DEFINITION AND DIAGNOSIS OF COBALT DEFICIENCY IN GOATS.

A thesis submitted to the Faculty of Science of the University of Glasgow to fulfil requirements for the award of the Degree of Doctor of Philosophy

by

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ABSTRACT

A review of the literature concerning cobalt, vitamin E, and selenium and their effects on immunity in ruminants is included, concentrating particularly on the definition and diagnosis of cobalt deficiency.

Five experiments are reported, the first three of which explore the requirement of goats for cobalt to prevent subclinical deficiency. Included in this is the effect of cobalt supplementation on immunity, and also a preliminary investigation of different forms of vitamin E supplementation and their suitability for treating vitamin E deficiency. In each experiment 24 feral cross goats were depleted of cobalt and then assigned to various levels of cobalt supplementation. In experiments 1 and 2 half the goats were supplemented with vitamin E in the last month of the experiment.

The symptoms of vitamin B_{12} deficiency observed included loss of weight, loss of appetite, pale mucous membranes and 'weepy eyes'. Concentrations less than 0.07 mgCo/kgDM in the diet are likely to lead to deficiency and this is between the comparable requirements for cattle (0.04-0.07) and sheep (0.07-0.08). Deficiency is best confirmed by measuring both MMA and vitamin B_{12} concentrations. Serum MMA concentrations between 15-20 μmol/l and serum vitamin B_{12} concentrations between 50-100 ng/l were associated with subclinical deficiency, and these differ from the levels associated with subclinical deficiency in sheep (MMA 10-15 μmol/l, B_{12} 200-400 ng/l) and cattle (MMA 2-4 μmol/l, B_{12} 150-200 ng/l). Serum succinate was not found to be reliable as an indicator of cobalt status. No effects on immune function were observed. Both oral and injectable vitamin E treatments raised plasma vitamin E concentrations, although the injectable treatment produced a rapid but short lived response, whereas daily oral supplementation produced a more gradual but long term response. Erythrocyte stability appeared to show some promise as a

method of detecting vitamin E deficiency, but results were not conclusive. Results of these experiments are discussed and compared with the relevant literature.

The fourth experiment investigated different methods of supplementing calves with vitamin E. Twelve calves were depleted of vitamin E and then randomly assigned to three groups, receiving either oral, injectable or no vitamin E supplement. Half the calves on each treatment also received injections of vitamin B_{12}. Again injection of vitamin E was found to produce a rapid, massive but short lived response in plasma vitamin E concentrations; however repeated injections produced an adverse reaction if insufficient time (< 3 weeks) was left between treatments. Oral supplementation produced a more gradual, but sustained response with no adverse reactions. Thus injection is highly suitable for rapid treatment of deficient animals, but oral supplementation is a more long term solution where instant effects are not required. In calves erythrocyte stability was unsuitable as an indicator of vitamin E status. A small increase in % kill of *E.coli* and *C.albicans* but not of *S.aureus* was observed in the vitamin B_{12} supplemented calves, but no effect of vitamin E status was observed. Results are discussed and compared to other results in this field.

The final experiment investigated the effect of cobalt deficiency on appetite preference in sheep, and by means of different treatments, oral cobalt, vitamin B_{12} injection, and methionine injection, attempted to gain some insight into the metabolic reason behind this phenomenon. Thirteen mature Suffolk cross castrates were maintained on a cobalt deficient diet, until they showed loss of appetite; they were then given one of the three treatments to see if this would restore their appetite. Vitamin B_{12} injections produced a rapid response, while cobalt treatment was also effective but took longer to work. Methionine treatment failed to improve appetite, suggesting that it is the failure of the propionate to succinate pathway which is responsible for the loss of appetite, and that it is absorbed vitamin B_{12} which reverses the symptoms and not free cobalt in the rumen. Results are discussed in the

light of other work.

LIST OF ABBREVIATIONS

CK	Creatine Kinase
CMIR	Cell mediated immune response
EDTA	Ethylenediaminetetraacetic acid
EMA	Ethylmalonic Acid
EST	Erythrocyte stability test
FIGLU	Formimino-L-glutamic acid
GC	Gas chromatography
GOT	Glutamic oxalacetic transaminase
GSH-Px	Glutathione Peroxidase
HIR	Humoral immune response
HPLC	High performance liquid chromatography
LWT	Liveweight
MMA	Methylmalonic Acid
NMD	Nutritional muscular dystrophy
NFT	Neutrophil Function Test
PBS	Phosphate buffered saline
PMN	Polymorphonucleocyte
PUFA	Polyunsaturated fatty acids
SAM	S-adenosyl methionine
SAH	s-adenosyl homocysteine
SCC	Somatic cell count
SRBC	Sheep red blood cells

CHAPTER 1

LITERATURE REVIEW

1.1. COBALT.

1.1.1. History and discovery of cobalt and cobalt deficiency.

The first recorded descriptions of cobalt deficiency date back to Robert Frazer of Devon and James Hogg of Scotland, around 1800. It was found that in certain areas ruminants suffered from a wasting disease but on movement to 'healthy pasture' they recovered and from this they deduced that a deficiency was involved. Towards the end of the 19th century 'Tauranga disease' in sheep and 'Bush sickness' in cattle in New Zealand were discovered to be similar. Due to the anaemic symptoms iron was first suspected, and some iron ores were indeed found to alleviate the disease, 'Limonite' being found to be both cheap and effective, (cited by Andrews 1970a).

However various other diseases, 'wasting disease' of western Australia, 'pining' in Britain, 'coast disease' also of Australia, 'Nakuruitis' in Kenya, 'salt lick' in Florida, 'Lechsucht' in Netherlands and Germany, 'Grand Traverse Disease' in Michigan and 'Mairoa dopiness' and a general unthriftiness observed in other areas of New Zealand, had similar symptoms, yet not all of these were associated with areas low in iron. In 1935 two separate groups in Australia, (cited by Andrews 1970a), identified cobalt as the missing link. The affected areas were either deficient in cobalt or it was unavailable to the animal for some reason.

Research (reviewed by Smith and Loosli 1956) indicated that the majority of the dietary cobalt requirement of ruminants was used for the synthesis of vitamin B_{12} by the rumen micro-organisms, and that it was a deficiency of vitamin B_{12} in the animal which caused the symptoms. Injection of vitamin B_{12} proved effective in preventing or curing the disease.

1.1.2. Role of cobalt/vitamin B_{12}

The major function of cobalt is as an essential component of vitamin B_{12} (cobalamin) and little is known of any functions carried out by cobalt itself but Ibbotson *et al* (1970) suggest that it is involved in stimulating erythropoietin and hence in both red and white cell formation in the bone marrow. However, as neither injected vitamin B_{12} nor cobalt alleviated the condition they suggest that cobalt might be the precursor in the rumen for a second essential factor.

Vitamin B_{12} is a coenzyme for several reactions of which two are well documented. During cobalt deficiency the activities of both these enzymes are impaired (Kennedy *et al* 1991a).

A. Methylmalonyl CoA Mutase.

Methylmalonyl CoA mutase is required for the conversion of propionate via methylmalonyl CoA to succinate in the liver (Marston *et al* 1961):-

PROPIONATE

↓ ATP, CoA — Propionyl CoA Synthetase

PROPIONYL CoA

↓ ATP, Biotin, CO_2 — Propionyl CoA Carboxylase

D-METHYLMALONYL CoA

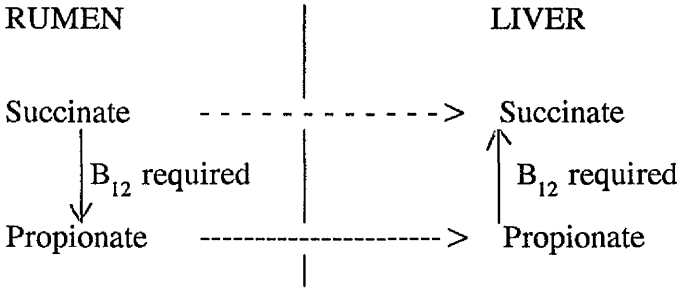
↓ — Methylmalonyl CoA Racemase

L-METHYLMALONYL CoA

↓ B_{12} coenzyme — Methylmalonyl CoA mutase

SUCCINYL CoA

The activity of methylmalonyl CoA mutase is limited by the availability of the coenzyme, vitamin B_{12}, rather than the enzyme itself (Peters and Elliot 1984). During vitamin B_{12} deficiency propionate and methylmalonic acid (MMA) concentrations in the blood are elevated, and many of the symptoms of cobalt deficiency have been attributed to impaired propionate metabolism. Recently succinate concentrations have been found to be elevated in the rumen, and in plasma during vitamin B_{12} deficiency (Kennedy *et al* 1991c & d, and Young *et al* 1991) and it has also been observed that succinate can be absorbed from the rumen. This would suggest a possible by-pass of propionate metabolism, due to lack of vitamin B_{12} in the rumen for conversion of succinate to propionate.



There is no evidence however that succinate from this source is as freely available to the host as succinate produced from propionate in the liver. Kennedy *et al* (1991c & d) and Young *et al* (1991) suggest that due to this by-pass, the block in the propionate to succinate pathway should not be critical, and no deficiency of glucose should result in the animal. However MacPherson *et al* (1973) found that glucose deficiency does occur in cobalt deficient ruminants, and Price (1991b) found that the breakdown in the propionate pathway occurs before the breakdown of 1-carbon metabolism. Thus although the by-pass may compensate to some extent, it is not sufficient to prevent cobalt deficiency from adversely affecting systemic metabolism of propionate.

Many experiments have been done to determine whether vitamin B_{12} deficiency is associated with the low milk fat syndrome in dairy cows. The accumulation of propionate due to decreased metabolism to glucose might upset the acetate : propionate ratio leading to decreased milk fat synthesis from acetate. However the results (cited by MacPherson 1982) failed to determine, conclusively, any relationship with vitamin B_{12} deficiency.

Lambs grazing pastures where many of the grasses were high in water soluble carbohydrates eg. fructan, had a greater requirement for cobalt if ovine white liver disease was to be prevented (Ulvund 1990/91).

In *in vitro* studies using rat liver supernatant fractions, methylmalonyl CoA was found to partially inhibit fatty acid synthesis and also to be incorporated into fatty acids in place of malonyl CoA (Cardinale *et al* 1970); the majority of fatty acids derived from methylmalonyl-CoA were branched, due to the presence of the additional methyl group. Further research (Frenkel *et al* 1973) revealed that propionyl CoA competed with acetyl CoA as a substrate for incorporation into fatty acids, providing a mechanism for odd chain fatty acid production. Again methylmalonyl CoA was found to partially inhibit fatty acid synthesis (Frenkel *et al* 1973). The presence of abnormal amounts of branched chain fatty acids, or odd chain fatty acids may be physiologically undesirable. Lambs born to vitamin B_{12} deficient ewes were found to have increased amounts of branched chain fatty acids. (Duncan *et al* 1981).

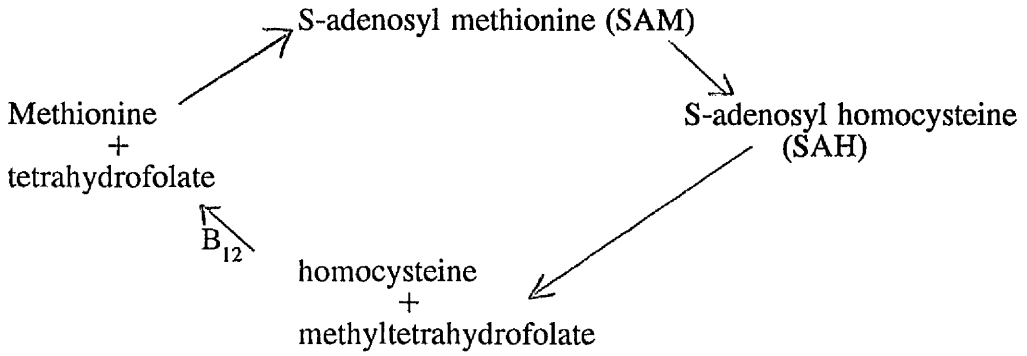
B. 5-Methyltetrahydrofolate-Homocysteine Methyltransferase.

The second enzyme, 5-methyltetrahydrofolate-homocysteine methyltransferase is involved in the recycling of methionine, catalysing the reaction:-

5-methyltetrahydrofolate + homocysteine $\xrightarrow{B_{12}\text{ coenzyme}}$ methionine + tetrahydrofolate

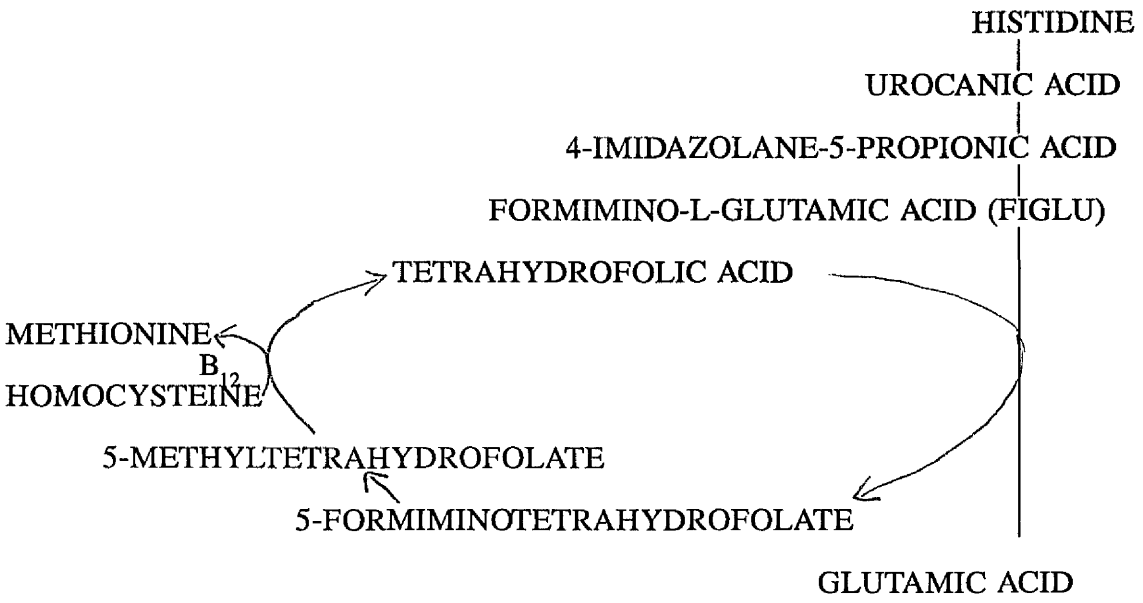
Methylcobalamin, the vitamin B_{12} coenzyme, is required for the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine.

Methionine recycling has been linked (Kennedy et. al. 1991e) to phosphatidylcholine metabolism:-



When methionine recycling is blocked this affects the ratio of SAM:SAH and SAM is involved in many transmethylation reactions (Stryer 1981) including conversion of phosphstidylethanolamine to phosphatidyl choline, conversion of noradrenaline to adrenalin, inactivation of catecholamines (noradrenaline and adrenalin) via catechol-O-methyltransferase, and methylation of RNA and DNA, for identification of self and non-self nucleic acids.

Methionine recycling is also linked to folic acid metabolism:-



Thus vitamin B_{12} deficiency leads to elevated concentrations of homocysteine and FIGLU. During vitamin B_{12} deficiency a secondary folic acid deficiency is also observed. The 'methyl trap' concept is based on the decreased activity of 5-methyltetrahydrofolate-homocysteine methyltransferase during cobalt/vitamin B_{12} deficiency. Metabolically active folates become 'trapped', and unavailable as methyltetrahydrofolates, because the formation of 5-methyltetrahydrofolate is essentially irreversible *in vivo*, thus a functioning methionine synthase is essential to reconvert methyltetrahydrofolate to tetrahydrofolate (Scott 1992, McDowell 1989). Increased proportions of methyltetrahydrofolate, and a decrease in tetrahydrofolate concentrations led to the methyl trap hypothesis. In particular 10-formyltetrahydrofolate is used as a cofactor for two of the enzymes involved in purine biosynthesis and 5,10-methylenetetrahydrofolate is involved in pyrimidine biosynthesis (Scott 1992) thus failure of methionine synthase to reconvert 5-methyltetrahydrofolate to tetrahydrofolate leads to decreased DNA and RNA biosynthesis.

Smith and Osbourne-White (1973) suggest that the depletion of methyltetrahydrofolates in the liver is caused by the failure of the liver to retain folates due to a failure to synthesise pteroylpolyglutamates from monoglutamates, a failure to completely reduce folates leading to their non retention and a failure to transport folates into the deficient cells. Supplementary methionine has been found to improve folate retention and Smith and Osbourne-White suggest that vitamin B_{12} may promote folate retention via methionine. As they point out, this would imply that a metabolic product was responsible for promoting its own synthesis, however in support of this hypothesis McGing & Scott (1980) found that methionine supplementation partially reversed the decrease in folate uptake by the liver, of vitamin B_{12} deficient rats. Methionine is thought to influence folate metabolism in vitamin B_{12} deficient ewes, by affecting the rate of transport of folates into the liver cells (Gawthorne and Smith 1974).

ATP, NADH and NADPH were found in lower concentrations in the livers of vitamin B_{12} deficient ewes, while the ratio of K^+/Na^+ was abnormally high (Smith *et al* 1974), which suggests that both respiratory derangement and failure of a transport process were occurring in the livers. Particularly high concentrations of methionine are required for the initiation of protein synthesis, and for maintainance of polyribosome integrity (McGown *et al* 1973, Park *et al* 1973), and the higher methionine requirement for folate retention than for lipid metabolism found by Smith *et al* (1974) may reflect a specific need of folate retention for methionine-initiated protein syntheisis.

In man an uninterrupted folate cycle appears to be a prerequisite for normal synthesis of methylcobalamin (Quadros *et al* 1976), which is the cofactor required for the homocysteine to methionine reaction, whereas 5-deoxyadenosylcobalamin is required for the isomerisation of methylmalonate to succinate. The high tissue

requirement of sheep for methylcobalamin, for methionine synthesis during wool production, exacerbates the shortage of 5-deoxyadenosylcobalamin available for propionate metabolism (Suttle 1988).

Folic acid deficiency in animals leads to anaemia and decreased growth, and the interference in tetrahydrofolate cycling affects purine synthesis, leading to decreased DNA synthesis. The methionine deficiency which results, leads to impaired nitrogen retention (decreased wool and body growth of sheep, Underwood 1981) and also to a decrease in available S-adenosyl methionine. Smith *et al* (1974) found that during cobalt deficiency the increase in lipids found in the livers of vitamin B_{12} deficient sheep were associated with a decrease in choline concentrations. Injections of methionine, into vitamin B_{12} deficient sheep, correct the decreased SAM concentrations and lead to normal liver fat content by increasing the activity of liver methyltransferase (Ramos Anton *et al* 1991). An alternative methionine salvage pathway via SAM, and 5-S-methyl-thioadenosine, has been postulated by Abeles (1992), in both mammals and plants. It is possible that in ruminants this pathway is not activated before SAM becomes limiting.

Apart from its function in these two enzyme reactions it has been suggested that vitamin B_{12} is also required for the synthesis of the thyroid hormones. In goats (Mgongo *et al* 1981) cobalt deficiency has been found to increase plasma thyroxine concentration, and the free thyroxine index, possibly by affecting the function of the hypothalamus.

1.1.3. Factors affecting the requirement of ruminant animals for cobalt.

Cobalt availability to the ruminant depends on the amount in the soil, the amount taken up by the plants, which depends on the type of plant, and may be affected by other compounds in the soil (Burridge *et al* 1983). Different parts of the plant may contain differing concentrations of cobalt, and soil contamination greatly affects the amount of cobalt ingested (Burridge *et al* 1983). Pasture improvements may lead to cobalt deficiency in grazing livestock, as the species planted tend to take up less cobalt, increased growth of the sward decreases incidental soil cobalt ingestion and liming decreases the availability of cobalt to plants by increasing the pH of soils. The faster growth of animals on more productive pasture also increases the demand for cobalt (Paterson 1988). Given the fact that 'limonite' was initially used to treat deficiencies, it would appear that even highly insoluble compounds provide a source of available cobalt when fed, and certainly appreciable quantities of cobalt oxide and carbonate appear to be available (Smith and Loosli 1956).

The ruminant uses cobalt very inefficiently as the rumen microbes synthesise physiologically inactive α-analogues of vitamin B_{12}, as well as 'true' vitamin B_{12}. The α-analogues of vitamin B_{12} are not thought to be absorbed in sheep, however there is some evidence that they may be in cattle (Paterson 1988). Some α-analogues of vitamin B_{12} are microbiologically active, and may be measured together with vitamin B_{12}, leading to confusion and overestimation when trying to quantify vitamin B_{12} using some of the microbiological assays.

The composition of the diet may affect the efficiency of vitamin B_{12} synthesis and also the ratio of vitamin B_{12}:α-analogue production. Alfalfa and high energy diets stimulate synthesis of all forms of vitamin B_{12}, but favour α-analogues. High feed intakes and high forage diets encourage vitamin B_{12} synthesis whereas cereals stimulate other derivatives (Sutton & Elliot 1972). Cobalt intake of the animal also

affects the ratio of vitamin B_{12} to other derivatives; in animals with a low cobalt intake up to 15% of vitamin B_{12} produced may be 'true', but when cobalt intake is high in sheep this can drop as low as 3% (cited by Underwood 1981). Parasitic infection may also affect vitamin B_{12} synthesis, relative to the synthesis of α-analogues (Ramos Anton *et al* 1991), and high concentrations of molybdenum may also decrease vitamin B_{12} synthesis in cows. At low feed intakes vitamin B_{12} production may be limited by feed intake before cobalt availability (Smith & Marston 1970b) because of the lack of precursors for vitamin B_{12} synthesis, or due to a decreased metabolic activity of the microbes involved. Supplementation of ruminants with the base 5,6-dimethylbenzimidazole (the precursor of 'true' vitamin B_{12}) leads to increased synthesis of 'true' vitamin B_{12} rather than α-analogues (Gawthorne 1970), and has a sparing effect on cobalt (Rickard *et al* 1975).

