00	109.00	114.30	(3.53)
Organic Matter	g/kg	939.00	941.00	939.00	940.00	(0.67)
Rumen Degradable Protein	g/kg	88.00	91.00	81.00	86.70	(2.96)
Undegradable Protein	g/kg	25.00	30.00	28.00	27.70	(1.45)
Calcium	g/kg	0.50	0.20	3.20	1.30	(0.95)
Phosphorous	g/kg	4.00	5.40	2.10	3.83	(0.96)
Magnesium	g/kg	1.30	1.40	1.30	1.33	(0.03)
Potassium	g/kg	8.24	2.46	8.49	6.38	(1.99)
Nitrogen	g/kg	18.30	19.40	17.80	18.50	(0.47)
Sodium	g/kg	0.20	0.10	0.08	0.13	(0.04)
Copper	mg/k	4.80	2.30	3.20	3.43	(0.73)

All values are expressed on a dry matter (DM) basis

TABLE I (Cont): Dietary Constituent Analysis - Experiment 2, Timothy Hay

PARAMETER MEASURED	UNITS	CONTENTS			MEAN	(Standard Error)
Selenium	mg/kg	0.036	0.039	0.033	0.036	(0.006)
Vitamin E	iu/kg	Trace	-	-	Trace	
Dry Matter	g/kg	861.00	860.00	863.00	861.30	(0.88)
Crude Protein	g/kg	59.00	58.00	60.00	59.00	(0.58)
Organic Matter	g/kg	940.00	946.00	946.00	944.00	(2.00)
Rumen Degradable Protein	g/kg	35.00	36.00	36.00	35.70	(0.33)
Undegradable Protein	g/kg	24.00	22.00	24.00	23.30	(0.67)
Calcium	g/kg	3.60	2.80	3.30	3.23	(0.23)
Phosphorous	g/kg	1.40	1.40	1.40	1.40	(0.00)
Magnesium	g/kg	0.80	1.20	0.90	0.97	(0.12)
Potassium	g/kg	10.60	10.60	10.90	10.70	(0.10)
Nitrogen	g/kg	9.10	9.60	9.50	9.40	(0.15)
Sodium	g/kg	0.89	1.00	0.91	0.93	(0.03)
Copper	mg/k	3.21	2.14	1.21	2.19	(0.58)

All values are expressed on a dry matter (DM) basis.

TABLE II: Mean Vitamin E Concentration ±SE (μmol/l) - Phase I

DAYS POST INF[N]	GROUP NO. (SE) 1		2		3		4	
-34	0.92	(0.07)	0.94	(0.08)	0.49	(0.06)	0.87	(0.06)
-17	0.66	(0.08)	0.79	(0.07)	0.69	(0.04)	0.64	(0.04)
2	0.52	(0.12)	0.60	(0.07)	0.48	(0.05)	0.45	(0.06)
9	1.06	(0.08)	0.90	(0.07)	0.65	(0.11)	0.76	(0.04)
16	0.67	(0.13)	0.67	(0.07)	0.59	(0.05)	0.47	(0.05)
23	0.91	(0.11)	0.90	(0.10)	0.51	(0.06)	0.47	(0.05)
30	0.87	(0.22)	0.91	(0.20)	0.57	(0.03)	0.50	(0.04)
37	1.46[a]	(0.23)	1.14[a]	(0.16)	0.46[b]	(0.07)	0.50[b]	(0.05)
44	1.51[a]	(0.19)	1.10[a]	(0.11)	0.76[b]	(0.05)	0.63[b]	(0.05)
51	1.22[a]	(0.35)	0.92[a]	(0.18)	0.55[b]	(0.08)	0.57[b]	(0.06)
58	1.81[a]	(0.20)	1.44[a]	(0.16)	0.78[b]	(0.06)	0.63[b]	(0.05)
65	1.21[a]	(0.23)	1.25[a]	(0.16)	0.77[b]	(0.09)	0.67[b]	(0.13)
72	1.67[a]	(0.22)	1.37[a]	(0.18)	0.86[b]	(0.19)	0.69[b]	(0.07)

Values with different superscripts differed significantly from other values in the same row ($P < 0.05$)

TABLE III: Mean GSH-Px Activity ±SE (U/ml at 30°C) - Phase I

DAYS POST INF[N]	GROUP NO. (SE) 1		2		3		4	
-34	189[a]	(10.1)	178[a]	(10.1)	141[b]	(7.4)	136[b]	(4.1)
-17	182[a]	(11.7)	162[a]	(6.5)	117[b]	(6.0)	106[b]	(7.8)
2	147[a]	(11.2)	153[a]	(8.6)	121[b]	(8.4)	113[b]	(8.5)
9	164[a]	(11.9)	177[a]	(14.0)	149[b]	(23.7)	135[b]	(12.2)
16	165[a]	(17.1)	176[a]	(13.2)	147[a]	(9.8)	145[a]	(10.8)
23	186[a]	(9.4)	192[a]	(7.6)	141[b]	(12.1)	142[b]	(9.8)
30	140[a]	(13.1)	115[a]	(3.7)	107[b]	(8.3)	91[b]	(8.4)
37	114[a]	(9.0)	116[a]	(7.8)	83[b]	(5.5)	80[b]	(6.8)
44	145[a]	(10.5)	106[a]	(9.8)	86[a]	(10.0)	75[a]	(10.7)
51	196[a]	(42.9)	176[a]	(36.9)	99[b]	(17.1)	83[b]	(7.4)
58	101[a]	(7.6)	106[a]	(7.3)	72[b]	(7.9)	78[b]	(6.9)
65	72[a]	(7.2)	78[a]	(4.5)	48[b]	(6.7)	47[b]	(5.0)
72	59[a]	(7.6)	57[a]	(6.0)	39[b]	(5.2)	41[b]	(3.8)
77	58[a]	(5.2)	75[a]	(10.8)	41[b]	(5.5)	47[b]	(4.6)

Values with different superscripts differed significantly from other values in the same row ($P < 0.05$)

TABLE IV: Mean Creatine Kinase Activity ± SE(iu/l at 30°C) - Phase I

DAYS POST INF^N	GROUP NO. (SE) 1	2	3	4
-34	303^a (42.5)	448^a (177.5)	1310^b(750.8)	1143^b(584.0)
9	705^a (172.6)	650^a (94.3)	2662^b(594.4)	2097^b(607.9)
16	400^a (190.5)	367^a (95.9)	1108^a(271.0)	1803^a(501.2)
23	312^a (106.5)	434^a (174.0)	1801^b(807.7)	1169^b(282.5)
30	600^a (239.7)	464^a (141.9)	1525^a(365.7)	899^a(266.4)
37	394^a (162.3)	152^a (151.7)	1068^b(313.5)	872^b(314.7)
44	265^a (74.6)	332^a (92.3)	2713^b(244.8)	1249^b(502.6)
51	325^a (100.3)	379^a (79.9)	864^b(201.0)	837^b(283.1)
58	173^a (54.0)	167^a (31.5)	679^b(304.3)	433^b (98.8)
65	247^a (76.1)	108^a (24.7)	771^b(301.8)	618^b(186.2)
72	191^a (40.0)	189^a (40.4)	1189^b(604.0)	1047^b(345.2)
77	105^a (31.0)	196^a (90.3)	1394^b(677.4)	1822^b(451.8)