Vitamin B_{12} administered orally was found to be utilised very inefficiently, in fact parenterally administered vitamin B_{12} is 35 times more effective (Smith and Loosli 1956). A group of closely related vitamin B_{12} compounds, the ß-analogues of cobalamin, have been found to be utilised equally efficiently as they are interconvertible. Absorption of 'true' vitamin B_{12} is poor and can be as low as 3-5% in cobalt deficient sheep, while absorption of supplemental cyanocobalamin given orally is even worse (Smith & Marston 1970b). Parasitic infection decreases the efficiency of absorption of vitamin B_{12} (Ramos Anton *et al* 1991).

Ruminants have been found to have a far larger requirement for cobalt than non-ruminants. Not only because of the inefficiency of incorporation of cobalt into vitamin B_{12}, or because they absorb vitamin B_{12} much less efficiently than non-ruminants, but in addition to this they actually have a greater requirement for vitamin B_{12} due to its important role in the metabolism of propionate, the synthesis of which is a feature of ruminant digestion. In ruminants carbohydrates are broken

down in the rumen into acetic, propionic and butyric acids, and a combination of these three volatile fatty acids is absorbed directly from the rumen. Propionate is the sole gluconeogenic volatile fatty acid produced during normal rumen fermentation.

A regular supply of vitamin B_{12} is required because large excesses in production rarely occur in the rumen, and because it is very poorly absorbed. It is stored in the liver, kidneys and muscle but not to any great extent, as supply rarely exceeds requirement, thus low intakes of cobalt are tolerated only in the short term since tissue stores are soon depleted when metabolic demand for the vitamin exceeds the amount absorbed from the gut. In the tissues vitamin B_{12} is always found bound to another molecule, cobalophilin, and in the gut vitamin B_{12} must be bound to intrinsic factor before absorption can take place in the lower intestine.

Sheep are more susceptible to vitamin B_{12} deficiency than cattle (Andrews 1970a), and thus have a greater requirement for cobalt; young ruminants are also particularly susceptible, possibly because they have no opportunity to build up any stores of vitamin B_{12} (Underwood 1981).

Pasture concentrations below 0.08 mgCo/kgDM (for sheep) or 0.04 mgCo/kgDM (for cattle) are likely to result in deficiency (Andrews 1970a) although concentrations slightly above 0.04 mgCo/kgDM were found to be inadequate for calves under certain conditions (MacPherson *et al* 1973). Alternatively 0.07mgCo/kgDM is the minimum requirement according to Underwood (1981) though he recommends an allowance of 0.11mgCo/kgDM. It is usually assumed that the 0.11 mgCo/kgDM recommended for cattle and sheep will be sufficient for goats also, although little research has been done specifically on goats.

Platten (1951) suggests that lactating goats require up to four times the daily cobalt intake recommended for sheep. However as the amount is not expressed per kgDM

intake the difference in feed intake may account for some of this. Goats maintained on 0.01 mg/kgDM became deficient (Mgongo *et al* 1984), while those receiving 0.035 mg/kgDM showed no symptoms during a four month period (Clark *et al* 1987). Serum vitamin B_{12} concentrations for the untreated goats decreased below 100 pmol/l which would be taken as indicative of marginal deficiency in sheep.

The requirement for cobalt is increased when livestock graze pastures containing the grass 'Phalaris tuberosa'. This contains the alkaloid tryptomena, a neurotoxin which can be neutralised by the rumen microbes. If the alkaloid is not neutralized the condition known as 'phalaris staggers' develops (Lee and Kuchel 1953). Cobalt leads to proliferation of the rumen micro-organisms and it is therefore the element itself that is required as opposed to vitamin B_{12} and the action is specific to cobalt, as copper, iron, zinc etc. do not produce the same effect (Lee *et al* 1957).

1.1.4. Deficiency symptoms

The designation 'enzootic marasmus' describes the deficiency which begins, as body stores of vitamin B_{12} become depleted, with a loss of appetite and decreased live weight gain. These accelerate and rapid muscle wasting (marasmus) begins and pica or depraved appetite may be observed before the onset of severe anaemia which eventually results in death, (Underwood 1981). Symptoms of cobalt deficiency may sometimes not become apparent (MacPherson 1982) in housed animals, suggesting that some form of stress is required to precipitate the clinical signs.

In animals with severe vitamin B_{12} deficiency, visual symptoms include pale mucous membranes, skin fragility and lachrymation in sheep and rough coats and increased nervousness in cattle. The animals become extremely emaciated and have virtually no body fat except for an accumulation in the liver. Smith and Marston (1970a)

attribute the loss of weight to metabolic inefficiency due to lack of vitamin B_{12} in addition to the resulting loss of appetite.

Clinical deficiency is easily recognised by the symptoms given earlier, but subclinical or marginal deficiency, which may well cause major economic loss, is difficult to detect. The decreased weight gain, general unthriftiness and increased susceptibility to disease are uneconomic but not specific to vitamin B_{12} deficiency. Delayed involution of the uterus, reduction in oestrus, and low sperm counts in mature animals (Hidiroglou 1979), and decreased viability of the young, particularly lambs between 11 and 17 weeks of age (Duncan *et al* 1981) also decrease profits, but again could be due to any number of causes. Only by measuring the response of the animal to cobalt or vitamin B_{12} can vitamin B_{12} deficiency be verified.

The pathological consequences of vitamin B_{12} deficiency include decreased appetite, polioencephalomalacia (cellular oedematous changes in the grey matter), neurological lesions, white liver disease (fatty degeneration of the liver), cellular infiltration, bile duct proliferation, and release of liver enzymes (aspartate aminotransferase, GOT). Some of these symptoms are similar to those produced by other deficiencies and this is thought (Suttle 1988) to be because they are the end result of a defect in the same biochemical pathway, eg. impaired gluconeogenesis produces lesions in the brain in both thiamine and vitamin B_{12} deficiencies, while impaired gluconeogenesis in the liver induces similar symptoms in both biotin and vitamin B_{12} deficiencies.

Ovine white liver disease, which is also thought to occur in goats, (Black *et al* 1988) is associated with cobalt/vitamin B_{12} deficiency and diets high in soluble carbohydrate (Sutherland *et al* 1979, Mitchell *et al* 1982, McLoughlin *et al* 1986 and Ulvund 1990/91). On such diets, sheep and goats (but not cattle and red deer) synthesise increased amounts of branched chain fatty acids from MMA in their

livers. A pale swollen liver with diffuse fatty hepatocytes results.

1.1.5. Diagnosis

Determination of levels of extractable cobalt in soil or total cobalt in the diet may give some indication of whether cobalt supply to livestock is likely to be adequate, but analysis of vitamin B_{12} concentrations in the animal takes absorption and inactive analogues into consideration.

Plasma cobalt concentrations could be misleading as they do not necessarily represent vitamin B_{12}. Liver vitamin B_{12} concentration is a useful indicator of vitamin B_{12} status, but liver samples are not readily obtained. Blood is more accesible for sampling and serum vitamin B_{12} concentrations are widely used for assessing vitamin B_{12} status in man and in the sheep.

Commonly accepted critical diagnostic concentrations (ng vitamin B_{12}/l) are (VIC Auchincruive):-

	Deficient	Marginal	Adequate
Sheep	<200	200-400	400-2500
Cattle	<150	150-200	200-2500

In sheep inactive α-analogs are not thought to occur to any great extent in serum making up only 8-14% of total corrinoids; in cattle however they are thought to constitute up to 50% of total corrinoids (Halpin *et al* 1984). In addition variation in serum vitamin B_{12} concentrations between individual animals can be large so it is best to sample more than a few animals.

Quantification of serum or plasma vitamin B_{12} concentrations is also complicated by the presence of binders. Vitamin B_{12} is never found free in the body, intrinsic factor is required for absorption and from there on it is passed from one binder to another. In sheep vitamin B_{12} is found bound to transcobalamin 2, in cattle however as well as this binder there are two others, transcobalamin 1, and transcobalamin 0 which is thought to be either a polymeric form of transcobalamin 1 or a complex of transcobalamin 1 with other plasma proteins. Price (1991) found that boiling in cyanide was sufficient to release vitamin B_{12} from transcobalamin 2, but not from the transcobalamins 1 or 0 found in cattle. This means that analysis of vitamin B_{12} in cattle plasma may be an underestimate of the total amount present, and could therefore lead to an overestimate of the prevalence of vitamin B_{12} deficiency in this species.

Liver diseases may affect vitamin B_{12} concentrations (Andrews 1970b), and also prolonged yarding can lead to increased serum vitamin B_{12} concentrations. In addition to these problems vitamin B_{12} itself is not a sensitive index of deficiency as it shows a storage type response to cobalt supply (Suttle 1986), therefore other parameters have been considered.

Serum or urinary MMA concentrations can be measured. However urine samples require special techniques for collection, and serum MMA concentration is a sensitive indicator of an existing vitamin B_{12} deficiency in sheep (Rice *et al* 1987).

Commonly accepted critical diagnostic serum MMA concentrations (μmol/l) are:-

	Deficient	Marginal	Adequate
Sheep	>15	10-15	<5 (10*)
Cattle	>4	2-4	<2

*(10) when fed concentrate rations.

Andrews (1970b) found MMA concentrations in urine increased rapidly in young lambs grazing cobalt deficient pastures, before serum vitamin B_{12} concentrations decreased to levels associated with deficiency. This suggests that low blood vitamin B_{12} concentrations are not always the first indicator of deficiency.

Rumen MMA concentrations in sheep are also elevated by a cobalt deficient diet (Rice *et al* 1989) but the MMA is not absorbed and does not affect plasma concentrations. According to Lough and Calder (1976) sheep fed a diet of rolled barley had elevated MMA concentrations between 10 and 20 mg/l in their urine and they also found evidence of high concentrations of ethylmalonic acid (EMA) in the urine. However O'Harte *et al* (1989a) found that the low concentrations found in plasma did not interfere with test results for vitamin B_{12} deficiency, although O'Harte *et al* (1989b) recommended that 10 μmol/l be regarded as the upper limit of normality for concentrate fed sheep. Inappetance due either to the cobalt deficiency itself or to parasite infestations may lead to decreased concentrations of propionate in the rumen and hence to decreased concentrations of MMA in serum, thus some false negatives make it advisable to sample several animals in any group (Paterson 1988, Paterson and MacPherson 1990).

Occasionally methylcobalamin deficiency might be of greater interest than adenosylcobalamin deficiency (which is what MMA concentration indicates) and so a separate marker for this has been considered. Formiminoglutamate (FIGLU), an intermediate in the degradation of histidine has been suggested as an indicator for impaired vitamin B_{12}-dependent methyltransferase activity. Gawthorne (1968) has reported that the urinary concentration of this metabolite was a sensitive indicator for cobalt/vitamin B_{12} deficiency in lambs and was elevated prior to plasma MMA concentrations. However other workers (Stebbings & Lewis 1983 and 1986, Skinner 1983) found that FIGLU concentrations were only elevated in severe vitamin B_{12}

deficiency after clinical signs of deficiency were apparent, and they concluded that FIGLU was unreliable as an indicator for the deficiency. Price (1990) compared MMA concentrations with various other parameters and found that plasma urocanate response to histidine infusions could be used to detect deficiency. However propionate metabolism was found to be affected earlier in vitamin B_{12} deficiency than 1-C metabolism hence it was concluded that MMA concentrations were a better indicator of deficiency than plasma urocanate response to histidine infusion.

Other parameters which decrease during vitamin B_{12} deficiency include plasma glucose (due to impaired propionate metabolism), alkaline phosphatase, ascorbic acid (due to liver damage) and thiamine concentrations (MacPherson *et al* 1973). Parameters which increase during vitamin B_{12} deficiency include aspartate aminotransferase (due to liver damage), and pyruvate concentrations (due to thiamine deficiency). These parameters are all non-specific and could also be due to many other factors.

Recently during experiments concerned with whether succinate concentrations, in both rumen and plasma are affected by cobalt deficiency Kennedy *et al* (1991d) postulated the following critical plasma concentrations (μmol/l).

	Deficient	Marginal	Adequate
Sheep	24-33	14-24	10-14

The consensus of opinion to date appears to suggest that the use of both vitamin B_{12} concentrations and MMA concentrations in conjunction leads to the most reliable diagnosis especially where sub-clinical vitamin B_{12} deficiency is concerned. (Fisher and MacPherson 1990, Paterson and MacPherson 1990).

1.1.6. Effects of cobalt on feed intake

Failure to metabolise propionate normally (Marston *et al* 1961), and hence its presence in increased concentrations, and proportions relative to the other volatile fatty acids in tissues, is thought to be the main cause of the lack of appetite seen in vitamin B_{12} deficient animals (Marston *et al* 1972). Increased concentrations of both acetate and propionate were found in the blood of vitamin B_{12} deficient sheep (Marston *et al* 1972), and acetate was metabolised at a slower rate than in control sheep. The vitamin B_{12} deficient animal also tends to have a preference for roughage, as opposed to concentrates which lead to a propionate type fermentation (MacPherson 1982). Ketelaars and Tolkamp (1992) in their recent review of feed intake regulation hypothesise that voluntary food intake is regulated to maintain maximum efficiency of oxygen utilization. This would be achieved by an optimum intracellular acid load (pH), which in turn would depend on an optimum volatile fatty acid concentration in all body compartments. Hence increases in propionate concentrations would be unfavourable.

Metabolites known to affect appetite include oestrogens, gastrin, secretin, bombesin, CCK and acetyl choline. However a link between these parameters and vitamin B_{12} deficiency has yet to be established.

Infusion of sodium propionate or sodium acetate into the rumen of lactating cows caused a depression in hay or silage intake in a dose related manner, however at physiological concentrations effects were not significant (Anil *et al* 1993). Propionate infusion into the hepatic portal vein of sheep has been found to decrease intake (Forbes 1983, Farningham 1990), and it is also known that propionate clearance from plasma during vitamin B_{12} deficiency is depressed (Marston *et al* 1972). Low doses of propionate (200kcal) have been found to produce an apparent increase in chaff intake, whereas higher doses (300 kcal) lead to decreases in intake

(Ulyatt 1965, cited by Church 1971). The initial increase might be due to a preference for roughage. Acetate is thought to exert its effects on feed intake via receptors in the rumen wall, while propionate receptors are thought to be located in the liver, alternatively both acetate and propionate may exert their effects via osmotic mechanisms (Anil *et al* 1993). Intraruminal propionate infusion has been found to increase plasma insulin (de Jong 1981), even when plasma propionate concentrations remained unchanged (Bhattacharyla & Alulu 1975, cited by Forbes 1986). This suggests that the effect of propionate on appetite may be complex, involving changes in insulin metabolism.

Non-ruminants suffering from a protein imbalance due to lack of methionine show loss of appetite. In ruminants this is unusual because microbial protein synthesis from non-protein nitrogen and sulphur corrects for any deficiencies in the diet. However if methionine recycling was upset this would result in an increased requirement for methionine, and might lead to an imbalance and loss of appetite. If this were the case then supplying the deficient animal directly with methionine would be expected to alter the preference of vitamin B_{12} deficient animals for forage rather than concentrates. The mode of action here is not known but might involve knock on effects to other amino acids such as tyrosine which are known to be precursors of neurotransmitters and so could exert an effect via the central nervous system.

1.1.7. Evidence Suggesting Cobalt/Vitamin B_{12} Status Affects the Immune System.

Cobalt deficiency is generally accompanied by decreased plasma concentrations of ascorbic acid. In 1976 MacPherson *et al* suggested this decrease might be responsible for the deficiency in the immune response, which resulted in far more cobalt deficient animals succumbing to infection than did cobalt adequate ones. However in 1982 MacPherson cited work that suggested ascorbic acid was not involved and that the observed effects of cobalt deficiency were due to changes in propionate and glucose metabolism.

The earliest work relating cobalt intake to resistance to infection investigated the resistance of lambs to worm (mainly *Haemonchus contortus*) infestations. These studies (Weir *et al* 1948, Shumard *et al* 1956, Threkeld *et al* 1956 and Downey 1965 and 1966b) suggested that the parasite had a cobalt requirement since supplementation apparently benefited the parasite, leading to increased fecundity of the worm and causing increased anaemia and mortality rate in the host. Certainly *Diphyllobothrium latum* has been found to cause pernicious anaemia in man (Nyberg 1963) as in this case the worm is capable of utilizing vitamin B_{12} bound to Intrinsic Factor in digesta.

However, other studies (Weir *et al* 1948, Richard *et al* 1954 and Downey 1966a) have indicated increased host resistance to infection, which appears to be a direct contradiction. Differences in worm burden used may however be responsible. The first group of experiments used in excess of 20,000, and in most cases 50,000 worm larvae, whereas the other experiments used fewer (15,000) worm larvae, or larger numbers administered in more than one dose. When large worm burdens were involved it would appear that the worms utilise the cobalt or vitamin B_{12}, and the host in effect receives little of the supplement. However when smaller worm burdens

were involved the host immune response may have been stimulated by increased vitamin B_{12} synthesis and absorption following cobalt supplementation.

Recent work by MacPherson *et al* (1987a) in cattle showed that in cobalt deficient animals, *Ostertagia ostertagi* had a shorter prepatent period and produced more eggs, also the increase in plasma pepsinogen concentrations of the host during infection was smaller in cobalt deficient cattle than in cobalt supplemented animals. Ferguson (1990) found similar effects on *Ostertagia circumcincta* infection in sheep, and also significantly higher mortality in the deficient sheep. In 1969 Michel and Sinclair showed that *Ostertagia ostertagi* infection is controlled immunologically, which suggests that the immune response of cobalt deficient animals is impaired. However Klesius (1988) reported that immunity to *Ostertagia ostertagi* is very slowly acquired and is weak even then. The response is mediated by antibodies (humoral) and also by IgE and mast cells (cellular immunity), and this is accompanied by an increase in eosinophils. The immune response can however be suppressed by the infestation itself, particularly in young animals.

In 1976 Kaplan and Basford looked at the effect of vitamin B_{12} deficiency on polymorphonucleocyte (PMN) activity in humans. They concluded that deficiency did not affect phagocytosis, but did decrease the % kill of the ingested *Staphylococcus aureus*, by the PMN, which was not so during folic acid deficiency. Vitamin B_{12} deficiency also decreased metabolic activation (Hexose monophosphate shunt activation) as determined from the evolution of ^{14}C carbon dioxide, after thirty minutes incubation in the presence of ^{14}C glucose.

Ludovici and Axelrod (1951) found no effect of vitamin B_{12} deficiency on the hemagglutination antibody response of rats to human erythrocytes. It is worth noting that pteroylglutamic acid deficiency severely impaired this response. Das and

Hoffbrand (1970) found enlarged lymphocytes and a different chromatin pattern, more finely reticulated and open, in pernicious anaemia patients, who cannot absorb vitamin B_{12} because they lack intrinsic factor. These lymphocytes had a greater incorporation of radiolabelled thymidine, which was less susceptible to inhibition by deoxyuridine. The lymphocytes were described as being more megaloblastoid. They concluded that the deficiency resulted in decreased methylation of deoxyuridylate to thymidylate, for which 5,10-methylene tetrahydrofolic acid is required. This is absent in both folic acid and vitamin B_{12} deficiency. Thymine, for incorporation into DNA, is generated by a second pathway, involving thymidine kinase, which has increased activity during vitamin B_{12} deficiency.

MacCuish *et al* (1974) found normal concentrations of B and T lymphocytes in pernicious anaemia patients, but radiolabelled thymidine uptake by lymphocytes was decreased, suggesting decreased lymphocyte transformation, when they were exposed to the PHA mitogen. Further work suggested that blastogenesis was unaffected but that labelled thymidine incorporation was decreased, which could be due to a defect in thymidine synthetase activity. Thus vitamin B_{12} seems to mediate its effects via the decrease in the availability of folate coenzymes required for thymine synthesis.

Wright, MacPherson and Taylor (reviewed by MacPherson 1982) found that neutrophils from cobalt supplemented calves were generally 50% more effective at killing *C. albicans* once ingested, than were neutrophils from cobalt deficient calves. MacPherson *et al* (1987a), Paterson and MacPherson (1987) and Fisher and MacPherson (1986) also found that cobalt deficiency led to decreased % kill of *C. albicans* in calves and lambs, and that this was only gradually restored on repletion (Paterson & MacPherson 1987). Fisher (1988) suggested that this was a direct effect of vitamin B_{12} deficiency as sub-clinical deficiency also affected % kill. Indeed the decreased neutrophil function was observed in these experiments prior to the

elevation in MMA concentrations, suggesting that it is a direct result of deficiency, not simply a result of the decreased appetite. Kaplan and Basford (1976) suggested that vitamin B_{12} deficiency in humans led to decreased activity of the hexose monophosphate shunt and this could also be the mode of action of vitamin B_{12} deficiency in ruminants.

Vitamin B_{12} deficiency in humans leads to decreased cell division, particularly of those cells which undergo rapid cell division, such as those of the bone marrow. Ibbotson *et al* (1970) reported decreased white cell counts in cobalt deficient ewes.

Cobalt deficient ewes were found to produce fewer lambs (Fisher & MacPherson 1991) and the lambs were found to have decreased vigour, there was also an increase in the numbers of stillborn lambs and in neo-natal mortality (Fisher and MacPherson 1986). Serum IgG concentrations and zinc sulphate turbidity as a measure of -globulin concentrations, were decreased in the cobalt-deficient lambs (Fisher & MacPherson 1991). Deficient lambs also had a decreased response to clostridial vaccination (MacPherson *et al* 1989).

Overall cobalt deficiency appears to decrease lamb resistance to parasites, to decrease % kill by PMN, but not to affect phagocytosis. The decreased killing ability seems to be due to decreased activity of the hexose monophosphate shunt, though how vitamin B_{12} affects this is not clear. Thymidine uptake by lymphocytes is affected, probably due to a defect in thymidine synthetase activity, but this does not seem to affect numbers of lymphocytes (blastogenesis), but does affect their appearance. Finally cobalt deficiency affects disease resistance, from which it can be concluded that supplementation may well be worthwhile financially.

Several suggestions as to the mode of action of vitamin B_{12} in these effects have been put forward. Folic acid has been found to impair cell mediated immunity and to decrease antibody response (Chandra 1991), and so folic acid has been suggested as the mediator; however it has already been noted that some of the effects of vitamin B_{12} on immunity appear prior to symptoms of functional deficiency, also (Kaplan & Basford 1976) that folic acid deficiency did not produce the same effects. Others suggest that the effects may be mediated via the action of the key anabolic enzymes, on protein and cell membrane biosynthesis in rapidly differentiating, and proliferating cells such as those in lymphoid tissue. Thus further investigation is required into other areas of the immune system, where cobalt might have other effects, and to clarify exactly how those effects that have been observed, are mediated.

1.1.8. Supplementation.

Supplementation can be used to prevent or cure cobalt deficiency. Many different methods have been used :-

a. cobalt top dressing of pastures.
b. oral dosing of the animal with cobalt.
c. injection of cobalt.
d. injection of vitamin B_{12}.
e. oral dosing with vitamin B_{12}.
f. cobalt bullets.
g. cobalt salt licks.
h. cobalt containing anthelmintics.
i. incorporation of cobalt in the ration.
j. incorporation of cobalt in piped water supplies.

The most efficient methods have been cobalt top dressing of pastures or the use of cobalt bullets (Whitelaw and Russel 1979). Both are long term treatments although some problems have occurred with rejection of the slow release cobalt bullet in young pre-ruminants and in dairy cows which are fed highly concentrated feeds.

Oral dosing with cobalt requires frequent handling, preferably once a week, although very large doses given monthly do provide some benefit (Stewart *et al* 1955). Incorporation in the anthelmintic is more practical but frequently the concentration used has not been large enough to act as the sole source of cobalt, although as Suttle *et al* (1990) point out, even a small amount of cobalt will provide some benefit and the efficacy of the anthelmintic for its primary purpose must remain the major concern. Salt licks provide adequate cobalt if they are consumed regularly, while inclusion of cobalt in the concentrate ration or water supply is only suitable if it can be incorporated into the existing routine.

Oral dosing with vitamin B_{12} is wasteful, because of the poor absorption of the vitamin, however injections work well, but are fairly expensive. Cobalt injections by-pass the rumen, hence incorporation into vitamin B_{12} does not occur, and the cobalt *per se* cannot be utilised by the animal.

Cobalt toxicity is unlikely to cause problems as sheep can withstand concentrations up to 3.5 mg/kg and cattle can withstand concentrations up to 1mg per kg body weight per day (McDonald *et al* 1988). Levels in excess of these are very rare but do cause anaemia and death.

1.2.VITAMIN E

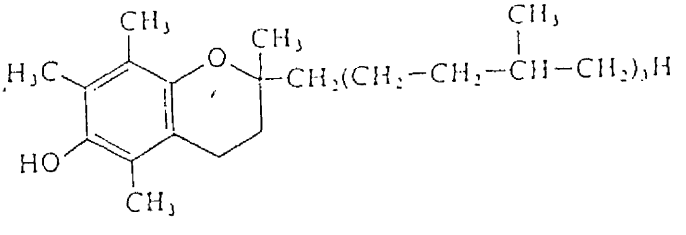
1.2.1. Introduction.

The original discovery of vitamin E began when certain milk-based diets were found to induce sterility in rats, and supplementation with wheat germ oil was found to restore reproductive ability. The unknown factor required was termed 'vitamin E'. A family of tocopherols and tocotrienols have since been identified of which the most biologically active form is α-tocopherol, but the term 'vitamin E' is still commonly used to designate the whole family of tocopherols and tocotrienols, or in place of α-tocopherol.

Since the discovery and purification of the different forms of vitamin E, many other symptoms of deficiency have been observed, involving changes in the nervous system, the muscles, the liver, the alimentary tract, and the fatty deposits, in addition to the reproductive system, suggesting a great diversity of functions for the vitamin. In conjunction with the selenium containing enzyme glutathione peroxidase, vitamin E is known to prevent the oxidative breakdown of tissue membranes which results from hydroperoxidation of polyunsaturated fatty acids.

1.2.2. Functions of Vitamin E.

The function of vitamin E is closely related to its structure :-



α-Tocopherol

The α structure (given here) is thought to be the main biologically active form, because it can sit in cell membranes. Some evidence (Chatterton *et al* 1961 & Chow 1985) suggests that the gamma form is also absorbed in calves and that it is absorbed and taken up by the tissues as effectively as the α form; however others (Putnam and Comben 1987) suggest that only the α form is absorbed, transported and utilised, and (Rice & McMurray 1982) that only α tocopherol has been found in the tissues. Vitamin E acts as a free radical scavenger as it donates a phenolic hydrogen to the initial free radical (or lipid peroxide) thus preventing a chain reaction (lipid peroxidation). Vitamin E is possibly reconverted by the action of ascorbic acid, or glutathione (Chow 1985), which cannot themselves act directly on the lipid because they are not lipid soluble and so cannot enter the membrane. Thus vitamin E exerts its free radical scavenging role inside the membrane, stabilizing it.

Vitamin E deficiency exacerbates exercise-induced damage to skeletal muscle, and this is accompanied by an increase in the formation of oxygen-centered free radicals (Jackson 1987), however it is unclear whether the radicals are responsible for the tissue damage, or whether damage to tissues results in free radical formation. Phoenix *et al* (1990) reported that vitamin E-deficient muscles had an increased susceptibility to intracellular calcium overload, but that addition of a calcium ionophore did not seem to have a direct effect on non-enzymic lipid peroxidation. They hypothesise that vitamin E may have a structural-membrane-stabilizing role in preventing skeletal muscle damage. These effects are thought to be mediated via the hydrocarbon phytyl chain, and to be unrelated to antioxidant ability. In particular vitamin E is thought to protect the mitochondria from damage by increased calcium ion concentrations (Phoenix and Guidoux 1991).

Vitamin E is also involved in arachidonic acid metabolism to thromboxane, leukotrienes, prostaglandins and prostacyclin. Many people have speculated that vitamin E may affect immunity by decreasing prostaglandin concentrations.

Increased PUFA concentrations might stimulate prostaglandin synthesis or they may directly affect adenyl cyclase. Vitamin E antagonises arachidonic acid peroxidation and hence limits formation of the precursors of prostaglandins. Anderson and Menzel (1975) found that intraperitoneal injection of vitamin E not only inhibited conversion of arachidonic acid to prostaglandins, but also directly inhibited the action of prostaglandin E_2.

In weanling rabbits fed a vitamin E deficient diet for 4 to 5 weeks, decreased concentrations of prostaglandin cyclooxygenase, which generates prostaglandins E_2 and $F_{2\alpha}$ in equal quantities, were found in semitendinosus muscle when compared to semitendinosus muscle from rabbits supplemented with 50 mg DL-α-tocopherol twice weekly (Chan *et al* 1980a). Vitamin E deficiency also increased prostaglandin dehydrogenase activity in skeletal muscle of rabbits, but not in heart and kidney (Chan *et al* 1980b). Overall this would give a low concentration of prostaglandins in muscles. This may, however, be a local effect as other workers have found decreased concentrations of prostaglandins in serum during vitamin E supplementation. Vitamin E reversed the adverse effects of adding heated fat to diets (Giani *et al* 1986), these included altering the balance of platelet thromboxane and arterial prostacyclin production.

Prostaglandins have been measured by radioimmunoassay, gas chromatography and high performance liquid chromatography. Granstrom and Kumlin (1987), however, state that due to the short half lives of prostaglandins in blood and tissues, particularly if they are handled, then measurement of certain metabolites of prostaglandins, which can be found in the urine, may be more reliable.

Other roles for vitamin E (Putnam and Comben 1987) are in DNA synthesis, because of observed increases in the concentrations of xanthine oxidase and creatine kinase during deficiency, and in protein synthesis (Chow 1985).

1.2.3. The Relationship between Vitamin E and Selenium.

A. Selenium

Selenium was first noted for its toxic effects on animals. This led to analysis of selenium concentrations in soils, feedstuffs and animal tissues. Later it was found to be an essential part of the diet and several diseases in selenium-deficient animals which respond to selenium supplementation were identified including liver necrosis, exudative diathesis and muscular dystrophy. The relationship between vitamin E and selenium was discovered due to their sparing action on each other in some diseases. eg. mulberry heart disease, white muscle disease and exudative diathesis. This led to the discovery of the role of selenium in free radical metabolism, and also to the discovery of the selenoprotein glutathione peroxidase (GSH-Px). More recently came the theory that supplementation with selenium slightly above the normal recomended concentration may stimulate the immune system (Spallholz *et al* 1973a, 1973b, 1974, 1975, Swecker *et al* 1987/88).

B. Functions of Selenium.

Selenium is chemically similar to sulphur, and is sometimes incorporated into proteins in its place. One particular protein, which contains selenium, is GSH-Px, this contains stoichiometric amounts (4g atoms /mole) of selenium, and catalyses the reaction:-

ROOH	2GSH	GSH-Px	ROH	GSSG
+		---------->	+	
peroxide	reduced glutathione		alcohol	oxidised glutathione

To regenerate reduced glutathione, NADPH, from the pentose phosphate pathway, (or possibly ascorbic acid) is required. The importance of this reaction is twofold, by reducing hydrogen peroxide to water GSH-Px prevents the possible formation of hydroxyl radicals (.OH), which can attack lipids, proteins and DNA. Secondly GSH-Px can act directly on lipid peroxides, preventing a chain reaction. The chain reaction that occurs in lipid peroxidation involves rearrangement of the double bonds which can seriously affect the properties of membranes, and may lead to cell lysis. In addition to 'classical' cytosolic GSH-Px two other forms of the enzyme have been discovered, phospholipid hydroperoxide glutathione peroxidase (PGSHPx) which is associated with cell membranes, and plasma glutathione peroxidase (Arthur 1992).

A second compound selenoprotein P has been found and its concentration correlates very well with some of the effects of selenium which are not due to GSH-Px (Burk 1989). Selenium is not thought to be involved in oxidative phosphorylation directly, but it may be involved in oxidation-reduction reactions. According to evidence cited by Underwood (1981) selenium may also be involved in fatty acid, glucose, and sulphydryl compound metabolism, and the oxidative reactions of the citric acid

cycle. Because of its tendency to complex with heavy metals selenium also protects against the toxic effects of cadmium and mercury.

A third selenoenzyme is iodothyronine 5'-deiodinase type I (IDI), which is involved in the conversion of thyroxine (T_4) to triiodothyronine (T_3) in the liver, kidney and thyroid (Arthur 1992). T_3 is required for growth hormone synthesis in the pituitary gland, and control of thyrotrophin production (Arthur *et al* 1988). This could lead to impaired growth in selenium-deficient animals. Selenium deficiency also affects the thyroid gland, possibly because the lack of GSH-Px allows a build up of hydrogen peroxide, which is rate limiting for the oxidative step during thyroid hormone synthesis and hence causes excess thyroid hormone synthesis and release leading to thyroidal depletion of both iodine and hormone. Alternatively it may be because of its effects on IDI, or because of the changes in T_3 : T_4 ratio's that result from this affecting circulating concentrations of thyrotropin; whichever mechanism is involved selenium deficiency exacerbates hypothyroidism. Additionally selenium plays an important role in non-shivering thermogenesis as T_3 is required for production of the uncoupling protein in brown adipose tissue (Arthur 1991).

C. Interrelationship.

The relationship between vitamin E and selenium was established because certain deficiency diseases could be cured by administration of either nutrient; they appear to have a sparing effect on each other. Further investigation into their functions revealed their roles in neutralising free radicals and preventing lipid peroxidation. They are not mutually replacable however, mainly because they act in different parts of the cell. Vitamin E being situated in the membranes while the selenium containing enzymes GSH-Px and PGSHPx are found in the cytosol, and membranes of cells, respectively.

Polyunsaturated fatty acids (PUFA) concentrations dictate the fluidity and hence the permeability of membranes, and this function requires the prescence of double bonds. The synthesis of prostanoids from arachidonic acid requires a certain controlled amount of lipid peroxidation, which is catalysed by enzymes eg. lipoxygenase. These enzymes must be prevented from peroxidising other lipids. Stray prostanoid synthesis would affect the balance of prostaglandins, prostacyclins and thromboxane, upsetting one of the control mechanisms of the body. Thus lipid peroxidation is an essential part of normal cell function, but unwanted side reactions must be prevented to stop cell damage (Rice and Kennedy 1988).

Free radicals such as superoxide and hydroxyl radicals can also cause lipid peroxidation, these are formed intentionally in phagocytes during the respiratory burst to kill pathogens; however they must be prevented from damaging the host cells.

1.2.4. Factors Affecting Vitamin E Requirement.

Mixtures of tocols are found in plants, and the concentrations tend to vary in different parts of the plant i.e. seeds (particularly in the germ) have more than the root. Sun curing decreases available tocols considerably, and preservatives such as propionic acid destroy α tocopherol. Addition of high concentrations of minerals such as copper and iron to feedstuffs leads to increased breakdown of vitamin E (Dove & Ewan 1990).

Different forms of vitamin E are not interconverted and neither is there any synthesis in the rumen or gut. Thus all animals require an adequate supply in the diet. If lipid absorption is upset i.e. by lack of bile salts, this will affect vitamin E, as this is absorbed along with fat, transported in high density lipoproteins (Chow 1985) and

stored in the fat reserves.

Vitamin E and fats diffuse through the intestinal mucosa and are combined with proteins (giving lipoproteins) which are transported by the lymphatic system (as chylomicrons), into the general circulation (McDonald *et al* 1988). Vitamin E is found in all lipoprotein fractions, but mainly in apo-B containing lipoproteins (Bjorneboe *et al* 1990). It is frequently secreted from the liver in very low density lipoproteins (VLDL).

In chicks, feeds contaminated with *Fusarium*, which produces the T-2 toxin, caused decreased plasma vitamin E concentrations. If micelle promoting substances are added to the diet, the problem is alleviated. Coffin and Combs (1981) considered the possibility of general lipid malabsorption, but found that duodenal lipase was not affected and no excess lipid appeared in the faeces, suggesting that general lipid digestion was not impaired. They suggested that the T-2 toxin may act on the liver reducing the output of plasma lipoproteins.

Because of their sparing action on each other, requirements for vitamin E and selenium are related. The requirement for vitamin E is further complicated by being associated with PUFA concentrations. If the peroxidisable index of PUFA in the diet is increased from low (linoleic acid free diet) to high (5% corn oil diet) the amount of vitamin E required to prevent 10% haemolysis of erythrocytes increases from 122ug/100ml packed cells to 196ug/100ml (Bieri & Poukka 1970). High concentrations of vitamin A or mycotoxins in the diet also decrease available concentrations of vitamin E, thus increasing the requirement.

For ruminants Putnam and Comben (1987) recommend 1mg/day/kg liveweight, plus 5mg/kg milk produced, plus 3mg/g PUFA in the ration, of vitamin E. In calves 2.4-3.4 mg/day/kg liveweight produced optimum live weight gain (Reddy *et al* 1987a), but the amount of PUFA in the diet was not specified.

If vitamin E deficiency is suspected, confirmation may be obtained by analysis of diagnostic parameters.

Normal concentrations of vitamin E are as follows:-

	Serum (μmol/l)	Liver (μmol/kg)
Sheep	1-6	5-45
Cattle	3-18	20-100
Goats		>10

To measure the vitamin E status, hydrogen peroxide used to be added to a blood sample, and the time taken for haemolysis to occur was related to the amount of the vitamin present (Chow 1985); more recently vitamin E has been measured directly by HPLC.

A. Factors Affecting Selenium Requirement.

The concentration of selenium in the soil affects the amount which can be taken up by plants, and the amount taken up by plants in turn affects the selenium intake of grazing animals. Some plants are better at extracting and concentrating it than others, which can be a problem in selenium rich areas, particularly if they convert it to an easily available form. This can lead to selenium toxicity (selenosis, blind staggers or alkali disease). This results in emaciation, lack of vitality, hair loss, soreness and sloughing off of hooves, stiff joints, cirrhosis of the liver, anaemia and

atrophy of the heart.This causes the animal to be unwilling to move, and so it may die of thirst or starvation. Growth rate and reproductive function are the first things to be impaired and deformed young may be born. The selenium is incorporated into the tissues in place of sulphur. High concentrations of dietary protein tend to decrease toxicity, possibly because of the sulphur containing amino acids. Thus when considering supplementing the diet with selenium great care must be taken that excess is not given.

Fishmeal tends to be a good source of selenium but most other protein supplements vary a great deal. Some forms of selenium are more easily utilized than others, there is also evidence that other compounds may interfere with absorption or metabolism of selenium. On comparison of fauna-free with faunated sheep, protozoa were found to decrease the bioavailability of selenium (Dayrell *et al* 1991).

Selenite is thought to be absorbed by diffusion, but it may be reduced in the lumen of the gut prior to absorption. Thiols (e.g. cysteine, cysteamine and mercaptopyruvate) have been found to increase the mucosal uptake of selenite (Scarrer *et al* 1989). L-leucine and L-glutamic acid inhibit the stimulatory effects of L-cysteine on selenite absorption, suggesting the formation of selenoamino acids (L-selenodicysteine and L-cystine selenopersulfide) which could be transported via the amino acid carriers of the brush border.

Selenate is absorbed faster than selenite, it is actively transported across the brush-border membrane against the concentration gradient by two mechanisms, sodium^{+}-selenate co-transport, and selenate transport by anion exchange. This is inhibited by other anions with two charges. Selenocysteine and selenomethionine are absorbed by the same carriers as cysteine and methionine (Wolffram *et al* 1989). Selenomethionine and methionine have the same affinity for neutral amino acid

carriers, whereas selenocysteine is a competitive inhibitor of cysteine transport.

Superphosphate fertilisers appear to cause an increased incidence of nutritional muscular dystrophy in lambs. Hudman *et al* (1988) infused soluble phosphate (6g/day) as sodium phosphate or sodium chloride intraruminaly into weaner sheep, and found that exess phosphate led to lower concentrations of selenium in the liver. Selenium concentrations in the blood, kidneys, heart and muscle were unaffected. Absorption of selenium from the gut was slower, suggesting a phosphate/selenate interaction in the gut. This is supported by the increased concentrations of selenium in the faeces and decreased concentrations in the urine of sheep fed the sodium phosphate. Selenium also had a faster turnover in phosphate fed sheep suggesting the possibility of a second interaction after absorption.

Lupins contain a mycotoxin, *Phomopsin leptostremiformis*, which causes myopathy, indistinguishable from nutritional muscular dystrophy, in sheep in Australia. However this myopathy does not respond to selenium supplementation, with or without vitamin E supplementation. Costa *et al* (1986) found that phomopsin disrupts microtubule assembly in the liver and hence affects hepatic processing of seleno compounds. Seleno compounds absorbed from the gut become more available to extrahepatic tissues, and in particular to the kidney, and so excretion of selenium in the urine increases. The mycotoxin appears to alter the type or concentration of seleno compounds in the plasma and hence the necessary metabolites for normal physiological functions are not available. A recent study however (Beetson *et al* 1993) has shown that an intramuscular injection of selenomethionine combined with an oral dose of α-tocopherol afforded significant protection for young sheep against the developement of myopathy.

High grain diets may decrease the availability of selenium, by lowering rumen pH and creating reducing conditions (Van Saun 1989), this increases the dietary requirement of dairy cows for selenium. Tissue selenium is linearly related to dietary selenium (Ku *et al* 1972), and for diets supplying 400-3100ug Se/day to non-lactating dairy cows, 51% of Se was absorbed and 41% retained (Harrison and Conrad 1984a).

If the calcium concentration of the diet varies much from 0.8%DM intake, apparent selenium absorption from natural feedstuffs is decreased in non-lactating cows (Harrison & Conrad 1984b). In calves dietary calcium leads to a small decrease in apparent selenium absorption at very low and very high concentration, but this was of little practical importance in selenium metabolism (Alfaro *et al* 1987).

Various values for selenium requirement are quoted (Underwood 1981), ranging between 0.03 and 0.12 mgSe/kgDM for sheep. The variation is thought to be due to different concentrations of vitamin E, sulphur, iron and copper (Van Saun 1989) in the diet, and also to different criteria being used to assess changes in function with selenium status, such as myopathy, or growth. Additionally different safety margins may be applied before quoting a recommendation. A general recommendation of 0.1-0.12 mgSe/kgDM for sheep and cattle is quoted (Underwood 1981). Van Saun (1989) stresses that due to the potential toxicity and expense selenium supplementation should not be general; diagnostic concentrations for various parameters are quoted and supplementation is only economic if deficiency or marginal deficiency states are present. Goats are thought to require more selenium than lambs or calves (Ramell *et al* 1989).

Diagnostic parameters used to define selenium deficiency are:-

Liver selenium concentration (nmoles/kg dry weight of tissue)

	Deficiency	Marginal	Adequate
Sheep	<200	200-450	>450
Cattle	<200	200-450	>450
Goats	<500	500-1100	>1100

More commonly because blood samples are easy to take whole blood GSH-Px activity (u/ml at 30°C) are used, (using the enzyme colourimetric method described in Chapter 2, based on Paglia and Valentine 1967 and Anderson *et al* 1978, in use at VIC, SAC Auchnicruive.)

	Deficient	Marginal	Adequate
Sheep	<27	27-42	>42
Cattle	<15	15-25	>25

Atroshi *et al* (1981) found that within a flock of Finn sheep some animals had a genetically lower whole blood GSH-Px, which they claim is an adaptation to low available selenium concentrations, suggesting that these sheep have a lower requirement for selenium than is normal. These sheep exhibited larger weight gain and wool production and a decreased lamb mortality rate, when compared to the other sheep in the flock, under the same production conditions. However Samson *et al* (1989) reported no such apparent adaptation in Rocky Mountain Bighorn sheep.

1.2.5. Deficiency diseases.

Many of the deficiency diseases associated with selenium and vitamin E are the same, but not all respond to either substance on its own. Symptoms vary between species, but most are caused by peroxidation, which attacks highly oxygenated tissues eg. muscles, liver, lungs, cerebellum, kidney, thyroid, adrenals and pancreas.