Values with different superscript differed significantly from other values in the same row ($P < 0.05$)

TABLE V: Mean Vitamin B_{12} Concentration ±SE (ng/l) - Phases I and II

DAYS POST INF[N]	GROUP NO. (SE) 1		2		3		4	
10	655	(63.5)	740	(93.8)	720	(67.1)	731	(60.9)
31	408	(61.9)	632	(99.3)	379	(20.3)	470	(44.0)
56	254	(16.9)	272	(13.0)	270	(12.1)	288	(15.0)
77	225	(37.1)	239	(38.0)	267	(30.3)	263	(20.0)
105	470	(36.8)	646	(93.0)	478	(34.7)	592	(77.3)

TABLE VI: Mean Serum MMA Concentration ± SE(µmol/l) - Phase I

DAYS POST INF[N]	GROUP NO. (SE) 1		2		3		4	
35	7.11	(1.22)	7.56	(1.11)	5.11	(0.76)	4.10	(0.47)
63	7.38	(1.03)	9.47	(2.08)	10.36	(1.58)	9.24	(0.99)

TABLE VII: Summary of Post-Mortem Results Findings from Casualties in Experiment 2

LAMB NO (Tube No)	GROUP	STATUS	TIME OF DEATH (Days PI)	REMARKS
7 (1)	1	Infected Vit E supplemented	4/8/88 (10)	Exentsive severe WMD affecting gastrocnemius plus quadricepts particularly. Kidneys swollen, pale and mottled. Haemorrhagic areas of ulcerations in the fundus and pyloris of the abomasum plus multiple white "parasitic nodules". **Histology:** Extensive lesions of subacute muscular dystrophy plus nephrosis and dilation of kidney tubules. Focal monomuclear cellular infiltration of abomasal mucosa. **Biochemistry:** GSH-Px = 153 U/ml; Vit E = 0.88 µmol/l; CK = 3405 iu/l
34 (24)	3	Infected non-supplemented	6/8/88 (12)	Lesions suggestive of myopathy affecting hind limb and longissumus muscles. Pulmonary congestion and pale soft kidneys were also evident. **Biochemistry:** BUN = 25.2 mmol/l; CK = >30000 iu/l; Vit E = 0.82 µmol/l; Liver Co = 0.38 ug/g
38 (22)	3	Infected non-supplemented	6/8/88 (12)	Enteritis and pulmonary congestion were the main features. Pale hind-limb musculature was also noted. Large numbers of coccidia were present in small intestine. **Biochemistry:** Liver Co = 0.4 µg/g; BUN = 23.0 mmol/l; CK = 4470 iu/l; Vit E = 0.35 µmol/l.
5 (32)	3	Infected non-supplemented	18/8/88 (24)	Pulmonary hepatic congestion and anaemia were the major findings. Muscles were pale and mottled particularly quadriceps and gastrocnemius. Oedema with pinpoint haemorrhage on abomasal mucosa were noted. Beta and epsilon toxins were demonstrated in gut contents. **Biochemistry:** Liver Cu = 2099 µg/kg; BUN = 14.2 mmol/l; SGOT = 80 iu/l; CK = 337 iu/l; Liver B_{12} = 0.52 µg/g; Vit E = 2.29 µmol/l.
4 (40)	4	Non-infected supplemented	20/8/88 (26)	This lamb grossly resembled a case of enterotoxaemia. Biochemistry indicated uraemia and elevated CK possibly due to nephrotic tubule damage. **Biochemistry:** BUN = 17.8 mmol/l; SGOT = 71 iu/l; CK = 336 iu/l.
43 (47)	4B	Non-infected Vit E deficient	23/10/90 (90)	Pneumonia was found in this lamb and P. haemolytica (A2) and Mycoplasma ovipneumoniae recovered from the lungs. Purulent material was present in the nose and the carcase lymph nodes were englarged and haemorrhagic. Pus was present in the pharynx and the trachea congested and contained froth. A well democated red pneumonia was present in the apical and ventral lobes of the lungs but no associated pleurisy. Muscles appeared normal. **Biochemistry:** Liver Cu = 2811 µmol/kg; Liver B_{12} = 0.1 mg/kg.

TABLE VIII(a): Pre-Treatment Clinical Cough Score Assessment (100 days PI)

ANIMAL NO.	COUGH	NASAL DISCHARGE	DYSPNOEA	TACHYPNOEA	HYPERN.	TEMP.	NON-SPECIFIC FINDINGS	TOTAL
1	-	-	-	-	-	-	-	-
2	3	1	0	0	0	1	0	5
3	0	0	0	0	0	0	0	0
4	1	1	1	1	0	1	0	5
5	2	0	1	1	1	0	0	5
6	0	1	1	1	0	1	1	5
7	3	1	1	0	0	0	1	6
8	3	0	1	0	0	0	0	4
9	0	0	0	0	0	0	0	0
10	1	1	1	1	0	3	1	8
11	1	0	1	0	0	0	0	2
12	1	0	1	1	1	0	0	4
13	3	1	0	1	0	0	0	5
14	0	0	1	0	0	0	0	1
15	0	0	0	0	0	0	1	1
16	0	1	1	1	1	1	0	5
17	2	1	0	0	0	0	0	3
19	0	0	0	0	0	0	0	0
20	1	0	1	0	0	0	0	2
21	2	1	1	0	0	0	0	4
23	0	1	0	0	0	0	0	1
25	1	0	0	0	0	1	0	2
26	0	1	1	1	0	0	0	3
27	0	1	1	1	1	1	1	6
28	0	1	1	1	0	0	0	3
29	0	1	1	1	0	0	0	3
30	1	0	1	1	0	0	0	3
31	1	0	1	1	0	1	0	4
33	3	0	0	0	0	0	0	3
34	1	0	1	1	0	0	0	3
35	0	1	1	0	0	0	0	2

TABLE VIII(a) (Cont)

ANIMAL NO.	COUGH	NASAL DISCHARGE	DYSPNOEA	TACHYPNOEA	HYPERN.	TEMP.	NON-SPECIFIC FINDINGS	TOTAL
36	2	0	1	0	0	0	0	3
37	0	0	0	0	0	0	0	0
38	2	0	1	0	0	1	0	4
39	0	0	1	0	0	0	0	1
41	1	0	0	0	0	0	0	1
42	1	0	0	0	0	0	0	1
43	0	1	1	1	0	1	0	4
45	0	1	1	1	0	2	0	5
46	1	0	1	1	1	0	0	4
48	0	0	1	1	0	0	0	2
49	0	0	0	0	0	0	0	0
50	0	1	1	1	1	0	0	4
51	1	1	1	1	0	0	0	4