Deficiency diseases associated with ruminants are :-

A. Nutritional myopathy or white muscle disease.

Sometimes animals are found to be deficient in vitamin E and/or selenium, and yet show no symptoms of disease particularly if housed (Arthur 1988). On turnout symptoms frequently appear which may indicate that 'stress' is required to initiate the clinical syndrome. Alternatively it may be due to increased concentrations of PUFA in the spring grass leading to an increased requirement for antioxidants such as vitamin E and glutathione peroxidase, or as Rice and Kennedy (1988) suggest it may be a 'conditioned deficiency' due to a diarrhoea inducing agent in young spring grass which leads to decreased vitamin E absorption. They speculate that this agent is protein in origin. Further evidence for some other factor being involved is that housed animals fed grass also demonstrated increased PUFA concentrations, but did not have elevated CK concentrations (Arthur 1988).

Nutritional myopathy is characterised by degeneration of muscles, whole groups are usually affected e.g.locomotor, thoracic, muscles of the upper digestive tract, or the myocardium (Bostedt 1980). If the heart is affected sudden death may occur, alternatively respiration and circulation may be impaired. Damage to skeletal muscles results in stiff unnatural postures (McDonald *et al* 1988). Muscle damage

leads to increased serum concentrations of aspartate aminotransferase (SGOT)(Blincoe & Dye 1958), creatine kinase (CK), and lactic dehydrogenase (LDH). Treatment with either vitamin E or selenium appears to be effective (McMurray & McEldowney 1977). The incidence of nutritional myopathy increases markedly when only farm produced feeds are fed (Bradley *et al* 1986), as these are frequently stored for long periods and so contain little vitamin E, and in certain areas of the country they will also be deficient in selenium. If concentrates are bought in separately they are frequently supplemented with vitamin E and selenium by the manufacturers.

Experimental myopathy due to vitamin E deficiency has the same symptoms as nutritional myopathy (Safford *et al* 1954) but the symptoms do not always develop. Protected linseed added to the diet (Kennedy *et al* 1987) exacerbates the condition emphasising the symptoms. It might be thought that any unsaturated fat would be hydrogenated in the rumen, however evidence (Rice & McMurray 1982) shows that this is not 100% efective, and even in fully ruminant calves on turnout to pasture there may be a 20 fold increase in plasma linolenic acid. For the same reason cod liver oil neutralised the beneficial effect of vitamin E on experimental myopathy (Blaxter *et al* 1952).

Paulson *et al* (1966) found increases in all 5 forms of serum LDH,with the percentage increase being highest in V then IV then III then II and least in I, although in absolute terms the increase in III was greatest. Vitamin E alone or with selenium restored concentrations but selenium alone only decreased concentrations temporarily. There is a suggestion that decreased ubiquinone concentrations are found in the tissues during nutritional myopathy, however Poukka (1968) failed to detect any significant differences in ubiquinone concentration between healthy and diseased calves.

Peroxide haemolysis of red cells increased in lambs fed maize oil, but not those fed cod liver oil, although signs of nutritional myopathy were apparent in cod liver oil but not maize oil fed lambs. α-tocopherol prevented nutritional myopathy but not peroxide haemolysis, therefore red cell fragility was not due to vitamin E deficiency but to the fatty acid composition of the unsaturated lipids in the diet (Boyd 1968). Addition of vitamin E to the erythrocytes *in vitro* was sufficient to prevent haemolysis. Supplemental vitamin E decreased red cell haemolysis in dairy cows fed a low vitamin E diet (Schingothe *et al* 1979). Calves fed maize oil developed nutritional myopathy if not supplemented with vitamin E (Poukka & Oksanen 1972), vitamin E inhibited desaturation but not chain elongation enzymes in the microsomes of liver and kidney, but had no apparent effect on the erythrocyte haemolysis test. The maize oil fed lambs (Boyd 1968) received 33.5 IU of vitamin E/kg, while the calves (Poukka and Oksanen 1972) were fed stripped maize oil. While this may have contained large amounts of residual vitamin E, (residual concentrations of vitamin E were not quoted) the chemical methods used for the removal of vitamin E frequently result in the formation of peroxides, which may explain why the calves developed nutritional myopathy but the lambs did not.

B. Unthriftiness.

This condition, found in Australia and New Zealand, leads to weight loss and mortality, but no muscle lesions are found and there is no increase in SGOT concentrations. Supplementation with selenium may lead to striking weight gains and decreased mortality (Underwood 1981). Subclinical cases probably result in large financial losses.

C. Reproductive disorders.

Selenium and vitamin E deficiencies lead to impaired reproductive performance. In ewes selenium deficiency causes high embryonic mortality, which is responsive only to selenium. The deficiency is associated with decreased fertility, and increased death rate of young lambs (Zachara *et al* 1989). In cows a mixture of vitamin E and selenium proved more effective than selenium alone in preventing premature births, weak and dead calves and retained placentae (cited by Underwood 1981). MacPherson *et al* (1987b) found that selenium supplementation significantly decreased the number of days between first insemination and calving, due to improved first and second service conception rates. Vitamin E and selenium interact in their effects on the number of retained placentae (Harrison *et al* 1984), however selenium alone decreased the incidence of metritis and cystic ovaries.

D. Weak Calf Syndrome.

Rice *et al* (1986) reported that a single dose of selenium prior to calving was found to have no effect on the incidence of weak calf syndrome. Sluijter *et al* (1990) hypothesise that one cause of weak calf syndrome might be selenium deficiency altering prostaglandin metabolism leading to premature placental separation.

During selenium deficiency various other parameters alter (Arthur *et al* 1988) eg. plasma urea and creatinine concentrations increase, plasma alkaline phosphatase activity decreases and the balance of the thyroid hormones is upset, thyroxine concentrations increase and concentrations of the more metabolically active triiodothyronine decrease. This leads to a decreased overall activity of the thyroid hormones, promoting anabolism and stimulating growth hormone synthesis in the pituitary, thus the imbalance may be responsible for some of the symptoms of deficiency.

1.2.6. Evidence that Vitamin E and Selenium Enhance Immunity.

Megadoses of vitamin E are thought to affect immunity, these may be 3-6 times the accepted requirement for the animal (Beisel 1982). Vitamin E exibits a general antioxidant effect, preventing peroxidation and free radical damage to sensitive lymphoreticular cells. It also specifically decreases prostaglandin synthesis and inhibits oxidation of arachidonic acid by its effects on key enzymes of oxidative phosphorylation, coenzyme Q and cytochrome synthesis, it alters receptor function of cell membranes of lymphocytes (Tengerdy 1986). Lower prostaglandin concentrations would in turn lead to a decrease in cAMP concentrations. High cAMP concentrations are known to suppress mast cell degranulation and mitogenic transformation. Prostaglandins are one of the possible mediators of vitamin E's action on the immune system as they have been found to increase during vitamin E deficiency and are known to modulate the immune system. Prostaglandins inhibit lymphocyte blastogenesis and via cyclic AMP they inhibit E-rosette formation, macrophage-migration inhibition and lymphocyte-mediated cytotoxicity (cited in Langweiler *et al* 1981).

A. Humoral Immune Response (HIR).

Increased antibody production in response to stimulation by both mitogens (primary immune response) and antigens (secondary immune response) has been recorded during vitamin E supplementation. Supplementation with vitamin E has frequently been found to increase antibody concentrations compared to those in vitamin E deficient animals. Addition of 47 or 200 mg/kg vitamin E to the diet of two different strains of rats enhanced T-cell mitogen responses to concanavalin A and phytohemagglutinin-P, and B-cell responses to lipopolysaccharide, compared to rats fed a vitamin E deficient semi-purified diet which resulted in undetectable plasma concentrations of vitamin E, (Bendich *et al* 1983). Marsh *et al* (1981) found that diets deficient in vitamin E or selenium (<0.02 mgSe/kg) caused impaired antibody response to ovine erythrocytes in two week old chicks, compared to chicks supplemented with selenium (0.1 mgSe/kg) and vitamin E (100IU/kg), while three week old chicks receiving either vitamin E or selenium supplementation maintained antibody responses.

In pigs Ellis and Vorheis (1976) found supplementary vitamin E (100,000 IU/ton of feed) increased anti-*E. coli* serum antibody titres threefold compared to unsupplemented pigs. Horses supplemented with vitamin E (600mg/day), or vitamin E (600 mg/day) and selenium (5 mg/day) had increased antibody titres after vaccination with novel antigens such as tetanus toxoid and equine influenza virus, but not to *E. coli*, compared to unsupplemented horses or horses given selenium alone (Baalsrud and 0vernes 1986).

In sheep (Tengerdy *et al* 1983) supplementation with 300 mg [dl]-α-tocopheryl acetate increased antibody production in response to *Clostridium perfringens* type C or D toxoids. Cipriano *et al* (1982) found large but nonsignificant increases in serum immunoglobulin (IgG_1, IgG_2 and IgA) concentrations between calves fed a vitamin E

deficient diet and calves supplemented with 1g vitamin E/day. Reddy *et al* (1986) found that supplementation of calves with 2800 mg vitamin E/week enhanced IgM concentrations but not IgG concentrations compared to unsupplemented calves. While Reddy *et al* (1987b) found that supplementation of calves with 125 IU vitamin E/day enhanced antibovine herpes-virus type 1 antibody production in response to a booster vaccination compared to unsupplemented calves, increased supplementation with 250 or 500 IU vitamin E/day did not significantly enhance the response. However Anderson *et al* (1986) found no increase in antibody titre to infectious bovine rhinotracheitis vaccine, tetanus toxoid or Johnes disease (*Mycobacterium paratuberculii*) in calves supplemented with 60 or 200 mg vitamin E/kg feed when compared to unsupplemented calves on a low vitamin E diet despite increasing plasma vitamin E concentrations from $<1\mu$g/ml to >1.5 and >3 μg/ml respectively.

Supplementation with excess vitamin E, compared to normal levels, has also been found to further stimulate antibody production. Guinea pigs supplemented with 33 IU DL-α-tocopherol/kg body weight by intramuscular injection, had significantly higher hemagglutination-inhibition antibody titres to Venezuelan equine encephalomyelitis virus than unsupplemented animals fed the same commercial ration containing 62 IU dl-α-tocopherol acetate/kg (Barber *et al* 1977). Likoff *et al* (1981) found that in chickens supplemented with 300 mg vitamin E/kg diet (six times current recommendations), antibody titres to *E.coli* lipopolysaccharide were enhanced compared to nonsupplemented chickens on the same diet containing 40 to 50 IU vitamin E/kg. Franchini *et al* (1987) found enhanced serum IgG and IgM responses in chickens supplemented with 300 mg vitamin E/kg feed immunized with emulsified and inactivated *Newcastle disease virus* and *Pasteurella anatipestifer*, compared to unsupplemented birds receiving only 20 mg vitamin E/kg feed from the diet.

Ritacco *et al* (1986) found that lambs fed 476 mg all-rac-α-tocopherol acetate/kg feed had a significantly enhanced peak primary serum antibody titre compared to lambs fed 33 mg all-rac-α-tocopherol acetate/kg feed, while lambs fed 3000 mg vitamin E, in three treatments given 3 days apart, produced greater secondary peak anti-*B. ovis* titres than unsupplemented controls. However no increase in antibody titre to SRBC was found in piglets supplemented with 550, 220, or 110 IU vitamin E/kg of feed compared to piglets receiving only 11 IU vitamin E/kg of feed (Bonnette *et al* 1990a & b), and cortisol concentrations were also unaffected by supplementation.

The number of plaque forming (ie. antibody producing) cells, and the concentrations of circulating antibody are increased. To determine whether the primary or secondary immune response is affected different substances are used. A mitogen to which the animal has not previously been exposed produces a primary immune response ie. initially IgM, whereas an antigen which has been met previously stimulates both IgM and IgG due to immunological memory (this is called a secondary immune response). To separate the immunoglobulins 2-mercaptoethanol is used to remove IgM. Vitamin E was found to stimulate the production of both IgM and IgG but it also favoured early conversion to IgG type production.

Attempts have been made to understand the mechanisms of action of vitamin E. Tengerdy (1980) suggested that the antioxidant action might create reducing conditions favourable for immunopoietic cellular developement and proliferation. Experiments comparing the action of vitamin E, versus antioxidants, suggest that this is so, but that vitamin E also has other effects. On separation of spleen into adherent (macrophage like cells) and non-adherent (lymphocyte like cells) Campbell *et al* (1974) found that vitamin E either bypassed the requirement for macrophage stimulation or enhanced it to such an extent that the few remaining cells were

sufficient to stimulate the lymphocytes, they suggest a role for vitamin E in cell cooperation. Corwin and Shloss (1980) support this by suggesting that vitamin E affects the HIR by enhancing T_h cell action and bypassing macrophage cooperation. In mice fed vitamin E deficient diets, macrophages were less able to present antigen to nonadherent cells than macrophages from vitamin E supplemented mice but they could act as suppressor cells instead (Gebremichael *et al* 1984).

Another possible effect of vitamin E, via its antioxidant action is the protection of ubiquinones, (or the stimulation of their synthesis). Ubiquinones are thought to stimulate the reticuloendothelial system leading to increased spleen weight due to increased lymphoproliferation and increased antibody production.

There is little doubt that selenium supplementation can also increase antibody synthesis. Spallholz *et al* (1973a, 1973b) found that supplementation of mice diets with selenium (0.7 or 2.8 mgSe/kg) increased antibody titres to SRBC 7-fold and 30-fold respectively compared to unsupplemented controls, but that unlike vitamin E supplementation, IgM synthesis was promoted and not IgG production; supplementation with 30 mgSe/kg depressed antibody production. Supplementation of mice with selenite (0,3,5,10,20, 60 or 100 μg), alone or with vitamin E (0, 0.15, 0.25, 0.5, 1,3 or 5 mg) stimulated anti-SRBC IgM and IgG, and differences in antibody titres were dependent upon the amount of selenium, and/or vitamin E administered (Spallholz *et al* 1974). Selenium injected prior to or simultaneosly with the SRBC antigen also enhanced antibody titres in response to SRBC, but not selenium injected 2 or 3 days later (Spallholz *et al* 1975). Marsh *et al* (1981) depleted chicks of vitamin E and/or selenium and found that the humoral response to SRBC was decreased in 2 week old chicks by a deficiency of either selenium or vitamin E but that in 3 week old chicks either vitamin E or selenium alone could prevent any adverse affects.

Sheffy and Shultz (1978) observed depression of antibody synthesis in response to vaccination in vitamin E/selenium-deficient dogs. In ponies (Knight & Tyznik 1990) on a low selenium diet (0.02 mg/kg) addition of supplemental selenium (0.22 mg/kg) enhanced IgG concentrations in response to SRBC.

Jelinek *et al* (1988) found supplementation of selenium deficient sheep with an intraruminal selenium pellet increased antibody titres to killed *Brucella abortus* cells and rabbit red blood cells, but not to *Corynebacterium pseudotuberculosis* toxoid. Selenium supplementation (1 mg Se/kg as sodium selenite) also enhanced antibody response to tetanus toxoid in sheep on a basal diet containing 0.13 mgSe/kg (Larsen *et al* 1988b, Moksnes et.al 1988). Selenium supplementation (0.2 mg/kg diet) enhanced antibody response to infectious bovine rhinotracheitis virus compared to unsupplemented calves (0.03 mg Se/kg diet) (Reffett *et al* 1988). In weaned beef calves with an average blood selenium of 67.3μg/l, free access to salt mineral licks containing 80, 120, 160 and 200 mg selenium/kg enhanced serum antibody titres to lysozyme compared to access to a lick containing only 20 mg selenium /kg (Swecker *et al* 1987/8). However selenium supplementation of marginally selenium-deficient lambs produced only a very slightly increased antibody response to *S. dublin* (Finch & Turner 1986 & Finch *et al* 1986) even when very large doses of selenium were given. Also increased IgG concentrations to sheep red blood cells were recorded (Knight & Tyznik 1990) with no effect on IgM, whereas Stabel *et al* (1989) found increased IgM concentrations following stress but decreased anti *P. hemolytica* titres.

Excess selenium decreased antibody production in the experiment by Marsh *et al* (1981), while Swecker *et al* (1987/8) found that concentrations of selenium optimal for antibody production were higher than those required to prevent nutritional myopathy. Selenium deficiency had no effect on IgG production in cattle (Stevenson

et al 1991) and they suggest that this is because antibody production has a high priority for selenium. Overall excess selenium does not always provide additional benefits so bearing in mind the danger of toxicity care needs to be taken in recommending dietary allowances.

Spallholz *et al* (1975) suggest selenium affects ubiquinones, while Reffet *et al* (1988) suggest it increases efficiency of antigen processing in the cell via T_h cells. These actions, and possibly an effect on prostaglandins are thought to be mediated via GSH-Px. Thus selenium and vitamin E affect the humoral immune system in different ways, as according to Larsen and Tollersrud (1981) and Reffet *et al* (1988), selenium favours IgM production, whereas vitamin E favours a fast shift to IgG production.

Langweiler *et al* (1981) measured antibody response to *Pasteurella haemolytica*. Injections of selenium or selenium and vitamin E increased antibody titre, but vitamin E alone had no effect.

Selenium and vitamin E increased IgM concentrations in sow colostrum (Hayek *et al* 1988), while IgA and IgG concentrations were unaffected. Initially IgM concentration in the piglets were increased by selenium, while either selenium or vitamin E led to elevated IgG concentrations 14 days after birth. Marsh *et al* (1982 & 1986) found deficiencies of either vitamin E or selenium to decrease bursa wieght in chicks, to affect the proportions of large and small lymphocytes, and to decrease the overall number of lymphocytes produced. Thus they think that vitamin E and selenium may exert many of their effects on the immune system by influencing the primary lymphoid organs and decreasing the number of lymphocytes. When given together vitamin E and selenium increased the antibody response of steers to *P.hemolytica* (Droke & Loerch 1989).

B. Cell Mediated Immune Response. (CMIR).

The actions of vitamin E and selenium on prostaglandins (including the coating of lymphocytes and suppression of cytotoxicity and T cell rosette formation, via cAMP) and ubiquinones, also affect the T lymphocytes. However generally the CMIR involves less cell cooperation than the HIR and this effect of vitamin E is not seen.

Vitamin E deficiency has been found to decrease T cell response to mitogens in rats (Bendich *et al* 1983 & 1986, Eskew *et al* 1985 and Gabriel *et al* 1984), guinea pigs (Bendich *et al* 1984), dogs (Sheffy & Shultz 1979), pigs (Bonnette *et al* 1987/88 & Larsen & Tollersrud 1981), and calves (Cipriano *et al* 1982, Reddy *et al* 1986, Reddy *et al* 1987b and Eicher-Pruiett *et al* 1992). Corwin and Shloss (1980) suggested that vitamin E affected T cell maturation. Vitamin E had no effect on mitogen induced stimulation indices in pigs (Bonnette *et al* 1990a), and no effect on cortisol concentrations. Increased concentrations of cortisol decrease PHA and ConA induced proliferation of porcine thymocytes, splenocytes and mononuclear cells *in vitro* (Westly and Kelley 1984).

Megadoses of vitamin E decreased mitogen induced lymphocyte transformations in humans (Prasad 1980), but had no effect on delayed hypersensitivity of skin to PHA compared to unsupplemented humans. In mice optimum stimulation was achieved with 5-20 IU/kg/day of vitamin E, 80 IU was inhibitory (Yasunaga *et al* 1982).

In vivo delayed type hypersensitivity skin tests suggest that selenium also modulates the CMIR. Selenium deficiency has been found to decrease T cell response to mitogens in rats (Eskew *et al* 1985), dogs (Sheffy and Shultz 1978), pigs (Larsen and Tollersrud 1981), lambs (Turner *et al* 1985 & 1986) and sheep, (Larsen *et al* 1988a, Jelinek *et al* 1988 & Moksnes *et al* 1988). Excess selenium however

decreased T cell response to mitogens (Moksnes *et al* 1988 & Larsen *et al* 1988a). Larsen *et al* (1988a) speculated that the effects of selenium on mitogen stimulation were not mediated via GSH-Px as results were not correlated with GSH-Px concentration. Immunological failure, including decreased mitogen responses in lambs, apparently resulted from nutritional myopathy and not simply selenium deficiency (Turner *et al* 1984 & 1986).

Neither vitamin E (150IU/kg) nor selenium (0.5 mg/kg) supplementation were found to affect macrophage function, killing of *S.aureus* or T lymphocyte blastogenesis in rats fed a diet deficient in both selenium and vitamin E (Eskew *et al* 1985).

Selenium was also found to affect soluble mediators, a deficiency in goats, lead to decreased concentrations of leucotriene B4 (Aziz and Klesius 1986), and the cytotoxic action of both T cells and NK cells (cited by Kiremidjian-Schumacher and Stotzky 1987). They suggest that selenium, by increasing the concentrations of GSH, H_2Se and HSe^-, could alter the disulphide bonding on the cell surface making it more sensitive to stimulation.

Sheffy and Shultz (1979) and Langweiler *et al* (1981) working with dogs and Reddy *et al* (1986) using calves, found that during vitamin E deficiency serum factors coat the lymphocytes inhibiting their activity, this inhibition is removed by washing and resuspending in serum from vitamin E-sufficient animals. Serum from supplemented calves inhibited IBR virus replication by week 12, this effect is thought to be mediated by a serum factor e.g. interferon, c-reactive protein, beta-lysins, lysozymes or serum transferrins. However Sheffy & Shultz (1979) and Langweiler *et al* (1981) speculate that prostaglandins may be the serum factor responsible for inhibiting lymphocyte activity during vitamin E deficiency.

C. Non-specific immunity, neutrophil responses.

Much of the work on immunity assesses changes in neutrophil function. Vitamin E deficiency leads to decreased chemotaxis to bacterial culture filtrate and decreased ingestion of IgG and C3b coated and unopsonized albumin coated paraffin oil droplets by rat PMN. Ingestion and killing of C3b coated *S.aureus* was not affected (Harris *et al* 1980). In large doses vitamin E decreased the bactericidal activity of human leucocytes (Prasad 1980). Vitamin E supplementation negated the adverse effects of vitamin C on neutrophil phagocytosis of *Staphylococcus aureus* in calves (Eicher-Pruiett *et al* 1992).