TABLE VIII(b): Pre-Treatment Clinical Cough Score Assessment (104 days PI)

ANIMAL NO.	COUGH	NASAL DISCHARGE	DYSPNOEA	TACHYPNOEA	HYPERN.	TEMP.	NON-SPECIFIC FINDINGS	TOTAL
2	2	1	0	0	0	0	0	3
3	2	1	1	0	0	0	0	4
4	2	1	1	1	1	0	0	6
5	2	0	1	0	0	0	0	3
6	0	1	1	1	0	0	0	3
7	0	0	1	1	1	1	0	4
8	2	0	0	0	0	1	0	3
9	2	0	1	1	1	0	0	5
10	1	0	1	1	1	0	0	4
11	2	1	1	1	0	0	0	5
12	1	1	1	1	0	0	0	4
13	3	1	1	1	1	1	0	8
14	2	0	0	0	0	0	0	2
15	1	1	0	0	0	0	0	2
16	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0
20	1	1	1	1	1	0	0	5
21	0	0	0	0	0	0	0	0
23	1	1	1	1	1	0	0	5
25	1	0	1	1	0	0	0	2
26	0	0	1	1	0	0	0	2
27	0	1	1	1	1	2	1 + 1	8
28	0	0	1	0	0	0	0	1
29	0	0	1	1	1	0	0	3
30	1	1	0	0	0	0	0	2
31	0	1	0	0	0	0	0	1
33	3	0	1	0	0	0	0	4
34	2	0	0	0	0	0	0	2
35	0	1	1	0	0	0	0	2
36	2	1	1	0	0	0	0	4

TABLE VIII(b) (Cont)

ANIMAL NO.	COUGH	NASAL DISCHARGE	DYSPNOEA	TACHYPNOEA	HYPERN.	TEMP.	NON-SPECIFIC FINDINGS	TOTAL
37	0	0	1	1	0	0	0	2
38	1	0	1	1	1	0	0	4
39	0	0	1	1	1	0	0	3
41	0	0	1	0	0	0	0	1
42	0	1	1	1	1	0	0	4
43	2	0	1	1	1	0	1	6
45	0	1	0	0	0	0	0	1
46	0	0	1	0	0	0	0	1
48	0	1	1	1	1	0	0	4
49	0	0	1	1	1	0	0	3
50	0	1	1	1	1	0	0	4
51	2	1	1	1	1	0	0	6

TABLE IX: Mean Liveweight ± SE (kg) - Phase I

DAYS POST INF[N]	GROUP NO. (SE) 1		2		3		4	
-31	14.8	(1.24)	14.2	(1.46)	14.3	(0.63)	14.7	(0.99)
-8	24.9	(1.00)	25.0	(1.20)	23.1	(0.85)	23.4	(1.35)
0	28.6	(1.42)	29.1	(1.52)	26.7	(0.98)	26.5	(1.44)
14	28.2	(1.44)	30.5	(1.43)	27.1	(1.52)	27.6	(1.72)
28	28.8	(1.28)	30.7	(1.50)	27.6	(1.66)	30.0	(1.84)
42	29.0	(1.10)	31.8	(1.81)	28.6	(1.62)	30.9	(1.98)
56	29.3	(1.49)	32.0	(1.43)	28.8	(1.56)	31.1	(1.95)
70	29.5	(1.52)	32.3	(1.56)	29.1	(1.62)	31.5	(2.02)
77	29.8	(1.00)	32.5	(1.43)	29.5	(1.67)	32.0	(1.77)

TABLE X: Mean Plasma Pepsinogen Concentration ±SE (mu/l at 37°C) - Phase I

DAYS POST INF[N]	GROUP NO. (SE) 1	2	3	4
-34	488[a] (26.3)	405[a] (22.4)	502[a] (28.4)	514[a] (27.0)
-17	468[a] (26.3)	398[a] (25.5)	499[a] (29.2)	498[a] (22.7)
2	395[a] (18.3)	- (-)	506[a] (46.0)	- (-)
9	853[b] (147.7)	- (-)	847[b] (64.1)	- (-)
16	1220[b] (152.2)	- (-)	1243[b] (85.6)	- (-)
23	721[b] (124.0)	- (-)	842[b] (82.3)	- (-)
30	714[b] (83.0)	- (-)	1037[b] (117.9)	- (-)
37	851[b] (108.8)	- (-)	1046[b] (120.0)	- (-)
44	1034[b] (135.0)	430[a] (37.1)	999[b] (173.5)	414[a] (32.7)
51	609[b] (115.7)	- (-)	1103[c] (164.1)	- (-)
58	687[b] (103.1)	- (-)	1304[c] (206.8)	- (-)
65	657[b] (105.5)	- (-)	1132[c] (151.8)	- (-)
72	716[b] (134.1)	342[a] (51.1)	1219[c] (189.1)	439[a] (46.6)
77	734[b] (156.7)	- (-)	1271[c] (161.8)	- (-)

Values with different superscripts differed significantly ($P < 0.05$)

TABLE XI: Mean WEC ± SE (EPG) - Phase I

DAYS POST INF[N]	GROUP NO. (SE) 1	2	3	4
0	0 (00.0)	0 (00.0)	0 (00.0)	0 (00.0)
22	221[a] (56.5)	- (-)	357[a] (71.6)	- (-)
30	343 (115.2)	- (-)	682[a] (171.7)	- (-)
35	325 (93.8)	- (-)	704[a] (162.2)	- (-)
42	221[a] (57.6)	0 (00.0)	731[b] (146.7)	0 (00.0)
49	457[a] (100.1)	- (-)	733[a] (168.4)	- (-)
56	210[a] (67.8)	- (-)	454[a] (115.9)	- (-)
63	264[a] (56.8)	- (-)	507[a] (232.7)	- (-)
70	192[a] (69.1)	0 (00.0)	665[b] (173.5)	0 (00.0)
77	236[a] (106.2)	- (-)	400[a] (105.8)	- (-)

Values with different superscripts differed significantly with other values in the same row ($P < 0.05$)

TABLE XII: Mean PCV Count ±SE (%) - Phases I and II

DAYS POST INF[N]	GROUP NO. (SE) 1		2		3/3B		4/4B	
-16	38.0	(1.35)	38.8	(0.82)	38.7	(0.51)	38.5	(0.72)
-2	36.4	(1.43)	36.8	(0.86)	36.9	(0.55)	36.1	(0.60)
3	37.7	(2.21)	37.5	(0.63)	36.8	(0.49)	37.9	(0.57)
10	38.6	(1.66)	37.3	(1.31)	38.4	(1.18)	38.2	(0.80)
17	38.3	(0.64)	37.1	(1.39)	37.4	(0.62)	38.0	(0.71)
24	35.6	(1.13)	34.8	(0.86)	35.8	(0.96)	35.6	(0.80)
31	36.4[a]	(0.48)	36.5[a]	(0.85)	33.9[b]	(0.412)	35.6[b]	(0.70)
38	33.4	(0.53)	33.9	(0.88)	33.1	(0.68)	34.3	(0.69)
44	34.1	(0.71)	34.5	(0.38)	33.7	(0.84)	33.9	(0.73)
51	33.1[a]	(0.63)	33.8[a]	(0.82)	30.7[b]	(0.71)	32.8[b]	(0.78)
58	30.9	(0.83)	31.9	(0.77)	30.1	(0.69)	30.8	(0.67)
65	29.4	(0.57)	30.5	(0.54)	29.9	(0.69)	30.1	(0.81)
72	30.4	(1.04)	29.8	(0.77)	29.2	(0.61)	29.5	(0.69)
79	30.0	(0.87)	30.8	(0.94)	30.4	(0.63)	30.9	(0.79)
86	31.6[a]	(0.75)	31.4[a]	(0.85)	29.4[b]	(0.50)	31.0[a]	(0.81)
93	32.0	(0.87)	31.0	(1.00)	31.5	(0.93)	31.9	(0.72)
100	32.3	(0.92)	32.6	(0.71)	31.9	(0.61)	32.6	(0.97)
107	31.7	(1.19)	32.3	(0.94)	30.1	(0.75)	30.7	(0.87)
114	31.1	(0.83)	32.3	(1.00)	31.1	(0.88)	32.9	(0.72)

Values with different superscripts differed significantly with other values in the same row ($P < 0.05$)

TABLE XIII: Mean Percentage Differential White Cell Count (±SE) Group 1 - Phases I and II

DAYS POST INF[N]	NEUTROPHILS	LYMPHOCYTES	MONOCYTES	EOSINOPHILS	BASOPHILS
-18	26.00 (2.12)	53.50 (2.75)	13.50 (2.10)	7.00 (1.00)	0.00 (0.00)
4	27.25 (2.93)	53.50 (1.50)	5.00 (1.10)	9.25 (1.38)	0.00 (0.00)
11	29.25 (3.12)	45.25 (1.76)	11.50 (2.00)	12.50 (2.69)	1.50 (0.51)
18	23.00 (4.97)	49.00[b] (3.15)	9.00 (1.19)	17.75[a] (4.10)	1.25 (1.00)
25	25.50 (1.03)	37.75[b] (2.93)	8.50 (1.11)	25.00[a] (3.03)	3.25 (1.75)
32	21.50 (1.85)	44.25[b] (2.12)	11.50 (1.08)	22.75[a] (1.80)	0.00 (0.00)
39	22.00 (2.50)	38.00[b] (1.76)	13.75 (0.94)	26.00[a] (1.38)	0.25 (0.50)
46	22.25 (1.25)	37.75[b] (2.47)	6.50 (1.49)	32.50[a] (2.53)	1.00 (0.50)
53	26.00 (1.04)	35.50[b] (6.17)	8.00 (2.06)	30.50[a] (2.95)	0.00 (0.00)
60	30.50 (2.02)	35.00[b] (2.06)	10.25 (1.76)	22.50[a] (1.85)	1.75 (1.00)
67	23.50 (2.36)	38.75[b] (3.62)	8.75 (0.85)	28.25[a] (1.32)	0.75 (0.25)
73	30.50 (2.56)	40.25[b] (3.93)	6.25 (1.70)	21.00[a] (1.65)	2.00 (0.60)
81	27.25 (1.38)	45.50 (4.55)	6.00 (1.25)	20.50[a] (1.11)	0.75 (0.50)
88	28.00 (4.26)	47.50 (0.946)	9.50 (0.75)	14.00[a] (2.00)	1.00 (0.50)
95	26.00 (1.93)	45.50 (2.11)	11.00 (0.28)	17.00[a] (1.08)	0.50 (0.25)
102	29.25 (1.25)	45.75 (2.68)	13.00 (1.04)	12.00 (1.32)	0.00 (0.00)
109	26.50 (2.95)	52.25 (2.29)	11.50 (1.17)	9.75 (1.19)	0.00 (0.00)

[a] = significant difference ($P < 0.05$) from corresponding value Table XIV, Appendix II
[b] = significant difference ($P < 0.05$) from corresponding value Table XIV, Appendix II

TABLE XIV: Mean Differential whilte Cell Count (±SE) Group 2 - Phases I and II

DAYS POST INF[N]	NEUTROPHILS	LYMPHOCYTES	MONOCYTES	EOSINOPHILS	BASOPHILS
-18	25.50 (1.25)	59.25 (2.08)	6.00 (1.71)	9.25 (1.97)	0.00 (0.00)
4	27.00 (2.14)	56.75 (3.26)	12.25 (1.49)	3.50 (0.85)	0.50 (0.25)
11	26.75 (2.02)	55.00 (2.73)	8.50 (2.01)	9.75 (2.29)	0.00 (0.00)
18	31.00 (1.76)	49.50 (2.11)	8.50 (1.35)	11.00 (0.63)	0.00 (0.00)
25	30.00 (1.25)	54.25 (6.71)	7.25 (0.84)	8.50 (1.18)	0.00 (0.00)
32	26.00 (1.04)	56.00 (1.96)	10.00 (1.26)	7.50 (1.55)	0.50 (0.25)
39	28.50 (0.95)	53.50 (3.01)	9.25 (0.91)	8.75 (0.65)	0.00 (0.00)
46	29.25 (1.11)	53.00 (2.10)	9.75 (1.13)	7.00 (1.32)	1.00 (0.50)
53	24.75 (1.08)	63.50 (3.71)	7.00 (0.87)	4.75 (1.60)	0.00 (0.00)
60	23.50 (2.12)	57.25 (4.91)	10.25 (0.25)	9.00 (0.81)	0.00 (0.00)
67	22.50 (1.93)	55.75 (2.78)	10.00 (1.49)	11.50 (2.03)	0.25 (0.25)
73	26.00 (1.75)	58.00 (1.94)	7.25 (3.21)	8.95 (1.41)	0.00 (0.00)
81	27.50 (0.923)	48.50 (2.75)	13.25 (1.22)	10.50 (1.65)	0.25 (0.25)
88	31.50 (1.55)	50.00 (4.79)	11.00 (0.707)	6.75 (2.06)	0.75 (0.25)
95	23.50 (1.08)	65.75 (7.53)	7.50 (1.36)	3.25 (1.47)	0.00 (0.00)
102	27.00 (1.38)	61.75 (2.86)	3.75 (1.41)	7.50 (1.08)	0.00 (0.00)
109	29.50 (3.34)	50.75 (3.72)	8.50 (1.20)	11.25 (0.85)	0.00 (0.00)