Vitamin E affects neutrophil lysosomal enzyme release and chemotaxis via prostaglandins and cAMP. Supplementation decreases prostaglandin concentrations leading to increased phagocytosis in chickens (Tengerdy and Brown 1977 and Likoff *et al* 1981). This may be due to an effect on membrane recognition of antigen and soluble mediators, or on binding and lysis of the cells. Impaired microtubules, due to membrane damage would alter receptor distribution and decrease chemotaxis. Ingestion of complement C_3 coated particles decreases during vitamin E deficiency; vitamin E maintains normal membrane function for direct movement and ingestion by phagocytic cells (Chow 1985). Vitamin E deficiency increases collagen induced platelet aggregation and serum PGE_2 and $PGF_{2\alpha}$ action via membrane fluidity.

The protection, or stimulation of synthesis, of ubiquinones also leads to enhanced phagocytosis, and ubiquinones may promote the peroxidation of bacterial lipid.

Selenium/vitamin E injections were also found to affect bovine polymorphonucleated leucocytes, increasing the percent kill of *Staphylococcus aureus* (Gyang *et al* 1984). Vitamin E was not given alone, and the controls were selenium-deficient, hence the effect was attributed to selenium.

Selenium deficiency leads to decreased migration and chemotaxis in goats (Aziz *et al* 1984), phagocytosis is not affected, but percent kill of *C. albicans* is decreased in rats, (Serfass and Ganther 1975) mice (Boyne & Arthur 1986) and cattle (Boyne and Arthur 1979 and 1981) and of *S. aureus* in cattle (Gyang *et al* 1984); repletion increases % kill. However selenium deficiency has no effect on % kill of *S. aureus* or *S. typhimurium* by neutrophils from selenium-deficient rats (Boyne *et al* 1985).

After incubation in a H_2O_2 generating system PMN from selenium-deficient rats had a decreased ability to ingest particles (Baker & Cohen 1984) suggesting that selenium may have an effect on phagocytosis when the cell is under severe oxidative stress.

In selenium-deficient goats degranulation was impaired. This coincided with a reduction in leukotriene B_4 production by PMN, which was thought to be responsible for the decreased neutrophil chemotaxis (Aziz & Klesius 1986) but was not thought to be mediated via GSH-Px.

The decreased killing ability of PMN is thought to be due to the decreased oxidative burst observed (Aziz *et al* 1984), the oxidative burst refers to the increased metabolic activity of neutrophils when they are phagocytosing foreign particles, which results in increased oxygen consumption. The neutrophils produce both hydrogen peroxide and superoxide during this phase of their metabolic activity, and these help to destroy ingested particles and microbes respectively. The decreased superoxide production is thought to be responsible for the decrease in reduction of the tetrazolium salt *in vitro* observed in the nitroblue tetrazolium test in neutrophils from selenium deficient steers (Boyne and Arthur 1979). This effect is thought to be mediated via GSH-Px, the decreased enzyme activity is insufficient to prevent

damage to enzyme systems including the hexose monophosphate shunt, this leads to inadequate superoxide production (Arthur *et al* 1981, Grasso *et al* 1987 and Gyang *et al* 1984). Phosphohydrolase activity is also impaired, decreasing hydroxyl ion formation (Boyne and Arthur 1985), and also NADPH oxidase activity (cited by Baker and Cohen 1984) and glucose-6-phosphate dehydrogenase (G6PD) (Serfass and Ganther 1975), which all leads to a decreased killing ability. Baker and Cohen (1984) speculated that bacteria and fungi may be killed by different mechanisms, because the reduction in % kill is species dependent.

Turner *et al* (1985) suggest that selenium acts directly (and not via GSH-Px) on lymphocytes, since factors in the circulation following selenium supplementation, or sodium selenite *in vitro*, had the same stimulatory effect on mononuclear cells.

The effects of selenium on macrophages are less well understood, but deficiency does decrease their candidacidal activity and GSH-Px activity, but not their phagocytic activity (Boyne and Arthur 1985b); however no effect of selenium deficiency on the very low capacity of macrophages to reduce nitroblue tetrazolium was demonstrated.

D. Resistance to disease.

Despite affecting many parameters used to asses immunity, eg. antibody concentrations, the only real test that supplementation will be worthwhile financially is whether it increases the animals resistance to disease.

Vitamin E supplementation (150-300 mg/kg, 3-6 times the normal requirement) decreased mortality in chicks infected with *E. coli* (Tengerdy and Brown 1977, Tengerdy and Nockels 1975 and Heinzerling *et al* 1974). Gill *et al* (1986) found that supplementation of recently transported, stressed cattle with 800 IU vitamin E/lb

feed decreased the number of days illness suffered by stressed cattle, while supplementation of lambs with 2182 IU vitamin E prior to chlamydia inoculation decreased the severity of pneumonia (Stephens *et al* 1979). Supplementation of lactating cows with 0.74g vitamin E/day decreased the incidence of mastitis compared to unsupplemented cows receiving only 0.32g vitamin E/day from the diet (Smith *et al* 1984).

Work cited by Tengerdy (1980) suggests that vitamin E does not protect against *M. tuberculosa*, which is controlled by the CMIR. However he suggests that host parasite interactions may still be affected, but by mechanisms other than the CMIR. Droke and Loerch (1989) found no effect of a single vitamin E injection (340 IU) on the number of sick days per steer in a feedlot environment, as assessed by an average rectal temperature greater than 39.4 °C, compared to unsupplemented animals, vitamin E status was not measured before or after supplementation.

Likoff *et al* (1978 and 1981) found that supplemental vitamin E or aspirin decreased mortality in chicks and also inhibited production of prostaglandins E_1 E_2 and $F_{2\alpha}$ in the spleen and bursa. Tengerdy and Brown (1977) found that vitamin E or vitamin A supplementation decreased mortality due to *E. coli* in chicks. Vitamin E depressed production of prostaglandins E_1 and E_2 in the bursa while vitamin A increased prostaglandin E_1 production. If both vitamins were given together their effects were antagonistic. Meydani *et al* (1984) found that vitamin E decreased prostaglandin E_2 production by the spleen and also enhanced the immune response of aged mice. Machlin *et al* (1975) found that vitamin E lowers platelet aggregation by inhibiting synthesis of prostaglandins from arachidonic acid. Vitamin E deficiency led to increased concentrations of platelet activating factor in rat PMN (Fukuzana *et al* 1989) by increasing synthesis. Increased PUFA concentrations proved detrimental to *E.coli* infected chicks, however vitamin E overcame this effect

(cited by Nockels 1988).

Tengerdy and Nockels (1975) suggest that vitamin E increased the protection of chicks against *E. coli* because of its effects on ubiquinone biosynthesis. Ubiquinones regulate redox processes in cells and have been found to increase phagocytic activity in rats. Vitamin E may protect ubiquinones from oxidation, thus raising their concentrations (Tengerdy 1980) and enabling them to promote peroxidation of bacterial lipid and hence the killing of bacteria, whereas vitamin E an antioxidant could not act directly.

Selenium supplementation in combination with vitamin E supplementation decreased mortality in chicks, due to coccidiosis (Colnago *et al* 1984), in pigs challenged with SRBC (Peplowski *et al* 1981) in preweaning lambs (Kott *et al* 1983) and decreased losses and increased weaning weights in calves (Spears *et al* 1986).

Atroshi *et al* (1986) suggested that as concentrations of prostaglandins in cows infected with mastitis are higher, prostaglandins might play a role in the control mechanisms, especially as prostaglandin concentrations are affected by vitamin E status of the host. They also noted that mastitic cows had lower concentrations of GSH-Px, which suggests a role for selenium in controlling mastitis. However in a separate experiment blood vitamin E concentrations were lower in cows suffering from mastitis (Atroshi *et al* 1988). Low selenium was correlated to chronic mastitis and muscle diseases (Braun *et al* 1991), but not to low fertility. Selenium supplementation (0.1 mg/kg bodywt) and/or vitamin E supplementation (0.74g/day) before parturition decreased the duration of mastitis infections (Smith *et al* 1984) but only vitamin E affected the incidence. Increased serum selenium correlated with decreased bulk tank somatic cell count (SCC), and clinical mastitis decreased as serum selenium and dietary vitamin E concentrations increased, but high intakes of selenium were associated with increased mastitis unless vitamin E intakes were also

high (Weiss *et al* 1990).

Combined vitamin E and selenium supplementation of dairy cows resulted in a 42% reduction in infected quarters at calving, a 59% reduction in infected quarters/day during lactation, a 32% reduction in clinical mastitis overall, and a 57% reduction in clinical mastitis within 4 days of calving. SCC were also decreased (Smith 1986). These effects were thought to be due to increased PMN function, increased immune responsiveness or altered arachidonic acid metabolism.

Selenium supplementation has been found to protect rats against *Staphylococcus aureus*, but not against *Salmonella typhimurium* (Boyne *et al* 1985 and 1986) and to increase chicks resistance to coccidial infection (Jenson and Johnson 1978). Adequate selenium status delayed *C.albicans* infection in mice (Boyne & Arthur 1985a &1986), and had a positive effect on *Treponema hyodysenteriae* infection in pigs (Teige *et al* 1982) but a single selenium injection (6 mg) had no effect on the incidence of pneumonia in housed calves (Phillipo *et al* 1987) nor did a single intraruminal selenium pellet increase resistance to *H. contortus* infection in sheep (Jelinek *et al* 1988).

Murray & Murray (1985) suggest that selenium repletion may increase susceptibility to infection in humans, and to *L. monocytogenes*, *P. bergeii* & *pseudorabies* in mice; they suggest a requirement for selenium by the pathogens for growth and pathogenicity. Unavailability of selenium for growth appeared to protect against *S.typhimurium* in rats (Boyne & Arthur 1986), but due to the importance of neutrophil function and microbicidal activity in controling *C.albicans* selenium deficiency impairs protection against this organism. Reffet *et al* (1988) considered various infections in calves and concluded that enhanced resistance depended on the organism used.

Selenium appears to play a role in lipid metabolism in platelets, GSH-Px is involved in the conversion of 12-HPETE to 12-HETE; the degradation products of 12-HPETE inhibit prostacyclin synthesis causing increased platelet aggregation (cited by Chesters & Arthur 1988).

E. Conclusions.

Both selenium and vitamin E affect the HIR by increasing antibody production to a variety of, but not all mitogens / antigens. Their effects appear to be slightly different, with selenium stimulating IgM and vitamin E stimulating early conversion to IgG production. Vitamin E stimulates cell cooperation, which has little effect on the CMIR, but probably via its action on prostaglandins and ubiquinone it affects not only the HIR but also the CMIR and phagocytes.

Selenium may have additional effects on the CMIR, affecting cell mediators and $T_k$cell cytotoxicity; some of these effects may be mediated via GSH-Px but on several occasions this apparently was not the case. Supplementation with selenium is also complicated because excess selenium proved inhibitory on several occasions, and so an optimum concentration needs to be established. Both vitamin E and selenium also affect phagocyte activity, motility and % kill, by protecting the enzymes involved in forming the toxic substances. However far more research has been done on the mechanism of action of selenium than for vitamin E.

Both vitamin E and/or selenium supplementation decrease mortality in a variety of species, when exposed to a variety of pathogens, but there are a few recorded cases where supplementation in excess of current recommendations seemed detrimental. Although this suggests that supplementation above the absolute requirement may often give financial returns, care must be taken particularly in the case of selenium,

and more work is required to find exactly which pathogens are affected. This may help to elucidate the exact mechanisms of action, and in which areas of the immune system selenium and vitamin E exert their effects.

1.2.7. Supplementation

Many experiments have been done to compare the effects of supplementation with vitamin E and selenium in different forms and by different routes.

The form and route of administration of vitamin E altered its effect on immunity in guinea pigs (Barber *et al* 1977). Oral administration of DL-α-tocopherol acetate had no effect. Intramuscular administration of an oil emulsion produced a severe local reaction whereas free α-tocopherol in an aqueous emulsion produced no such reaction, and increased antibody titres significantly. This suggests that guinea pigs have difficulty hydrolysing the ester form of vitamin E, to produce free α-tocopherol.

When given in bolus form less vitamin E is absorbed than if given by continuous infusion. Medium chain triglycerides increase vitamin E absorption, while long chain polyunsaturated fatty acids (PUFA) and retanoic acid are inhibitory. Long chain PUFA and alcohol may lead to loss of vitamin E activity due to oxidation. (Bjorneboe *et al* 1990). Addition of high concentrations of copper to diets also decreases the vitamin E concentration in serum (Dove & Ewan 1990).

A slow release pellet of vitamin E supplement, administered to pregnant ewes provided partial protection to the lambs against nutritional muscular dystrophy; when administered directly to the lambs protection was almost complete (Hidiroglou *et al* 1972).

The biological availability of D-α [5-Me-^{3}H]-tocopherol to sheep was greatest via the intravenous route, followed by the intramuscular route, the oral route and finally via intraruminal injection. Rate of elimination followed the same trend except that it was slowest for intramuscular administration (Hidiroglou & Karpinski 1987).

Free D-α-tocopherol was absorbed more efficiently than the DL- form or the D- or DL-acetate, from the gut, as measured by peak absorbencies and the area under the α-tocopherol plasma concentration curve (AUC) (Hidiroglou & Karpinski 1988, Hidiroglou *et al* 1988, Hidiroglou *et al* 1989, Reddy *et al* 1985). D-α-tocopheryl acetate was the more effective when given intravenously; because of the rapid elimination of free DL-α-tocopherol on intramuscular injection the unesterified form was superior (Charmley *et al* 1992) but the physiological state of the animal influenced the relationship of circulating vitamin E to stored reserves, thus plasma vitamin E may be an indicator of plasma lipid status rather than vitamin E status. When vitamin E was administered orally, very high supplementation concentrations led to a decreased proportion of the vitamin E being absorbed.

Intraperitoneal administration of DL-α-tocopheryl acetate led to increased vitamin E in tissues and greater AUC relative to intraruminal and Hidiroglou & Charmley (1990) suggest that this could be due to the large numbers of mesenteric lymph vessels in the peritoneum, and deacetylation may also occur in the peritoneum. Alternatively it may be hydrolysed in the liver, by a hepatic vitamin E esterase. Transport to the liver would be rapid via the portal circulation.

Peak uptake of a single intraperitoneal administration by the tissues, especially the liver occured on day three, and therafter there was a steady decline (Hidiroglou *et al* 1990), while on intramuscular administration the amount of vitamin E absorbed was proportional to the dose, although the increase in the tissues was greater than the

increase in the plasma. Muscles demonstrated the slowest elimination rate, and Hidiroglou & Karpinski (1991) hypothesise that control of vitamin E concentrations in the blood and in the liver are mediated separately.

Lewis and Kirkwood (1990) administered 12,500 i.u./day DL-α-tocopheryl acetate, for 8 months, followed by 12,500 i.u./day free DL-α-tocopherol in vegetable oil, for 6 months, to a black rhinoceros. Neither supplement produced a rise in plasma vitamin E. This suggests either that rhinoceroses have a high requirement for vitamin E or that they are unable to utilise and/or absorb it in these forms. Later oral D-α-tocopheryl polyethylene glycol 1000 succinate was found to be an effective source of vitamin E for black rhinos (Kirkwood *et al* 1991) which lack bile and so fats and vitamin E are not properly emulsified and absorbed.

Overall D-α-tocopherol was absorbed fastest; however when smaller daily supplemental doses are concerned, hydrolysis of the acetate ester might not be rate limiting. For immediate response intraperitoneal or intravenous administration would be most efficient, but this would not be suitable on a day to day basis.

Calves from hay fed cows had decreased vitamin E status and elevated GOT concentrations relative to calves from silage fed cows (Lynch 1983) suggesting that the loss of vitamin E by oxidation was decreased in silage.

On administration of SeO_3^{2-} and SeO_4^{2-} to dairy cows, 90% of the selenium was bound to TCA precipitable components within 2 minutes, this was cleared by the liver (40%) bound to α and gamma globulins and released within 30 minutes. SeO_3^{2-} was more readily metabolisable (Symonds *et al* 1981a). At low doses (5ug) selenium was more readily absorbed into tissues from SeO_3^{2-} in steers (Symonds *et al* 1981b).

The absorption of selenium from organic versus inorganic sources has recently been considered. The bioavailability of selenium from organic sources (selenomethionine and a selenium containing yeast product) was twice that from inorganic sources (sodium selenite and cobalt selenite) (Pehrson *et al* 1989). Cobalt selenite was used because it is insoluble in the rumen but soluble in the abomasum, where pH<5, as it was hoped that this might protect it from reduction in the rumen. However no differences in availability of selenium to cattle from cobalt and sodium selenites were observed. On comparison of sodium selenite with plant selenium (produced by spraying the crop with sodium selenite) Ekholm *et al* (1991) found that plant selenium was more effective at increasing the selenium content of all tissues, particularly muscle. Similar results have been found in sheep (Van Ryssen *et al* 1989) however the percentage of selenium incorporated into GSH-Px was lower for the selenium treated wheat than for sodium selenite.

When incubated with rumen microbes, selenite was converted to selenocysteine, whereas selenomethionine (the main selenium containing component in the wheat) was unchanged. In goats (Aspila *et al* 1988) organic selenium as selenomethionine is far more readily absorbed and incorporated into milk than inorganic selenium. However Aspila (1991) concluded that true absorption from inorganic verses organic selenium was similar but organic selenium increased plasma selenium more efficiently. Twice as much selenium was excreted in the urine after inorganic supplementation; overall organic supplementation was more efficient and safer.

Addition of selenium to the feed is only feasible if a concentrate ration is being fed. If the animals are housed, selenium could be added to the water or sprinkled on the food. Treatment of the pasture with selenium, or salt licks have been used for grazing animals. A more direct method involves using an intraruminal bullet or direct subcutaneous injection. Oral dosing causes increased handling of the animals,

however incorporation of selenium into anthelmintics has proved fairly successfull (MacPherson *et al* 1987c and MacPherson 1988). The choice of method depends on what fits into the current management programme. Care must be taken if selenium is sprayed onto the land, as initially highly toxic concentrations may occur, before selenium is taken up by the plants and soil.

Year round supplementation of dairy cows is required and particularly precalving as selenium does cross the placenta and protects the foetus whereas vitamin E does not (Van Saun 1989). Adequate selenium status leads to improved transfer of dietary tocopherol to milk (Nicholson *et al* 1991), although this only led to improved flavour of milk when copper was added to the milk to catalyse oxidation.

Jenkins and Hidiroglou (1972) suggested that animal feeds in Canada should be routinely supplemented with selenium and vitamin E, or that cattle and sheep should have free access to a selenized salt lick containing 26ppm Se. Other possibilities include fertilizers, boluses, implants, and injections.

CHAPTER 2

MATERIALS AND METHODS

2.1. Sampling Techniques.

2.1.1. Collection of Blood Samples.

Blood samples were taken by jugular venepuncture and collected into appropriate evacuated tubes. These were silicone coated for serum, coated with 143 USP units of lithium heparin for plasma or whole blood, or with 15% EDTA for the differential cell counts, and 10 ml tubes to which 1 ml of 1.5% EDTA solution (pH 6.8) had been added, for whole blood for use in the neutrophil function tests.

Serum was obtained by retracting the clots onto wooden sticks in a waterbath at 37°C. The sticks were then withdrawn along with the clot and the serum centrifuged at 3,000 r.p.m. for 10 minutes to separate any remaining debris.

Serum and plasma were stored frozen (at -20°C) for analysis, but the neutrophil function tests, differential cell counts and the erythrocyte stability test were carried out on the day the samples were taken. SGOT and plasma CK determinations were done within a week of sampling.

2.1.2. Rumen sampling.

Rumen samples were taken from goats and sheep by passing a tube down the oesophagus and into the rumen. A perforated metal weight on the end of the tube filtered the fluid removing any large particles. Rumen fluid was drawn through the tube by suction using a syringe and a valve. 30 ml of this fluid was then preserved by adding to 750μl of saturated mercuric chloride solution and centrifuging at 3000 rpm. for 20 minutes to remove any debris before being frozen for determination of succinate and VFA's at a later date.

2.2. Analytical Procedures.

2.2.1. Serum Vitamin B_{12}.

Serum vitamin B_{12} concentrations were determined using the microbiological method of Taylor and Greer (1982). Test samples, aqueous vitamin B_{12} standards or standard sera of known vitamin B_{12} content, were diluted 1:9 in acetate buffer (pH 4.5) containing 20 mg/l potassium cyanide, and heated for 30 minutes at 108°C. After cooling all samples were 'ringed' with a wooden applicator to release particles stuck to the sides of the tube, and then centrifuged at 3,000 rpm. for 10 minutes. This caused the precipitated serum proteins to form a pellet while the vitamin B_{12}, which had been converted to the stable cyanocobalamin form remained in the supernatant. The supernatant was then diluted 1:4 with assay broth (B_{12} Assay Medium USP, Difco Laboratories, Detroit, Michigan) and sterilised at 121°C for 5 minutes prior to inoculation with the vitamin B_{12} requiring bacteria, *Lactobacillus leichmanii* ATCC7830. After incubation at 37°C for 18-24 hours, growth was measured spectrophotometrically and a standard curve plotted from the results, sample concentrations were obtained by reading off from the curve.

2.2.2. Methylmalonic Acid and Succinic Acid.

Standards were prepared by diluting methylmalonic acid, MMA (Purum, Fluka) and ethylmalonic acid, EMA (Purum, Fluka) initially to give a 10 μmol/ml stock in acetone (AnalaR BDH) and then by further dilution to give a 0.5 μmol/ml MMA working standard and a 1 μmol/ml EMA working internal standard.