TABLE XV: Mean Differential White Cell Count (±SE) Group 3/3b - Phases I and II

DAYS POST INF[N]	NEUTROPHILS	LYMPHOCYTES	MONOCYTES	EOSINOPHILS	BASOPHILS
-18	21.50 (2.36)	59.00 (4.76)	6.00 (1.08)	12.50 (1.49)	1.00 (0.50)
4	26.00 (1.04)	53.00 (6.16)	13.50 (1.76)	7.50 (1.11)	0.00 (0.00)
11	22.25 (2.62)	54.50 (2.59)	11.50 (0.75)	11.75 (0.96)	0.00 (0.00)
18	26.00 (1.25)	39.25[b] (2.34)	10.75 (2.29)	19.00[a] (1.25)	1.50 (0.50)
25	24.25 (2.14)	38.50[b] (2.06)	6.50 (1.24)	27.50[a] (1.04)	3.25 (1.00)
32	25.00 (0.82)	34.75[b] (4.11)	9.75 (2.68)	30.00[ac] (2.06)	0.50 (0.25)
39	25.5 (2.91)	34.00[b] (1.93)	8.00 (0.55)	31.50[ac] (1.32)	1.00 (0.00)
46	21.25 (1.30)	39.00[b] (2.71)	10.25 (1.37)	29.50[a] (2.56)	0.00 (0.00)
53	26.00 (1.55)	34.50[b] (3.02)	9.50 (0.98)	28.25[a] (1.85)	1.75 (1.00)
60	23.50 (1.11)	39.00[b] (3.31)	6.00 (1.13)	30.72[ac] (3.21)	0.75 (0.25)
67	25.50 (2.29)	38.75[b] (2.68)	9.50 (1.25)	25.50[a] (1.78)	0.75 (0.25)
73	27.29 (3.33)	30.25[b] (3.12)	9.25 (1.04)	21.25[a] (0.78)	2.00 (0.60)
81	26.00 (1.75)	40.25[b] (2.06)	7.00 (0.85)	26.75[ac] (1.11)	0.00 (0.00)
88	30.50 (2.00)	40.25[b] (2.28)	8.75 (0.99)	20.00[ac] (2.14)	0.50 (0.25)
95	31.00 (0.92)	40.75[b] (1.95)	13.50 (2.17)	14.75[a] (1.47)	0.00 (0.00)
102	30.50 (2.86)	39.25[b] (2.70)	11.50 (1.50)	17.75[ac] (1.18)	1.00 (0.50)
109	29.50 (2.36)	46.50 (4.01)	11.50 (1.38)	12.50 (0.94)	0.00 (0.00)

[a] = significant difference ($P < 0.05$) from corresponding value Table XVI, Appendix I
[b] = significant difference ($P < 0.05$) from corresponding value Table XVI, Appendix I
[c] = significant difference ($P < 0.05$) from corresponding value Table XIII, Appendix II

TABLE XVI: Mean Differential White Cell Count (±SE) Group 4/4b - Phases I and II

DAYS POST INF[N]	NEUTROPHILS	LYMPHOCYTES	MONOCYTES	EOSINOPHILS	BASOPHILS
-18	26.50 (1.08)	56.75 (2.75)	9.75 (1.49)	7.00 (1.58)	0.00 (0.00)
4	22.50 (1.25)	60.25 (4.04)	8.50 (0.84)	8.75 (0.94)	0.00 (0.00)
11	23.25 (2.11)	62.00 (6.25)	10.00 (1.76)	4.75 (1.60)	0.00 (0.00)
18	28.75 (3.10)	48.25 (3.75)	12.50 (1.55)	10.50 (2.00)	0.00 (0.00)
25	30.50 (1.61)	52.25 (2.14)	9.25 (1.91)	7.75 (1.19)	0.25 (0.25)
32	27.50 (2.44)	54.50 (2.22)	7.00 (0.714)	11.00 (0.68)	0.00 (0.00)
39	27.00 (1.75)	54.75 (1.98)	10.25 (1.75)	7.50 (1.32)	0.50 (0.25)
46	22.50 (3.13)	60.25 (3.75)	9.75 (1.26)	7.50 (0.81)	0.00 (0.00)
53	21.50 (1.38)	64.50 (2.99)	6.00 (0.65)	8.00 (1.44)	0.00 (0.00)
60	28.00 (2.12)	50.50 (4.71)	10.25 (2.14)	11.25 (2.29)	0.00 (0.00)
67	31.50 (1.00)	57.50 (2.86)	7.50 (0.45)	3.50 (1.40)	0.00 (0.00)
73	23.50 (1.22)	62.50 (4.01)	7.50 (1.41)	6.25 (1.17)	0.25 (0.25)
81	26.00 (1.74)	51.75 (1.45)	11.00 (1.53)	11.25 (2.06)	0.00 (0.00)
88	30.00 (2.08)	47.75 (3.11)	12.25 (1.33)	10.00 (1.00)	0.00 (0.00)
95	30.50 (1.91)	56.50 (2.84)	6.25 (1.04)	6.75 (1.04)	0.00 (0.00)
102	28.75 (3.24)	52.00 (3.15)	8.00 (1.00)	11.25 (2.41)	0.00 (0.00)
109	29.25 (0.94)	48.75 (2.50)	11.50 (2.04)	10.50 (1.55)	0.00 (0.00)

TABLE XVII: Mean NFT Score ± SE(KI) - Phases I and II

DAYS POST INF[N]	GROUP NO. (SE) 1	2	3/3B	4/4B
-18	36.3[a] (1.89)	40.8[a] (1.25)	36.3[a] (3.18)	40.0[a] (1.96)
11	41.0[a] (2.86)	42.8[a] (1.89)	48.8[a] (3.54)	48.5[a] (2.99)
25	53.3[a] (1.80)	52.0[a] (1.78)	50.8[a] (1.11)	45.5[a] (2.02)
39	49.3[a] (1.03)	48.3[a] (1.44)	42.3[b] (2.10)	43.3[b] (1.30)
53	49.0[a] (1.78)	49.5[a] (1.19)	42.3[b] (0.75)	46.8[b] (1.60)
67	50.0[a] (1.87)	50.0[a] (1.73)	43.8[a] (2.75)	48.3[a] (2.46)
81	53.3[a] (2.66)	47.3[a] (2.06)	46.3[b] (1.75)	47.3[b] (2.73)
95	54.0[a] (2.94)	51.5[a] (2.63)	44.5[b] (0.96)	46.0[b] (2.04)
109	59.3[a] (3.33)	56.5[a] (1.85)	47.5[b] (1.04)	44.5[b] (3.52)

Values with different superscripts differed significantly from values in the same row ($P < 0.05$)