500 μl of sample, or 100 μl of standard made up to 500 μl with distilled water, was placed in a 5 ml glass tube, 100 μl of internal standard, (EMA in acetone) was added

followed by 500 μl of acetone while vortex mixing, to precipitate the proteins. To this 2 ml of saturated sodium chloride (AnalaR BDH) containing 0.5 M sulphuric acid (AnalaR BDH) was added and the tubes were capped and inverted. 1 ml of ethyl acetate (AnalaR BDH) was added and the tubes were once more capped and mixed by inversion for 20 seconds before centrifugation at 2000 rpm. for 10 minutes. The ethyl acetate layer was then transferred to a glass 1 ml microtube and evaporated to dryness on a Dri-block heater at 60°C under oxygen free nitrogen (BOC gases). This stage was repeated with a further 1 ml of ethyl acetate. This process extracted the EMA and MMA from samples into ethyl acetate under acidic conditions. The extracted acids were then converted to their butyl esters by addition of 100 μl of a 1:10 mixture of acetyl chloride (AnalaR BDH):Butanol (Aristar BDH), followed by incubation in a waterbath at 70°C for 20 minutes. After being allowed to cool 320 μl of hexane (Rathburn) and 600 μl of distilled water were added and the tubes were mixed by inversion for 10 seconds. The hexane layer containing the butyl esters was then transferred to a 300 μl autosampler vial.

The esterified MMA was measured by capillary gas chromatography, as in McMurray *et al* (1986), using a 'Packard 439' GC and a flame-ionisation detector. The column was a 25 by 0.32mm i.d. RSL150BP (Alltech). 2.5 μl of the esterified sample was injected onto the column using a splitter injector set at a split ratio of 1:30, with helium as carrier gas. The injector temperature was 200°C. and the detector was at 250°C. The oven temperature profile during the run was as follows:-

1. 120°C. for 2 mins.
2. Rise of 5°C. min^{-1} for 7 mins.
3. Rise of 40°C. min^{-1} until 230°C. reached.
4. 230°C. for 10 mins. (to remove any components with higher boiling points from the column).

Results were collected and integrated by a Shimadzu CR6A Chromatopac integrator and the concentration of MMA in the samples calculated from that in the standards as follows:-

Area EMA in Std./Area MMA in Std x Conc. MMA in Std. = RF (response factor)
Area MMA in sample/Area EMA in sample x RF =Conc. MMA in sample.

This method was later adapted for measurement of both MMA and succinic acid in both serum and rumen fluid; changes involved the inclusion of succinic acid (AnalaR, BDH) as a standard at 10 μmol/ml in the stock. The working standards were all more dilute, giving concentrations of 0.1μmol/ml MMA and succinic acid in the mixed standard and of 0.1 μmol/ml EMA for the internal standard. The sample preparation remained the same except that the hexane for the final extraction was replaced with the same quantity of octane (Purum, Fluka) to shorten the run time. This was because its lower boiling point required less cooling of the column before it would condense. For rumen samples 100μl sample and 400μl distilled water were treated as for serum.

The GC separation was also modified considerably as follows :-
2.5 μl of esterified sample was injected onto a capillary column (30m by 0.32 mm i.d. SE-54, Alltech U.K., 6-7 Kellet Rd. Industrial Estate, Kellet Rd. Carnforth, Lancashire) with a 5m by 0.32mm precolumn (Restek, supplied by Belmont Instruments, 4 Park Gardens, Glasgow.) using a splitless injection technique. The injector temperature was increased to 250°C the same as the detector, and after 30 seconds a vent flow of 50 ml/minute (Helium, BOC gases) flushed the liner. The oven temperature profile was as follows:-

1. 105°C for 1 minute
2. Rise 17.5°C/minute for 2 minutes,
3. Rise 2°C/minute for 8.5 minutes
4. Rise 40°C/minute until final temperature reached 230°C.
5. 230°C for 10 minutes.

The lower initial temperature was to allow the octane to condense in the precolumn focusing the injection into a narrow band. The slightly longer rise time was to allow the succinate to elute. Concentrations were calculated by the same technique.

2.2.3. Vitamin E.

Plasma sample preparation:-

Vitamin E standard (Kodak Chemical Co. Liverpool) 2 μg/ml in ethanol (0.5 ml) and internal standard (100μl of 2.5 μg/ml tocol in ethanol) were diluted with (0.5 ml) distilled water. Plasma samples (0.5 ml) and internal standard (as above) were precipitated with ethanol (0.5 ml). The vitamin E was extracted from samples and standards into hexane (Rathburn) (2.5 ml) by vigorous mixing followed by centrifugation at 1500 rpm. for 5 minutes. As much as possible of the hexane layer was taken off and dried on a Dri-block heater under oxygen free nitrogen (BOC gases) at 50°C and then redissolved in methanol (BDH)(300μl) before being transferred to autosampler vials for vitamin E determination.

Feed samples were prepared as follows:-

1.5 g of milled sample was weighed into a 50 ml polypropylene conical bottomed centrifuge tube to which was added 10 ml of 10% pyrogallol (AnalaR BDH) in ethanol (BDH). The tubes were placed in a 70°C waterbath for 5 minutes to

equilibrate before addition of 2.5 ml 60% aqueous potassium hydroxide (BDH) and then left for 30 minutes at 70°C to saponify. Finally 22.5 ml of distilled water and 10 ml of hexane (Rathburn) were added and the tubes were mixed by inversion to extract the tocopherol. Once the layers separated 1 ml of the hexane layer was transferred to a 5 ml glass tube and evaporated to dryness on a Dri-block heater at 50°C under oxygen free nitrogen (BOC gases). The residue was redissolved in 300 μl methanol (Rathburn) and transferred to an autosampler vial.

Separation of the end products of both the plasma and the feed sample preparations was achieved by HPLC, using a 15cm by 4mm i.d. Superspher 4μm RP18 column with a Lichrocart 4-4 guard column. Degassed Methanol: water 98:2 was used as eluent, flowing at 1.5 ml/min from a Spectra Physics SP8770 isocratic pump. A Gilson 232Bio autosampler was used to inject 110 μl through the 20μl loop the contents of which were then flushed onto the column. Detection was achieved using a Jasco 821-FP fluorimeter with 295 nm excitation and 330 nm emission wavelengths and results were collected and integrated using a Spectra Physics ChromJet integrator. Retention times for tocol and vitamin E were about 3 and 5 minutes respectively. To validate the results obtained a large number of duplicate samples were also analysed at the Animal Diseases Research Institute (Moredun) and the results compared. The correlation between our vitamin E results and those of the Moredun was 0.976 (or R^2=95.3), and the relationship between the two values was,

Auchincruive result = 0.017 + (1.1 x Moredun result).

2.2.4. Serum Glutamic oxalacetic transaminase (SGOT).

Serum glutamic oxalacetic transaminase (SGOT) concentrations were measured using a colourimetric kit obtained from SIGMA (procedure No. 505). The kit is based on the reaction:-

Aspartic Acid +	GOT	Oxalacetic acid +
alpha-ketoglutaric acid	---------->	Glutamic acid

The oxalacetic acid can then be determined colorimetrically at 505nm, after reaction with 2,4-dinitrophenylhydrazine.

2.2.5. Plasma Creatine Kinase (CK).

Plasma CK activity was measured using a colourimetric kit (Boehringer Mannheim cat. No. 126322). This method is based on the N-acetylcysteine activated UV-system, using CK to convert creatine phosphate and ADP to creatine and ATP; the ATP generated is reconverted to ADP by reacting with glucose in the presence of hexokinase, producing glucose-6-phosphate. This in turn reacts with $NADP^+$ in the presence of glucose-6-phosphate-dehydrogenase forming gluconate-6-phosphate, H^+ and NADPH. The production of NADPH can be measured spectrophotometrically at 365nm in a kinetic assay at 30°C and related back to the activity of CK.

2.2.6. Whole blood Glutathione peroxidase.

Glutathione peroxidase was measured in heparinised whole blood, using an enzyme coupled colourimetric method, (based on Paglia and Valentine 1967 and Anderson *et al* 1978) as used by the Veterinary Investigation Centre, SAC. This assay relies on the activity of GSH-Px to convert cumene hydroperoxide substrate to free radicals, in the presence of reduced glutathione, glutathione reductase and NADPH, and the

rate of NADPH oxidation can be measured spectrophotometrically at 365nm, in a kinetic assay at 30°C. This can be related to the activity of GSH-Px in the red cell fraction.

2.2.7. Serum Copper, Calcium and Magnesium.

Serum copper, calcium and magnesium were analysed by atomic absorption spectroscopy according to standard methods used by the Biochemical Sciences Department SAC.

2.2.8. Rumen fluid Volatile fatty acids.

Separate stock standards were made up by weighing Acetic acid (3g AnalaR, BDH), Propionic acid (3.7g Sigma), Butyric acid (4.4g Sigma), Isobutyric acid (0.44g Sigma), Valeric acid (0.51g Sigma), and Isovaleric acid (0.51g Sigma) and making each up to 50ml with distilled water. A single mixed working standard was then prepared from 5ml acetic, and 2ml of each of the other stock standards, made up to 100 ml. This gave final molarities for the acids of 0.05, 0.02, 0.02, 0.002, 0.002 and 0.002 respectively.

Thawed samples were centrifuged at 1000 r.p.m. for 10 minutes to remove any remaining debris, 2.5 ml of sample was then added to a screw capped centrifuge tube and 1.1 ml of 1M sodium hydroxide (AVS) was added. For the standards 1.1 ml of water was added to the 2.5 ml of working standard. 0.5 ml of pivalic acid solution (0.22g pivalic acid Sigma, in 100 ml water) and 2ml oxalic acid solution (4.725g oxalic acid AnalaR, in 500 ml water) were added to each tube. The tubes were then capped and mixed before being centrifuged at 3000 r.p.m. for 10 minutes, and the supernatants were transfered to 2ml autosampler vials.

Separation was achieved by injecting 1 μl onto a 2m by 2mm i.d. glass column packed with 4% carbowax 20M on carbopack B-DA 80/120 mesh (Supelco Inc.) using an AMS GC with a flame-ionisation detector. Column flow rate was 24 ml/minute with hydrogen at 30 ml/minute and air at 300 ml/minute. Injector and detector temperatures were both 200°C and the oven temperature was 175°C.

2.2.9. Neutrophil Function Test.

The neutrophil function test used was a modification of the candidacidal test used by Boyne and Arthur (1979). The neutrophils were isolated by a series of washing and centrifugation steps, according to the method of Carlson and Kaneko (1973). Fresh blood with EDTA as anticoagulant, was centrifuged at 1000G for 20 minutes and the plasma portion was removed by aspiration. 4 ml of isotonic (0.8%) PBS was added to resuspend the remainder before transferring to a 50 ml conical based centrifuge tube. Distilled water (20 ml) was added, mixed gently and left for 1 minute to lyse the red cells. To prevent lysing of the more resistant white cells isotonicity was restored by the addition of 10 ml 2.7% sodium chloride in PBS. The tubes were then centrifuged at 500G for 10 minutes to form a white cell pellet, the remaining supernatant being discarded by aspiration. The cell pellet was washed three times by suspending in 25 ml PBS mixing by gentle inversion and recentrifuging and aspirating the supernatant as before. Finally the cell pellet was resuspended in 1ml PBS.

For the *C.albicans* neutrophil function test the neutrophils were then incubated for an hour at 37°C. with the yeast *Candida albicans*. Dead *C. albicans* were stained blue by incubating in the presence of methylene blue dye for 15 minutes, which enabled them to be identified. Using a haemocytometer, the number of neutrophils containing dead *C. albicans* were then counted and expressed as a percentage of the number of neutrophils containing ingested *C. albicans*, which is then referred to as

the percentage kill.

For the other neutrophil function tests incubation with *E.coli* and *S.aureus* was done by a modification of the method described by Hogan et al. (1990). Neutrophils were incubated with either *E.coli* or *S.aureus* at 37°C. for 90 mins. After incubation the assay suspension was diluted 2:1:1 with acridine orange (14mg/100mlPBS) and crystal violet (50mg/100ml PBS). Wet mount slides were prepared and examined under a fluorescence microscope, using a 1,000 x oil immersion lens. % kill was calculated as for the Candida.

2.2.10. ELISA for *Clostridium tetani* antitoxin.

This ELISA was carried out according to the method of Keith Wood (1990 personal communication, unpublished) using pre-coated Dynateck M129B micro-ELISA plates (coated with *Cl. tetani* toxin; K.Wood). These were washed three times with phosphate buffered saline containing 0.1% Tween (PBST) before being incubated with 250 μl PBS containing 0.1% bovine serum albumen (BSA)(BDH)/well, for 1h at 37°C. The plates were then re-washed three times.

A standard dilution series was set up using goat serum known to contain 2.5 IU/ml *Cl. tetani* antitoxin (K.Wood). The unknown samples were also diluted, either 1/250 for pre-vaccination samples, or 1/2000 or 1/3000 for post-vaccination samples. The standards and samples were plated out and a row of blank wells was also included on each plate. The plates were then incubated for 1hr at 37°C and thereafter washed a further three times. Rabbit anti-goat IgG-horseradish peroxidase conjugate (K.Wood) diluted 1/500 in PBST was added to each well and incubated for 1hr at 37°C. The plates were then washed three times again.

250 μl of substrate solution, made up per plate from:-

25 ml of 0.1M sodium-acetate-citric acid buffer (pH 6.0)

25 μl H_2O_2 (30%)

250 μl tetramethyl benzidine soln. (4.2mM TMB in DMSO from K.Wood)

was added to each well and the colour was allowed to develop for 5-10 mins. The reaction was then stopped by adding 50 μl sulphuric acid (AnalaR BDH) (1M) to all wells, and gently agitating.

The absorbance at 450 nm was measured using a Titertek plate reader, and the concentrations in the samples calculated from the standard calibration curve. All samples and standards were measured in duplicate and mean values calculated.

2.2.11. Erythrocyte stability test.

Detergent sensitivity was assessed essentially as described by Stevenson and Jones (1989), using Polyoxyethylene Sorbitan Monolaurate, Tween 20 (Sigma). Whole blood (0.2ml) was washed in phosphate buffered saline (5ml PBS) pH7.4 containing 0.5mmol/l ethylenediaminetetraaceticacid (disodium salt) (PBS/EDTA). After centrifugation (1000G for 10 mins) the supernatant was aspirated and the cell pellet was resuspended in PBS/EDTA (5ml). Phosphate buffered saline contained 6.8g sodium chloride (AnalaR, BDH), 1.48g di-sodium hydrogen orthophosphate (anhydrous, AnalaR, BDH), 0.43g potassium dihydrogen ortho-phosphate (AnalaR, Hopkin and Williams Ltd. England) per litre adjusted to pH 7.4 using 0.1N sodium hydroxide (GPR BDH).

0.5 ml aliquots of the above suspension were mixed 1:1 with dilutions (0.5, 2 and 6%) of Tween 20 in 0.9% saline. This mixture was incubated in a waterbath at 37 °C for 15 mins. Ice cold PBS (2 ml) was added, and the mixture recentrifuged. Supernatant absorbances were read at 415 nm. Percentage haemolysis was calculated

by comparison with the absorbance of a 100% haemolysate, obtained by adding 10 ml distilled water to 50 μl of whole blood.

This method was modified for use with the calves to overcome several problems that were experienced with the goats.

Modifications.

1. Tween 20 dilutions used were 1, 3 and 5%.
2. Incubation time was increased to 18 minutes.
3. 10 ml of ice cold PBS was added after incubation.
4. 100% haemolysed sample was obtained by adding 0.5 ml of washed cells (resuspended in PBS/EDTA) to 10.5ml of distilled water.

The effect of these changes was to give a larger end volume, which increased precision of absorbance measurements. The supernatant was more dilute and so had a lower absorbance which meant that readings were now in the more sensitive range of the instrument and no longer in danger of going off scale. Another unexpected effect of dilution was to decrease the percent haemolysis. This suggests that previously the reaction was not entirely stopped by the addition of PBS. Incubation time was extended slightly to compensate for this.

During the modification process, very small differences in incubation time were found to affect results greatly which may explain the large week to week differences observed previously.

2.2.12. Differential cell counts.

Whole blood was drawn into a vacutainer containing EDTA to prevent clotting. Differential cell counts were carried out as described by Scholm *et al* (1975). The blood was inverted gently to obtain a homogeneous sample, one drop of which was placed on a standard glass slide, a second slide modified to have a narrow edge was then used to smear the drop to give a film approximately one cell thick. The slide was then allowed to dry before being flooded with Wright's Giemsa stain (Sigma) for 15 seconds, this was followed by flooding with distilled water for 2 minutes, and then a thorough rinse in distilled water. Once the slide was dry it was then examined under a light objective microscope (Kyowa Opticals, Japan) using a x 550 magnification, and one hundred white cells were counted by the battlement procedure. The number of each cell type was recorded and expressed as a percentage of the total.

2.2.13. Single Radial Immunodiffusion (SRID) for goat IgG.

Standards were prepared by reconstituting 10 mg of lyophilised salt free goat IgG (product N°.15256 Sigma) in 250 μl distilled water to give a concentration of 4000 mg/100 ml. Serial dilutions 1:2, 1:4, 1:8, 1:16 and 1:32 were prepared in normal saline.

SRID plates were prepared as follows:-

Sodium barbitone (5g AnalaR BDH) and sodium acetate (3.25g AnalaR BDH) were dissolved in 32.2 ml of HCl (0.1 N) and the solution made up to 500 ml by addition of distilled water. 150 ml of this 0.1M barbitone-acetate buffer (pH8.6), was then used to prepare a 1.2% agar solution (1.8g Agar-Purified Difco) incorporating 6g polyethylene glycol 4000 (BDH) and was boiled to dissolve the agar. 5.6 ml aliquots

were transferred to test tubes. On cooling to 56°C an aliquot (amount depending on the concentration of antibody used) of anti-goat IgG whole molecule (product N°. G5518 Sigma; lyophilised form raised in rabbits, titre 2.0 mg antibody/ml), was diluted with sodium barbitone buffer to 1 ml and this was added to the agar and mixed thoroughly before pouring into a plate. Plates were allowed to set on a perfectly flat surface and then stored at 4°C before use.

Seventeen 5 μl capacity wells were cut using a template and 2mm well cutter. 4 μl of standard or sample was added to each well, standards being distributed among the plates which were then incubated overnight at room temperature.

Results were obtained from a standard curve plotting IgG concentration on the log (y-axis) of semi-log paper, against ring diameter in mm (x-axis).

CHAPTER 3

THE REQUIREMENT OF GOATS FOR COBALT, TO MAINTAIN GROWTH AND IMMUNE FUNCTION.

3.1. Introduction.

Very little is known to date about the precise requirements of goats for micronutrients. Recommendations are based on the requirements of sheep and cattle and are generous enough to allow for some error. However distinct differences are apparent between sheep and cattle, and although goats have survived to date on this system, a better understanding of their specific needs would be beneficial. This is true not only from the scientific viewpoint but also from an economic one, as it could lead to saving on mineral/vitamin supplements.

With the prospect of an open European market goats could become far more popular. They are highly suited to poor grazing conditions, being small and having a split upper lip, which allows them to be highly selective about the vegetation they eat and enables them to derive more nutrients than the average analysis of a crop would suggest. They are also leaner than sheep, which, in this calorie conscious age, should increase their popularity. Currently it is the consumer resistance which the British public has to eating goat meat that prevents them from being marketable.

For research purposes goats are more suitable than sheep as a model for cattle. A model is generally required for economic reasons because the cost of purchasing and feeding large animals like cows is high. Goats are generally very similar to sheep in their metabolism. However, possibly because they have also been bred for milking purposes in many areas, in some characteristics they resemble cows more closely than sheep do.

Finally very little of the research on relating micronutrients supply to immunity has been done on goats and so it is not known whether the effects of deficiencies or excesses are common to all ruminants, or are species specific.

The first objective was to investigate cobalt requirement in the goat, and to attempt to establish a precise dietary cobalt requirement. There is not a great deal of literature on this subject. However Platten (1951) suggests that the cobalt requirement of the goat may be greater than that of sheep, while Clark *et al* (1987) suggest that it may be as low as 0.035 mg/kg DM, and Mgongo *et al* (1984) found that levels as low as 0.01 mg/kg DM definitely produced symptoms of vitamin B_{12} deficiency. Thus initially it was necessary to consider a range of dietary cobalt levels based around the known requirements of sheep and cattle for this element.

To establish the dietary requirement for cobalt the minimum dietary concentration which caused no symptoms of deficiency such as loss of appetite, weepy eyes or decreased liveweight gain would be identified, with an attempt being made to assess the relationship between dietary cobalt level and immune function as this has been shown to be affected by cobalt/vitamin B_{12} deficiency before liveweight gain in sheep and cattle (Fisher 1988 & Paterson and MacPherson 1987 & 1990). It is also necessary to establish parameters for diagnosing deficiency. In sheep serum vitamin B_{12} and MMA concentrations used in conjunction give the best indication of deficiency and concentrations indicative of marginal vitamin B_{12} status are well established. It is well known that vitamin B_{12} concentrations can be misleading in cattle as much of the vitamin B_{12} is bound to transcobalamin 1 and is consequently not measured by most conventional methods. Thus as well as establishing the critical concentrations for these parameters, it was necessary to assess the usefulness of each for diagnosing cobalt/vitamin B_{12} deficiency. More recently (Kennedy *et al* 1991d) suggested that serum succinate concentrations become elevated during vitamin B_{12} deficiency and this could be used as an aid to diagnosis. Increased concentrations of the liver enzyme aspartate aminotransferase (or glutamic oxalacetic transaminase, GOT) in serum are indicative of liver damage such as occurs during white liver disease, or during fatty infiltration of the liver without white liver disease, thus

SGOT concentrations might also help to identify animals suffering from cobalt deficiency.

The effect of vitamin E status, and intake on the health of goats is also unrecorded and so we aimed to consider the effects of vitamin E supplementation with two different forms of vitamin E. Vitamin E deficiency in other ruminants leads to elevated concentrations of the liver enzyme GOT in serum and also to elevated concentrations of the muscle enzyme creatine kinase in plasma. Because of the close link between the effects of vitamin E, and selenium, an indicator of the selenium status of the goats was required, Glutathione peroxidase (GSH-Px) activity of whole blood is commonly used as an indicator of selenium status.

Another long term aim of this work was to study effects of cobalt on the immune system, using, in the initial experiment, a neutrophil function test similar to that described by Boyne and Arthur (1979). This assesses neutrophil function as the percentage of *Candida albicans* killed, (as opposed to those merely ingested). This does ensure that all aspects of phagocyte function, chemotaxis, phagocytosis, and microbicidal ability, are functioning. It is however an *in vitro* test and so cannot be said to represent exactly what would occur *in vivo*, and it also considers only those aspects of immunity which involve phagocytes. It does not inform us of any effects on lymphocytes or antibodies etc.

Other aspects of immunity which might be affected by vitamin B_{12} and/or vitamin E deficiency include changes to the white cell populations, which can be assessed by differential cell counts, and direct effects on the humoral immune response which can be measured by antibody titres and antibody response to vaccination.

3.2. Experimental Design.

In each of the three trials a very similar experimental procedure was used and differences between them will be discussed in each section.

3.2.1. Animals.

Twenty four six month old goats of feral x Tasmanian, Russian or Australasian cashmere strain, were used in each trial. They were wormed, deloused and vaccinated as required, in experiment 1, wormed on day -27 and vaccinated with Heptavac P on either day 64 or 70. In experiment 2 they recieved Fasinex for fluke on day -89, one goat died very early on and was replaced before supplementation commenced, a second goat allocated to group 2 died later on in the trial, but was not replaced. In experiment 3 all goats were treated with Parosol pour-on for lice on day 42. The goats were housed throughout the trial.

3.2.2. Treatments.

The goats were fed a cobalt-deficient diet for two to three months until their serum vitamin B_{12} concentrations suggested that they were depleted of vitamin B_{12}. They were stratified on the basis of comparable liveweight and vitamin B_{12} status and randomly allocated into groups, 4 groups of 6 (experiments 1 and 3) and 6 groups of 4 (experiment 2). The date of the first cobalt treatment has been termed day zero for all experiments, and from this time all groups received the appropriate supplement weekly; in each case group one was the control and received no additional cobalt.

The first experiment was aimed at gaining a general idea of the dietary cobalt concentration required to maintain growth and normal immune function and so the differences in cobalt concentrations received by the different groups were quite

large. The following two experiments attempted to use these initial findings to pinpoint the requirement much more precisely. Treatment effects were monitored by weekly liveweight recording, and analysis of fortnightly blood samples.

EXPERIMENT 1 During this experiment the goats were randomly allocated into 4 groups of 6 and supplementation was as follows:-

Group 1, the control group, received no cobalt supplementation.

Group 2, the low cobalt group, received 0.35 mg of cobalt per head per week.

Group 3, the medium cobalt group, received 0.525 mg of cobalt per head per week.

Group 4, the high cobalt group, received 0.7 mg cobalt per head per week.

These are equivalent to dietary concentrations of:-

Group 1 -	0.04			mgCo/kgDM
Group 2 -	0.11	falling to	0.09	"
Group 3 -	0.14	" "	0.12	"
Group 4 -	0.18	" "	0.15	"

changes are due to increasing feed intake (particularly of maize) during the trial.

The cobalt was in the form of hydrated cobalt sulphate, which contains 20.96 % cobalt by weight, from which a stock solution containing 0.7 gCo/l was made. 0.5, 0.75 or 1 ml of this was further diluted to approximately 15 ml and administered to all goats in groups 2, 3 and 4 respectively, orally using a dosing bottle once a week.

Groups 3 and 4 thus received a dietary supply which was greater than that recommended for sheep (0.11 mg Co/kg DM, Underwood 1981). The concentration received by group 2 was approximately equal to the requirement of sheep as given by Marston (1952), between 0.08 and 0.1 mg/kgDM. While the basal diet supplied group 1 with less than the requirement of cattle (0.05 to 0.07 mg/kgDM (MacPherson, Moon and Voss 1973).

On day 163 group 1 were given 1ml of Neo-Cytamen (Glaxovet Ltd) injection (containing 1mg of hydroxocobalamin BP.) intramuscularly. By this stage they were all losing weight and some of them were showing other signs of vitamin B_{12} deficiency, (not eating concentrates, pale mucous membranes and weepy eyes). From then on they received the same cobalt dose as group 4, to try and correct their condition by the end of the trial.

From day 169 until the end of the trial on day 182, half the goats (numbers 1, 2, and 4 from group 1, number 8 from group 2, numbers 15, 16, 17, and 18 from group 3 and numbers 20, 21, 22, and 24 from group 4) were given 100 IU DL-α-tocopheryl acetate supplement (as Rovimix E50 Roche) per head sprinkled on the food daily.

All goats received 3mg selenium as sodium selenite solution on day 105.

EXPERIMENT 2 During experiment 2 the goats were randomly allocated into six groups of four and supplementation was as follows:-

Group 1 again received only the basal diet, containing on average 0.055 mgCo/kgDM.

Groups 2-6 received an oral supplement weekly which supplied 0.01-0.05 mgCo/kgDM respectively (thus after taking the diet into consideration they were receiving a total of 0.055-0.105 mgCo/kgDM).

The exact amount of cobalt in the dose was increased monthly to take the increasing DM intakes into consideration as follows:-

A stock solution of cobalt sulphate was made up monthly, as DM intakes increased the concentration of cobalt in the stock solution was increased such that 0.5 ml contained 0.01 mgCo/kgDM intake/week. Groups 2-6 received 0.5, 1, 1.5, 2, and

2.5 ml respectively.

DM Intake (kg/day)	Stock Cobalt Solution (mgCo/ml)
0.7	0.10
0.75	0.105
0.9	0.126
0.95	0.134
1.0	0.142

It was decided to increase the level of cobalt given to group 5 to 0.07mg/kgDM (3.5 ml of stock solution) on day 97, in case we had underestimated the requirement of the goats. On day 104 half the goats (numbers 3, and 4 from group 1, numbers 5, 6, and 8 from group 2, numbers 11, and 12 from group 3, numbers 17, and 19 from group 5, and numbers 21 and 23 from group 6) received an injection of vitamin E (3ml injectable vitamin E preparation, Stuart Products Inc) containing 300 IU D-α-tocopherol/ml. Towards the end of the experiment on days 146 & 147 goat 1, from Group 1, received 1ml of injectable vitamin B_{12} (1mg/ml hydroxocobalamin Neo-Cytamen, Glaxovet Ltd.), and goats 1-4, in Group 1, received oral cobalt doses from day 154, containing 0.05 mgCo/kgDM intake.

All goats received 3mg selenium as sodium selenite solution on day 35.

EXPERIMENT 3 During Experiment 3 the goats were randomly allocated into 4 groups of 6, offered the basal diet and supplemented orally as follows:-
Group one received no additional cobalt.
Groups 2,3 and 4 received additional supplements of 0.01, 0.02 and 0.06 mgCo/kgDM respectively. Thus in total the combined dietary and supplementary cobalt received by each group was:-
group 1, 0.04, group 2, 0.05, group 3, 0.06, group 4, 0.10 mgCo/kgDM.

Again a stock solution of cobalt sulphate was made up monthly, and as DM intakes increased the concentration of cobalt in the stock solution increased such that 0.5 ml contained 0.01 mgCo/kg DM intake per week. Thus groups 2-4 received 0.5, 1 and 3 ml respectively.

DM Intake (kg/day)	Stock Cobalt Solution (mgCo/ml)
0.5	0.07
0.55	0.08
0.65	0.09
0.7	0.098
0.75	0.1

All goats received 3 mg of selenium as sodium selenite solution on day 154.

3.2.3. Diets.

The diet used in these experiments aimed to mimic the normal ruminant diet as closely as possible, and consisted of concentrate (maize except for the second half of the first trial which incorporated propcorn barley) with forage (hay, except for trial three when a hay :straw, 1:1 mix was used). SAC rationing software was used to formulate diets containing adequate protein and energy, but to be deficient in cobalt. Rations were calculated to allow for liveweight gains of 50g/day. Urea was added to the forage to provide adequate nitrogen, and calcium, magnesium and sodium were supplemented when required. Details of the nutritional composition of the feeds are included in tables 3.2.1 to 3.2.6, Appendix I. In the first trial the overall diet supplied 0.04 mg Co/kg DM, for the second trial the basal diet contained more cobalt, 0.06 falling to 0.05 mg/kg DM as intake of the concentrate increased during the trial, with an average of 0.055 mgCo/kgDM. In the third trial, because of the high levels of cobalt in the hay it was mixed with straw, and the ratio of hay:straw:maize was maintained at 3:3:4 throughout the trial giving an overall concentration of 0.04mg/kg DM. The goats were housed in two pens and group fed,

so individual feed intakes were not measured.

3.2.4. Experimental Parameters.

Serum vitamin B_{12}, MMA, succinate and GOT concentrations were monitored to investigate their relationship to cobalt intake of the animal. Plasma vitamin E, creatine kinase (CK) activity and whole blood GSH-Px activity were monitored to investigate their relationship with the vitamin E intake of the goats. Additionally from day 163 of experiment 1, erythrocyte stability was monitored weekly to see if there was a link between this and vitamin E supplementation.

To ensure that the results observed were due to the imposed treatments, and not to confounding effects, concentrations of various other micronutrients were also monitored. When these fell below accepted levels, as was the case with selenium in all three trials, suitable remedial action was taken. Concentrations of copper, magnesium and calcium, were also measured for this reason.

Vitamin B_{12} concentrations and other blood parameters were measured fortnightly, while liveweights (LWT)were recorded weekly, using a weigh crate (Ritchie Farm Equipment) zeroed before use.

Neutrophil function was assessed fortnightly during experiment 1, and blood samples were taken before and after vaccination with Heptavac-P for determination of the antibody response of the goats by ELISA. During experiment 2 differential cell counts were carried out fortnightly and at the end of experiment 3 total IgG titre was measured by single radial immuno diffusion (SRID).

3.2.5. Statistical analysis.

Statistics were carried out on the results using the Genstat V programme (Lawes Agricultural Trust 1988). Analysis of variance or covariance was used unless otherwise stated.

3.3. Results:- Experiment 1

3.3.1. Serum Vitamin B_{12} Concentrations.

The basal diet proved inadequate to maintain serum vitamin B_{12} concentrations above 100 ng/l, see Fig.3.1. and Table 3.7 in Appendix I. Concentrations in the control group fell as low as 30 ng/l. All three oral cobalt supplementation levels showed a significant increase in serum vitamin B_{12} concentrations maintaining them above 100 ng/l but not, except in the case of group 4, raising them above 200 ng/l. Concentrations for group 4 were significantly higher than those for groups 2 and 3 on 4 occasions. Vitamin E treatment did not affect vitamin B_{12} concentrations. The injection of vitamin B_{12} administered to the animals in the control group on day 163 increased serum vitamin B_{12} concentrations beyond the range of the assay (>2500ng/l) by day 168.

3.3.2. Serum MMA Concentrations.

The control group MMA concentrations rose continually from below 2 μmol/l at the outset to over 120 μmol/l just prior to supplementation, see Fig.3.2. and Table 3.8 Appendix I. Concentrations for the other groups rose prior to supplementation but decreased rapidly thereafter returning to below 10 μmol/l. Concentrations for groups 2,3 and 4 were consistently significantly lower than those for group 1 but there were

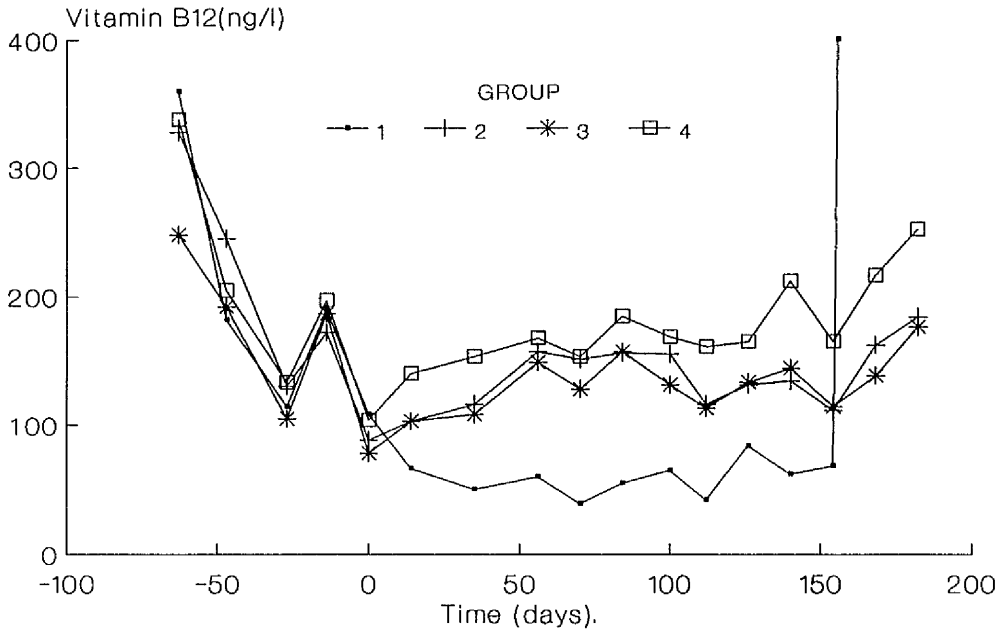


Fig 3.1 Serum Vitamin B12 Concentrations of Goats, Expt 1

Day 0 for all figures was the day on which cobalt supplementation was initiated

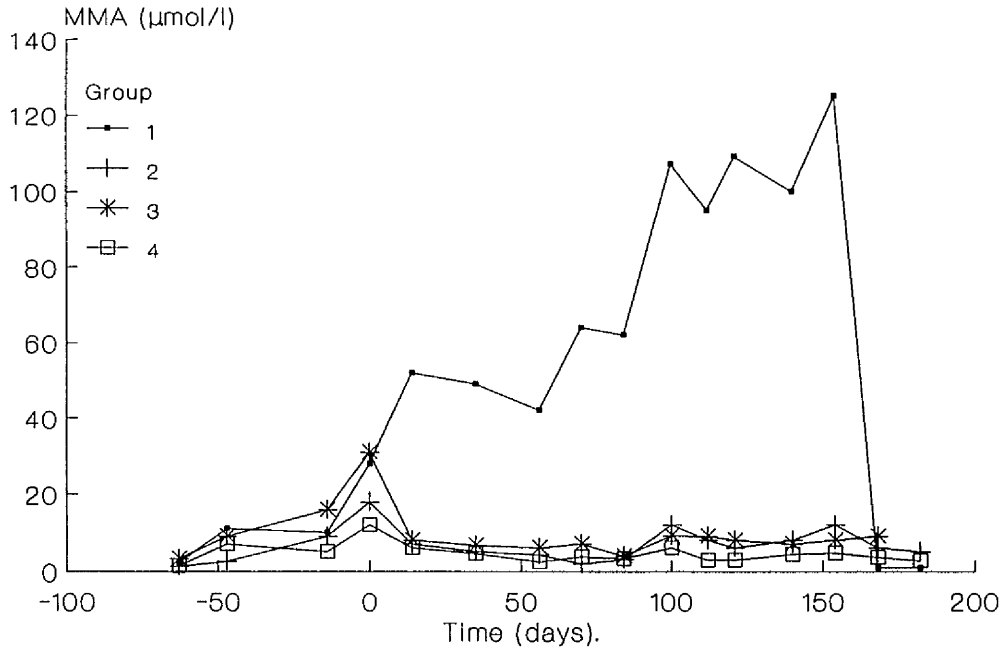


Fig 3.2 Serum MMA Concentrations of Goats, Expt 1

Group 1 goats received Cytamen on day 163

no significant differences in concentration among groups 2,3 and 4. The vitamin B_{12} injection administered to group 1 on day 163 caused serum MMA concentrations in these goats to decrease from 121 to <1 μmol/l by day 168.

The relationship between vitamin B_{12} concentration and MMA concentration can be seen in Fig.3.3. Despite the low correlation coefficient (-0.292) it can be seen that for most values of vitamin B_{12} above 200ng/l, MMA concentrations are low $<10\mu$mol/l. While for all MMA concentrations >10 μmol/l, vitamin B_{12} concentrations are, with one exception below 200 ng/l. There are a great number of points showing instances where both vitamin B_{12} concentrations and MMA concentrations are low (<200ng/l and $<10\mu$mol/l respectively). Vitamin E treatment had no significant effect on MMA concentration.

3.3.3. Serum GOT Activity.

Results, seen in Fig.3.4. and Table.3.9. in Appendix I, show significantly elevated GOT concentrations in group 1 animals at the end of the trial, suggesting that they were suffering some liver damage. Vitamin E treatment had no significant effect on serum GOT.

3.3.4. Liveweight.

The basal diet failed to maintain liveweight gain in the control group; liveweights of the other three groups increased steadily and by day 105 they were all significantly heavier than those animals in group 1,(Fig.3.5. and Table 3.10 Appendix I). There were no significant differences however among the 3 supplemented groups even when using liveweight on day zero as a covariate in the analysis. On regressing the results for each goat individually and comparing the derived constants there were no