TABLE XVIII: Mean NBT Score ±SE (dOD) - Phases I and II

DAYS POST INF[N]	GROUP NO. (SE) 1	2	3/3B	4/4B
4	0.071 (0.017)	0.040 (0.026)	0.048 (0.016)	0.028 (0.009)
18	0.174[a] (0.028)	0.135[a] (0.023)	0.104[b] (0.024)	0.073[b] (0.008)
32	0.052 (0.017)	0.051 (0.017)	0.065 (0.009)	0.048 (0.007)
46	0.196[a] (0.005)	0.120[b] (0.038)	0.255[a] (0.057)	0.134[b] (0.047)
60	0.094[a] (0.009)	0.086[a] (0.021)	0.045[b] (0.010)	0.064[b] (0.009)
74	0.058 (0.012)	0.060 (0.016)	0.036 (0.007)	0.066 (0.032)
88	0.117 (0.010)	0.111 (0.014)	0.073 (0.013)	0.058 (0.011)
102	0.084 (0.021)	0.125 (0.052)	0.110 (0.014)	0.089 (0.007)

Values with different superscripts differed significantly from other values in the same row ($P < 0.05$)

TABLE XIX: Mean ELISA Titre to *O. circumcincta* ±SE (% Reference Sample) - Phase I

DAYS POST INF[N]	GROUP NO. (SE) 1	2	3	4
-17	12.8[a] (1.66)	13.7[a] (2.19)	10.6[a] (1.45)	6.2[a] (1.63)
9	17.7[a] (3.48)	18.5[a] (2.43)	18.0[a] (2.74)	28.4[a] (3.57)
23	20.9[a] (3.41)	17.5[a] (2.02)	30.2[a] (3.42)	25.1[a] (2.26)
37	32.5[a] (10.20)	21.1[b] (4.17)	33.8[a] (3.63)	27.0[b] (5.75)
51	45.4[a] (13.20)	21.1[b] (3.12)	46.6[a] (4.41)	25.9[b] (5.08)
65	69.4[a] (12.20)	21.1[b] (1.77)	67.5[a] (7.39)	27.8[b] (4.39)
77	73.9[a] (8.67)	23.4[d] (2.73)	59.6[b] (5.16)	33.6[c] (4.44)

Values with different superscripts differed significantly from other values in the same row ($P < 0.05$) or less

TABLE XX: Pre- and Post-*Clostridial* Vaccination Titres for Individual Animals - Phase I, Experiment 2

Group 1 - Vitamin E Supplemented

TUBE NO	ANIMAL NO	PRE-VACCINATION	POST-VACCINATION
1	R7	0.48	18.02
2	R21	0.74	0.86
3	R30	0.34	2.22
4	R51	2.12	0.82
5	R13	0.74	4.79
6	R45	-	0.709
7	R49	0.21	1.08
8	R50	0.25	3.56
9	R24	-	5.73
10	R48	0.38	0.794
11	R42	-	0.91
12	R40	0.82	0.266
13	R53	-	3.87
14	R46	-	0.94
15	R10	0.60	9.16
16	R9	0.65	44.52

Group 2 - No Supplement

TUBE NO	ANIMAL NO	PRE-VACCINATION	POST-VACCINATION
17	R41	0.202	2.17
18	R44	1.29	-
19	R37	0.93	10.48
20	R29	-	3.96
21	R39	-	2.17
22	R38	-	3.91
23	R2	-	6.96
24	R34	-	9.09
25	R15	0.34	0.552
26	R28	-	7.98
27	R17	0.25	17.89
28	R25	-	4.02
29	R36	0.19	4.31
30	R19	0.23	26.15
31	R31	0.35	0.42
32	R5	-	-
33	R27	0.30	6.89
34	R22	0.61	8.45
35	R11	-	-
36	R12	2.82	4.34
37	R3	0.65	-

Group 2 - No Supplement (Cont)

TUBE NO	ANIMAL NO	PRE-VACCINATION	POST-VACCINATION
38	R18	-	1.77
39	R23	-	-
40	R4	1.57	-
41	R8	-	-
42	R33	-	3.06
43	R35	-	2.06
44	R14	1.24	0.44
45	R47	0.28	2.06
46	R71	-	-
47	R43	1.08	-
48	R6	-	-
49	R26	-	9.58
50	R16	-	-
51	R32	0.40	3.55
52	R52	-	-

- = No sample

All units are iu activity per ml serum (iu/ml)

TABLE XXI: Mean GSH-Px Activity ±SE (U/ml at 30°C) - Phase II

DAYS POST INF^N STATUS	GROUP NO. (SE) 1	2	3A	3B	4A	4B
86	48.7^a (3.77)	55.9^a (7.86)	26.9^c (2.95)	53.0^a (5.28)	25.0^c (2.55)	47.2^a (5.07)
93	53.0^a (4.71)	57.3^a (4.98)	22.5^c (0.85)	58.2^a (3.78)	24.5^a (3.87)	54.9^a (3.48)
100	70.4^a (8.99)	70.0^a (7.73)	11.0^c (1.26)	56.0^a (3.63)	19.8^c (0.20)	53.1^a (7.77)
107	63.3^a(12.72)	73.2^a (6.89)	15.1^c (2.06)	61.9^a (4.91)	21.2^c (3.46)	63.8^a (3.82)
114	68.4^a (5.11)	91.0^b (8.21)	15.7^{ac}(2.91)	74.2^a (4.23)	22.5^{bc}(3.52)	71.0^b (6.84)

Values with different superscripts differed significantly from other values in the same row ($P < 0.05$)

TABLE XXII: Mean Vitamin E Concentration ±SE (µmol/L) - Phase II

DAYS POST INF^N STATUS	GROUP NO. (SE) 1	2	3A	3B	4A	4B
86	1.38^a (0.16)	1.37^a (0.29)	0.79^a (0.14)	0.64^b (0.14)	0.62^a (0.09)	0.60^b (0.05)
93	1.11^a (0.12)	0.84^a (0.16)	0.88^a (0.18)	0.43^b (0.07)	0.74^a (0.11)	0.47^b (0.06)
100	1.32^a (0.12)	1.51^a (0.26)	0.98^a (0.13)	0.58^b (0.09)	1.01^a (0.17)	0.55^b (0.11)
107	1.69^a (0.16)	1.87^a (0.33)	1.32^b (0.26)	0.57^c (0.11)	0.82^b (0.29)	0.69^c (0.09)
114	1.96^a (0.13)	1.77^a (0.27)	1.35^b (0.23)	0.70^c (0.10)	1.50^b (0.23)	0.76^c (0.10)

Values with different superscripts differed significantly from other values in the same row ($P < 0.05$)

TABLE XXIII: Mean CK Activity ±SE (iu/l at 30°C) - Phase II

DAYS POST INF^N STATUS	GROUP NO. (SE) 1	2	3A	3B	4A	4B
86	101^b (55.9)	214^b (120.9)	483^a (261.5)	349^b (52.6)	254^a (31.1)	316^b (100.3)
93	144^b (32.9)	137^b (16.9)	427^a (174.2)	176^b (37.2)	255^a (41.6)	149^b (16.2)
100	135^b (15.9)	180^b (55.2)	383^a (72.2)	222^b (44.3)	564^a (193.4)	277^b (67.4)
107	91^b (11.0)	75^b (7.1)	209^a (42.8)	128^b (35.8)	289^a (130.0)	121^b (16.4)
114	131^b (33.9)	144^b (62.0)	346^a (90.2)	308^b (130.3)	287^a (73.2)	137^b (23.7)

Values with different superscripts differed significantly from other values in the same row ($P < 0.01$)

TABLE XXIV: Mean Liveweight ±SE (kg) - Phase II

DAYS POST INF^N STATUS	GROUP NO. (SE) 1	2	3A	3B	4A	4B
98	30.5^b (0.99)	32.4^b (1.53)	20.7^a (3.02)	30.5^b (1.18)	22.0 (2.27)	31.9^b (1.14)
105	31.9^b (1.02)	34.6^b (1.60)	20.8^a (2.56)	32.2^b (1.13)	24.9^a (2.07)	34.2^b (0.92)
112	32.3^b (2.65)	35.6^b (1.75)	21.3^a (2.31)	33.0^b (1.63)	25.0^a (1.91)	34.9^b (1.03)
119	33.7 (1.14)	36.8^b (1.55)	22.0^a (1.96)	33.8^b (1.51)	25.4^a (1.87)	35.7^b (1.13)

Values with different superscripts differed significantly from other values in the same row ($P < 0.05$)

TABLE XXV: Mean Plasma Pepsinogen Concentration ±SE (mU/l at 37°C) - Phase II

DAYS POST INF^N STATUS	GROUP NO. (SE) 1	2	3A	3B	4A	4B
86	616 (121)	- (-)	1078 (179)	811 (164)	- (-)	- (-)
93	549 (90)	- (-)	928 (138)	775 (114)	- (-)	- (-)
100	781 (108)	- (-)	1153 (54)	1057 (154)	- (-)	- (-)
107	854 (138)	- (-)	1158 (165)	976 (91)	- (-)	- (-)
114	717 (145)	- (-)	1343 (225)	934 (157)	- (-)	- (-)

TABLE XXVI: Mean Worm Egg Count ±SE (epg) - Phase II

DAYS POST INF^N STATUS	GROUP NO. (SE) 1	2	3A	3B	4A	4B
84	221^a (70.6)	- (-)	863^a (216.4)	485^a (121.4)	- (-)	- (-)
91	467^a (128.2)	- (-)	1000^a (102.1)	486^a (157.0)	- (-)	- (-)
98	107^b (25.4)	- (-)	425^a (32.3)	272^b (121.4)	- (-)	- (-)
105	164^b (38.9)	- (-)	638^a (229.5)	238^c (71.5)	- (-)	- (-)
112	257^b (69.4)	- (-)	825^a (180.8)	340^b (110.6)	- (-)	- (-)

Values with different superscripts differed significantly from other values in the same row ($P < 0.05$)

TABLE XXVII: Mean ELISA Titre to *O. circumcincta* ±SE (% Reference Sample) - Phase II

DAYS POST INF^{N} STATUS	GROUP NO. (SE) 1	2	3A	3B	4A	4B
93	76.9^{a} (5.92)	22.6^{a} (2.84)	56.0^{b} (5.48)	67.6^{a} (3.24)	33.0^{c} (2.89)	38.3^{c} (5.05)
107	72.8^{a} (6.66)	26.4^{c} (4.69)	51.7^{b} (4.83)	65.2^{a} (4.54)	23.6^{c} (2.29)	36.6^{c} (8.15)
114	99.4^{a} (4.45)	- (-)	69.1^{b} (5.13)	86.5^{a}(10.51)	- (-)	- (-)
121	102.5^{a} (7.11)	- (-)	66.9^{b} (9.94)	94.0^{a} (5.45)	- (-)	- (-)

Values with superscripts ^{c} differed significantly from others in the same row ($P < 0.001$)
Other superscripts differed significantly from values in the same row ($P < 0.05$)

APPENDIX III

TABLE I: Mean Post-Treatment Vitamin B_{12} Concentration ±SE (ng/l) - Experiment 1

DAYS POST INFN STATUS	GROUP NO. (SE) 1	2	3	4	5	6
PRE-TREATMENT	475 (-)	180 (-)	171 (46.0)	279 (76.4)	585 (80.5)	810 (84.6)
0	370 (-)	190 (-)	156[a] (30.8)	241[b] (26.0)	540[a] (52.9)	739[b] (65.3)
7	1250 (-)	1250 (-)	1250 (0.0)	1102 (78.7)	1212 (24.5)	1203 (33.8)
14	- (-)	860 (-)	1250 (0.0)	1214 (35.7)	1117 (71.5)	1229 (21.1)
56	- (-)	1140 (-)	2148 (147.1)	1691 (210.0)	1826 (141.6)	2034 (123.1)

Values with different superscripts differed significantly from others in the same row ($P < 0.05$)

TABLE II: Mean Post-Treatment GSH-Px Activity ±SE (U/ml at 30°C) - Experiment 1

DAYS POST INFN STATUS	GROUP NO. (SE) 1	2	3	4	5	6
PRE-TREATMENT	140 (14.1)	151 (21.4)	180 (32.5)	179 (21.9)	193 (40.0)	179 (37.1)
0	129 (-)	132 (-)	177 (21.4)	165 (30.0)	168 (22.4)	141 (40.5)
7	179 (-)	157 (-)	199 (30.1)	226 (23.6)	201 (38.7)	184 (23.3)
14	- (-)	98 (-)	159 (18.9)	176 (34.1)	191 (56.4)	167 (35.5)
28	- (-)	141 (-)	147 (18.7)	180 (44.6)	207 (35.0)	171 (34.3)

TABLE III: Mean Post-Treatment Liveweight ± SE(kg) - Experiment 1

DAYS POST INF^N STATUS	GROUP NO. (SE) 1	2	3	4	5	6
PRE-TREATMENT	21.0 (-)	26.5 (-)	20.2 (1.11)	27.9 (1.58)	28.5 (1.65)	26.5 (1.15)
0	21.8 (-)	26.5 (-)	22.8^a(1.20)	28.1^b(1.63)	31.5^b(1.29)	27.1^c(1.14)
7	22.3 (-)	27.0 (-)	24.2^a(1.42)	29.9^b(2.02)	32.2^b(1.32)	27.3^c(1.56)
14	- (-)	28.5 (-)	25.1^a(1.55)	31.0^b(1.58)	32.4^b(1.82)	28.1^c(1.22)
28	- (-)	31.0 (-)	26.3^a(1.66)	32.9^b(1.85)	33.4^b(1.77)	29.1^c(1.21)

Values with different superscripts differed significantly from other values in the same row ($P < 0.05$)

TABLE IV: Mean Post-Treatment Vitamin B_{12} Concentration ±SE (ng/l) - Experiment 2

DAYS POST INF^N STATUS	GROUP NO. (SE) 1	2	3A	3B	4A	4B
PRE-TREATMENT	470 (36.8)	646 (93.0)	478 (34.7)	478 (34.7)	592 (77.3)	592 (77.3)
0	401 (55.6)	520 (73.1)	347^a(67.4)	441 (21.5)	544^b(51.6)	571 (102.0)
7	1163 (381.0)	1907 (193.0)	1255 (200.0)	1521 (611.0)	2283 (110.0)	1156 (243.0)
14	904 (318.0)	1008 (122.0)	494 (68.0)	1107 (596.0)	1106 (215.0)	765 (139.0)
28	299 (29.6)	388 (51.9)	344 (91.9)	372 (90.9)	335 (55.0)	665 (189.0)

Values with different superscripts differed significantly from other values in the same row ($P < 0.05$)

TABLE V: Mean Post-Treatment GSH-Px Activity ±SE (U/ml at 30°C) - Experiment 2

DAYS POST INF^N STATUS	GROUP NO. (SE) 1	2	3A	3B	4A	4B
PRE-TREATMENT	68.4^a(5.1)	91.0^a (8.2)	15.7^b (2.9)	74.2^a(4.2)	22.5^b (3.5)	68.5^a (6.8)
0	45.8^a(15.4)	53.5^a (4.3)	21.9^b (5.5)	38.0^a(10.9)	22.1^b (2.8)	44.6^a (3.6)
7	42.7^a(0.7)	51.5^a (5.9)	22.8^b (3.6)	53.3^a(7.8)	20.1^b (3.0)	39.5^a (2.9)
14	58.5^a(0.3)	52.0^a (5.6)	19.9^b (5.1)	34.9^a(4.3)	21.2^b (3.0)	43.9^a (5.2)
28	45.1^a(5.8)	56.7^a (5.2)	32.1^b (7.5)	40.7^a(0.8)	34.5^b (5.6)	45.4^a (3.1)

Values with different superscripts differed significantly from other values in the same row ($P < 0.01$)

TABLE VI: Mean Post-Treatment Vitamin E Concentration ±SE (µmol/l) - Experiment 2

DAYS POST INF^N STATUS	GROUP NO. (SE) 1	2	3A	3B	4A	4B
PRE-TREATMENT	1.93^a(0.13)	1.77^a(0.27)	1.35^a(0.23)	0.70^b(0.10)	1.50^a(0.23)	0.76^b(0.10)
0	1.84^a(0.47)	0.99^a(0.18)	1.51^a(0.44)	0.64^b(0.27)	1.17^a(0.54)	0.82^b(0.22)
7	2.40^a(0.18)	2.94^a(0.29)	2.03^a(0.18)	0.84^b(0.34)	2.11^a(0.40)	1.48^b(0.26)
14	2.04^a(0.12)	2.56^a(0.31)	1.77^a(0.32)	1.13^b(0.47)	1.88^a(0.33)	1.55^b(0.34)
28	2.35^a(0.31)	2.93^a(0.42)	2.33^a(0.41)	1.40^b(0.41)	2.10^a(0.37)	1.29^b(0.15)

Values with different superscripts differed significantly from other values in the same row ($P < 0.05$)

TABLE VII: Mean Post-Treatment Liveweight ± SE (kg) - Experiment 2

DAYS POST INF^{N} STATUS	GROUP NO. (SE) 1	2	3A	3B	4A	4B
PRE-TREATMENT	32.3^{a}(2.65)	34.0^{a}(1.75)	25.8^{b}(2.58)	32.2^{a}(1.13)	31.6^{b}(3.56)	30.9^{a}(1.03)
0	33.6 (1.41)	34.2 (1.16)	33.9 (1.44)	33.4 (1.43)	32.5 (2.36)	31.6 (1.58)
7	34.5 (1.26)	35.3 (1.76)	36.0 (1.00)	34.1 (1.51)	33.3 (3.87)	32.1 (1.23)
14	35.2 (1.30)	37.5 (1.75)	38.5 (0.50)	37.5 (2.10)	35.8 (3.94)	34.1 (1.53)
28	37.1 (1.44)	39.3 (1.84)	39.2 (1.32)	38.7 (2.04)	36.9 (2.76)	36.2 (1.70)

Values with different superscripts differed significantly with other values in the same row ($P < 0.05$)

A STUDY OF THE ROLE OF MICRONUTRIENT NUTRITION IN THE PATHOGENESIS OF HELMINTH DISEASE AND IMMUNE FUNCTION IN SHEEP

A thesis submitted for the Degree of Doctor of Philosophy by Edward George Whitten Ferguson

ERRATA

Page Number	Line Number	Word or Phrase	Amended to read As follows:
vi		Montoring	Monitoring
xx	13	by	but
xxiii	10	as	was
xxiv	20	throught	thought
3	15		*resulting
	20	of	a
4	6		*who
11	6	exucation	exudation
12	7	phsophatase	phosphatase
	11	palsma	plasma
38	14	still	stiff
48	22	nitrogen	antigen
49	12	Recognisition	Recognition
50	1	nitrogen	antigen
52	21	plague	plaque
54	1	(HBV-1)	(BHV-1)
55	20	Co A	Con. A
	22	not	no
		Co A	Con. A
71	21	recongised	recognised
75	19-20	O. circumcincta	O. ostertagi
78	13	coloubriformis	colubriformis
81	23		*an
82	23	circimcincta	circumcincta
85	5	studies	studied
87	13	Ostertagie	Ostertagia
88	13	singificantly	significantly
90	2		*antigens
95	24	roduction	production
98	6	immuneresponse	immune response
100	19	Kenyes	Keynes
107	21	to	at
122	17	(Downey, 1966a), however,	Downey (1966a)
126	16		*week
137	20	Pastuerella	Pasteurella
147	6	Nigrogen	Nitrogen
150	4	respresentative	representative
179	21	haemotological	haematological

ERRATA continued

Page Number	Line Number	Word or Phrase	Amended to read As follows:
180	6	netrophils	neutrophils
	3	et at	et al
181	8	acitivity	activity
182	27	than	that
184	21	but	and
185	17	non-supplemented	supplemented
219	Table 28	23.01	53.01
		29.18^{a}	29.18^{ab}
221	Table 30	39.5	69.5
223	2	fo	for
241	21	of deficiency	on immunity
268	8		*and
275	25	coparison	comparison
282	12	secretor	secretory
283	4	titre	titres

* denotes that a missing word should be included in the text.