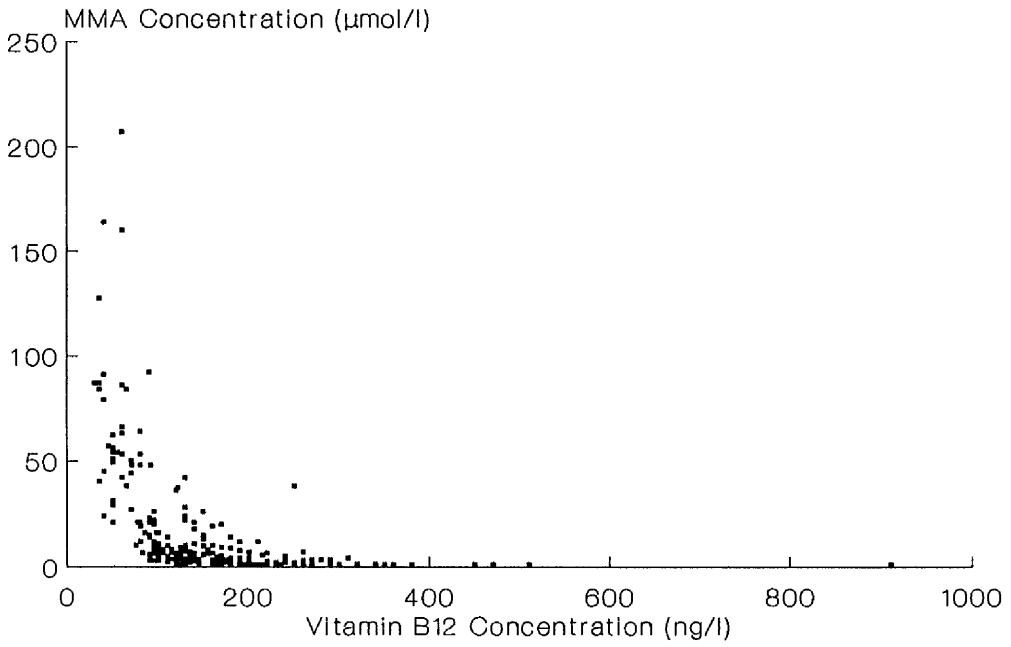


Fig 3.3 Serum Vitamin B12 versus MMA Concentrations of Goats

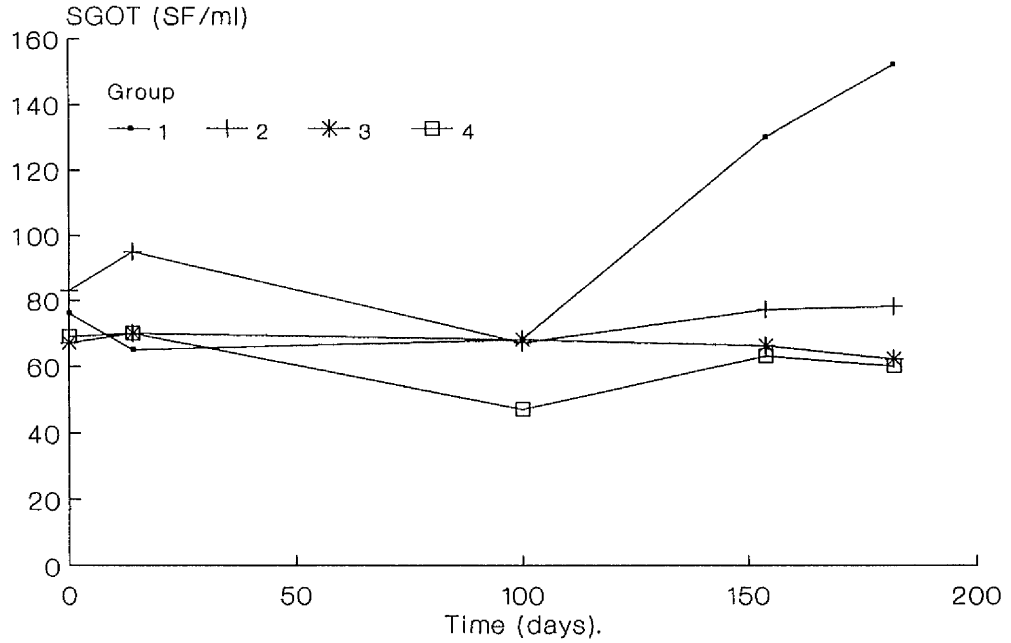


Fig 3.4 Serum GOT Activity of Goats, Expt 1

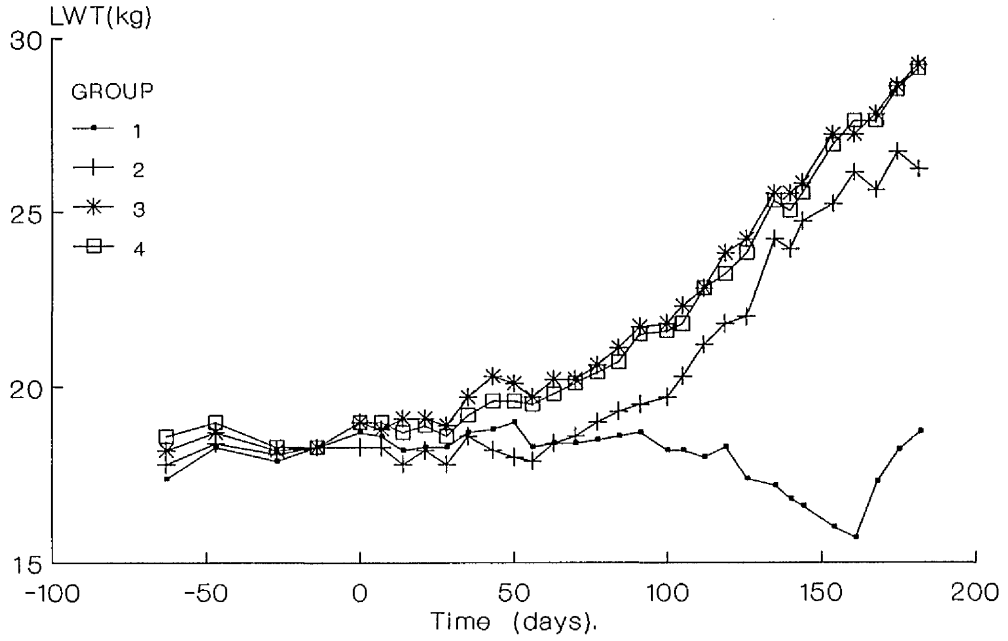


Fig 3.5. Liveweights of Goats, Expt 1

Group 1 goats received Cytamen on day 163

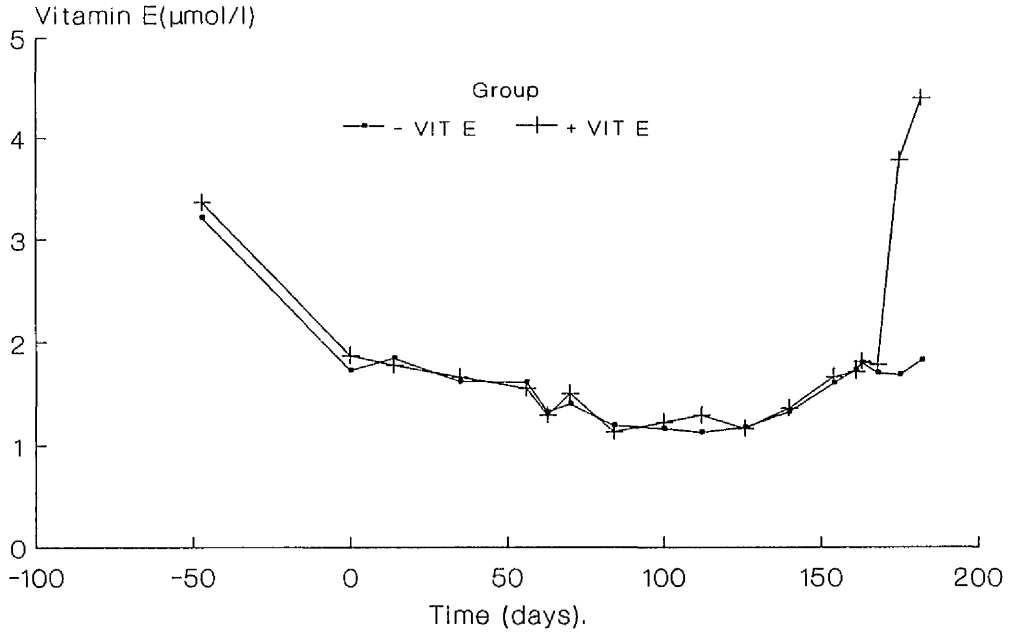


Fig 3.6 Plasma Vitamin E Concentrations of Goats, Expt 1

Vitamin E treatments started on day 169

significant differences, this may be partly due to the poor fit of the lines representing group 1, as initially they lost weight, and then began to gain weight after supplementation. On day -63 the animals later supplemented with vitamin E, which were unevenly distributed amongst the cobalt treatment groups (as described in section 3.2.2, experiment 1) had a significantly greater liveweight than those which were not later supplemented with vitamin E, however this was a chance effect as vitamin E treatments did not start until day 169. The difference in size, at the end of the trial, between a group 1 goat and a group 4 goat can be clearly seen (Plates 1 and 2).

3.3.5. Plasma Vitamin E Concentration.

Supplementation with vitamin E produced a significant effect, raising the plasma vitamin E concentration from 1.8 μmol/l to 4.4 μmol/l, see Fig.3.6. and Table 3.11. Appendix I. Plasma vitamin E concentration was unaffected by cobalt treatment.

3.3.6. Whole Blood GSH-Px Activity.

Despite some significant differences in whole blood GSH-Px activity among groups, and some values (day -14) indicating deficiency, all remained adequate until day 56 when all groups were low (although group 2 was significantly less so), see Table 3.12 Appendix I. After supplementation with selenium on day 105 concentrations rose until all groups were again sufficient. The vitamin E supplemented group had significantly lower GSH-Px activity on days 70, 84 and 100, however as vitamin E supplementation did not commence until day 169 this is unlikely to have affected response to vitamin E treatment.


Commander


Cavalier Commander

3.3.7. Plasma CK Activity.

Group 1 showed elevated CK activity compared to the cobalt supplemented groups but this was only significant on day 161 due to the large spread in values, (Fig.3.7. and Table 3.13 Appendix I). These results suggest that goats in group 1 had some muscle damage. However, vitamin E treatment did not significantly affect plasma CK activity.

3.3.8. Serum Copper, Calcium and Magnesium Concentrations.

Despite some differences among groups all had adequate concentrations of copper, calcium and magnesium, see Table 3.14. Appendix I.

3.3.9. Neutrophil Function.

There was no clear significant effect of treatment on neutrophil function, see Fig.3.8. and Table 3.15. Appendix I.

3.3.10. ELISA for *Clostridium tetani*.

There was no significant effect of cobalt treatment on antibody titre in response to vaccination, see Table 1. There were small apparent differences in response according to the day of vaccination, see Table 2, but once the difference in initial titre was taken into account this was barely significant even at the 10% level. Thus it is unlikely that a difference between cobalt treatment groups was masked by the different vaccination dates.

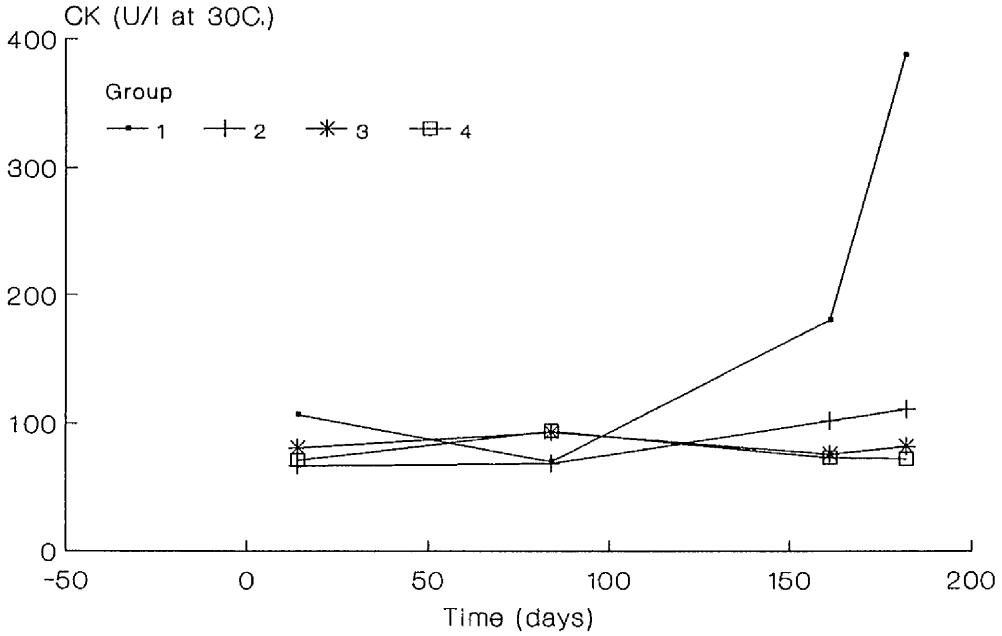


Fig 3.7 Plasma CK Activity of Goats, Expt 1

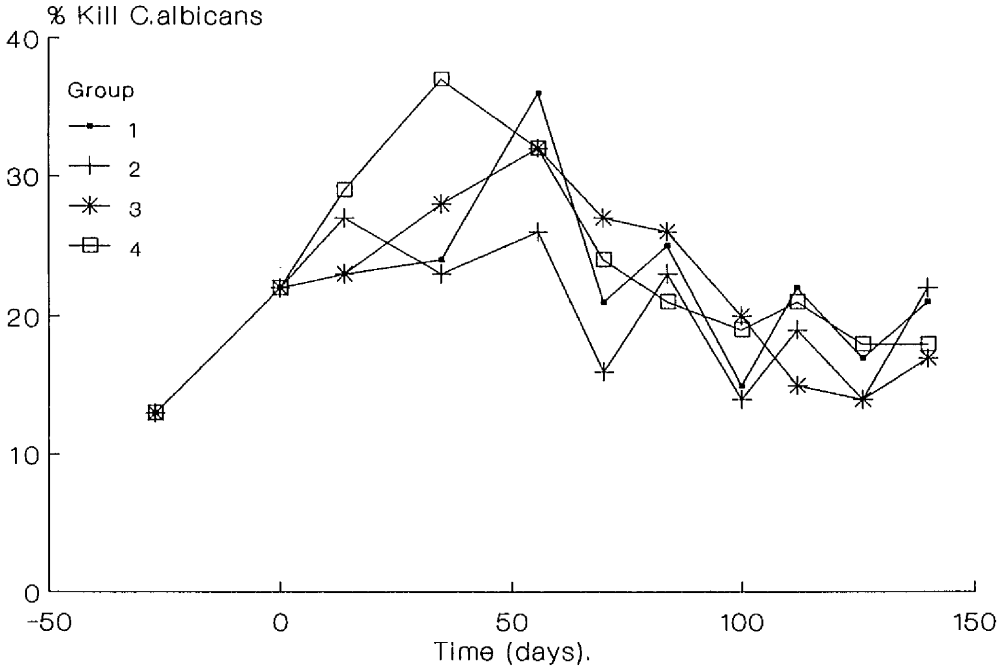


Fig 3.8 Neutrophil Function of Goats, Expt 1

Table 1. *Clostridium tetani* Antibody Titre (IU/ml)

Day No.	Cobalt Treatment Group Number				S.E.D	p
	1	2	3	4		
63	0.182	0.133	0.253	0.383	0.1929	0.601
84	9.345	5.348	5.710	7.905	3.24	0.575
154	2.775	0.653	0.717	1.00	1.08	0.193

Table 2. *Clostridium tetani* Antibody Titre (IU/ml)

Day No.	Vaccination Date		S.E.D	p
	63	69		
63	0.149	0.327	0.1308	0.188
84	5.116	8.99	2.13	0.084
154	0.572	2.00	0.758	0.073

Table 3. Erythrocyte Stability (% Haemolysis)

Day No.	Vitamin E Treatment		S.E.D.	p
	+E	-E		
0.5% TWEEN				
163	5.9	6.4	1.858	0.767
168	1.9	1.2	0.459	0.197
175	9.1	11	3.16	0.482
182	7.0	10	2.78	0.292
2% TWEEN				
163	33	19	10.68	0.197
168	2.0	1.4	0.716	0.411
175	12	21	6.76	0.191
182	4.8	6.2	1.881	0.455
6% TWEEN				
163	65	75	12.99	0.453
168	47	54	14.4	0.638
175	99	99	0.442	0.528
182	48[a]	82[b]	12.16	0.011

Table 4. Erythrocyte Stability (% Haemolysis)

Day No.	Cobalt Treatment Group Number				S.E.D	p
	1	2	3	4		
0.5% TWEEN						
163	5.6	6.2	5.7	7.0	2.739	0.955
168	1.2^{a}	2.6^{b}	1.4^{ab}	1.0^{a}	0.590	0.063
175	13	13	7	7	4.34	0.309
182	4.4^{a}	15^{b}	8.8^{ab}	5.5^{a}	3.29	0.016
2% TWEEN						
163	31	42	19	13	14.93	0.261
168	1.2^{a}	3.7^{b}	1.0^{a}	1.0^{a}	0.791	0.005
175	20^{ab}	30^{b}	8.8^{a}	11^{a}	9.04	0.103
182	3.2	8.7	4.5	5.0	2.525	0.196
6% TWEEN						
163	70^{ab}	87^{b}	77^{ab}	46^{a}	17.09	0.142
168	53^{ab}	78^{b}	34^{a}	37^{a}	18.3	0.092
175	99	99	100	99	0.654	0.407
182	55^{a}	92^{b}	72^{ab}	40^{a}	16.99	0.036

3.3.11 Erythrocyte Stability.

Vitamin E supplementation significantly decreased % erythrocyte haemolysis in the presence of 6% Tween on day 182, there were no other apparent effects of vitamin E treatment, see Table 3. Significant differences between the response of the different cobalt treatment groups were observed, see Table 4, with group 2 showing an increased % erythrocyte haemolysis.

3.4. Results:- Experiment 2

3.4.1. Serum Vitamin B_{12} Concentration.

Once again the basal diet failed to maintain serum vitamin B_{12} concentrations above 100 ng/l; and in the present trial, values fell as low as 30 ng/l, (Fig.3.9. and Table 3.16 Appendix I). In relation to values for group 1 receiving no supplementary cobalt, concentrations for the supplemented groups were slightly greater but group 2 was only significantly greater on 2 occasions, groups 3 and 4 on 3 occasions, group 5 on 5 occasions and group 6 on all occasions after supplementation. Group 5 was also significantly greater than groups 2 and 3 on 2 occasions, and group 6 was significantly greater than all other groups on three occasions. All these differences were significant when concentration at day 0 was used as a covariate. Supplementation of group 1 goats with oral cobalt from day 154, (one animal also recieved vitamin B_{12} injections) increased serum vitamin B_{12} concentrations in these animals to >200 ng/l within a week. Vitamin E treatment had no significant effect on vitamin B_{12} concentration.

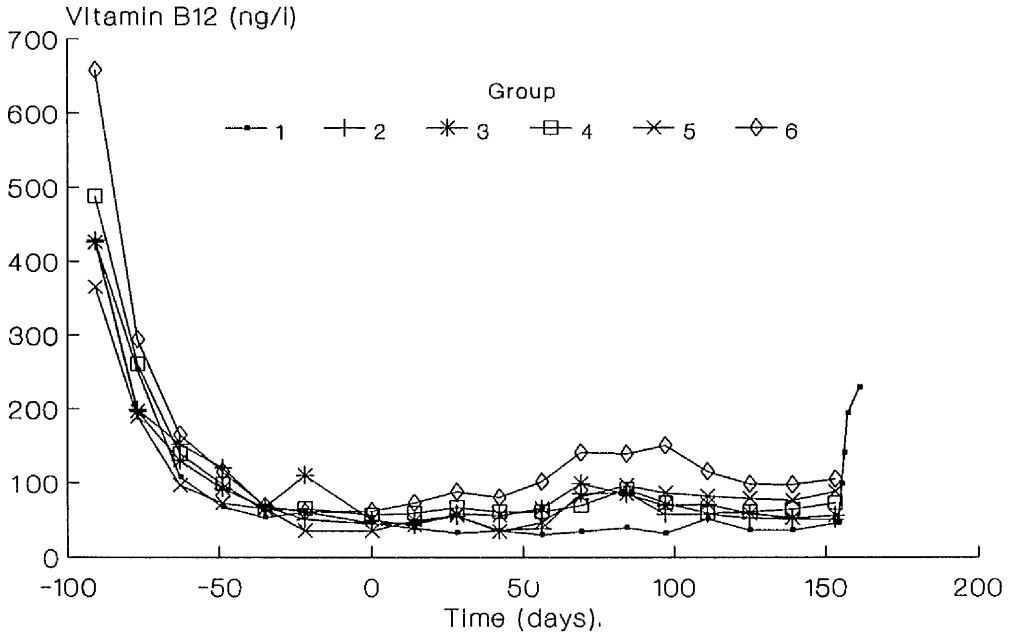


Fig 3.9 Serum Vitamin B12 Concentrations of Goats, Expt 2

Group 1 received cobalt from day 154

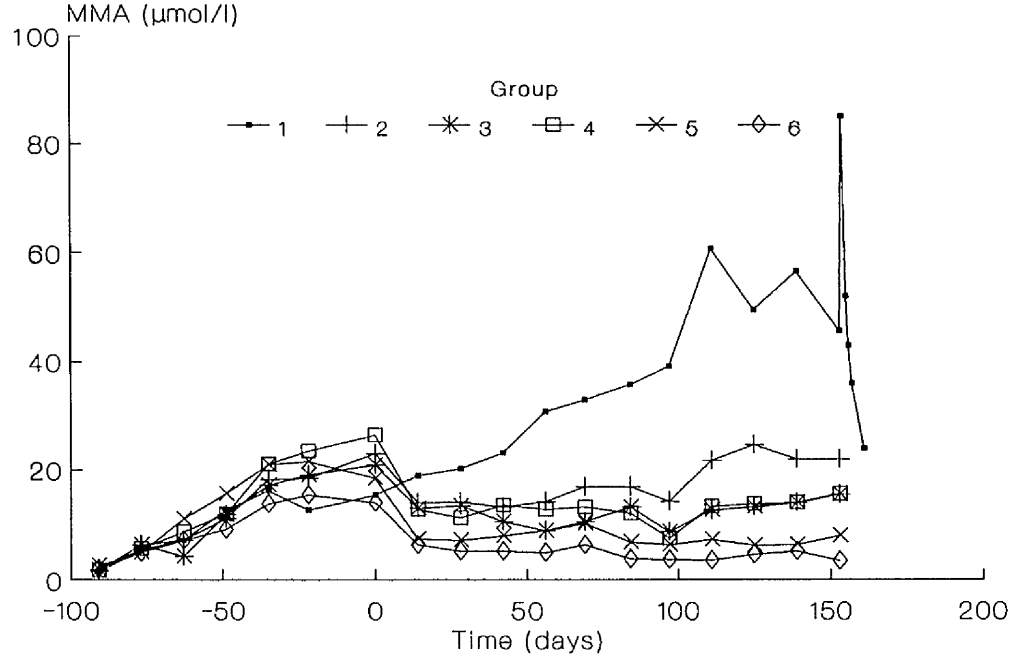


Fig 3.10 Serum MMA Concentrations of Goats, Expt 2

3.4.2. Serum MMA Concentration.

MMA concentrations for all groups rose slowly until day 0, see Fig.3.10. and Table 3.17. Appendix I. Concentrations for group 1 continued to rise and reached a peak of 85 μmol/l on the day they received their first oral cobalt supplement. For groups 2 to 6 concentrations returned to <20 μmol/l, for groups 2 and 3, and $<10\mu$mol/l for groups 4 to 6. Even when concentrations on day 0 were used as covariates the differences between groups 2 to 6 were not significant. The difference between group 1 and all other groups was highly significant from the first supplementation. Supplementation of the group 1 goats with oral cobalt (and of one animal with vitamin B_{12} injections) returned their serum MMA concentrations to <30 μmol/l within a week.

The relationship between vitamin B_{12} and MMA concentration is shown in Fig.3.11 and shows a similar pattern to that for the first experiment, except that this time elevated MMA concentrations ($>5\mu$mol/l) seem to be associated with vitamin B_{12} concentrations below 100 ng/l, however in this experiment there was only one vitamin B_{12} concentration between 100 and 200 ng/l. The correlation coefficient on this occasion was -0.283.

3.4.3. Serum Succinate Concentration.

Serum succinate concentrations for all groups seem to follow similar trends with a large peak between days 40 and 100, see Fig.3.12. and Table 3.18. Appendix I. When concentrations on day 0 were used as covariates significantly lower concentrations were observed for group 1 than for all other groups on at least 2 occasions. No other group was consistently higher or lower than the rest.

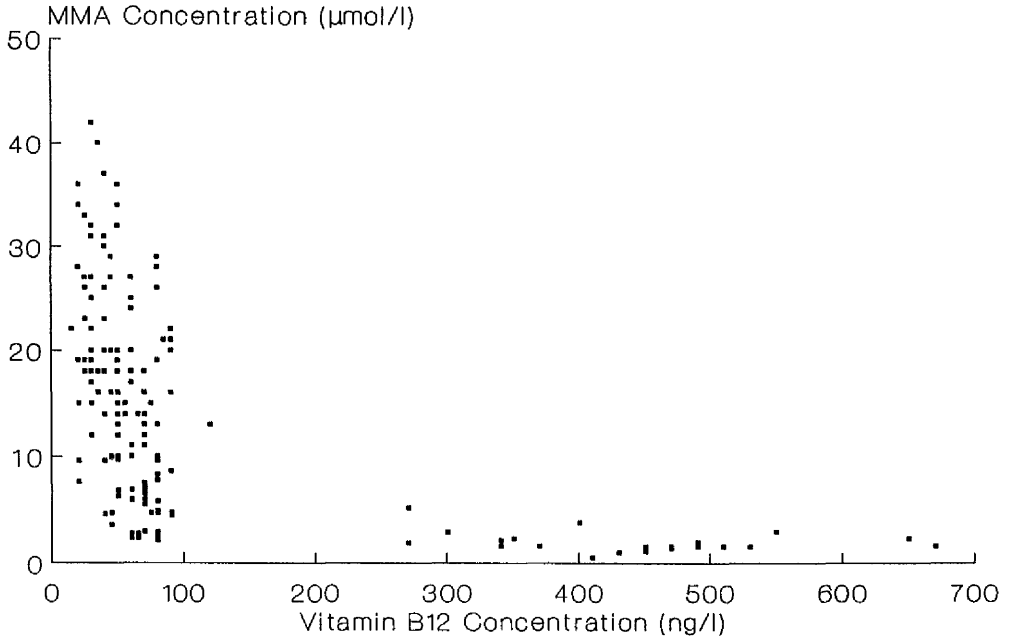


Fig 3.11 Serum Vitamin B12 versus MMA Concentrations

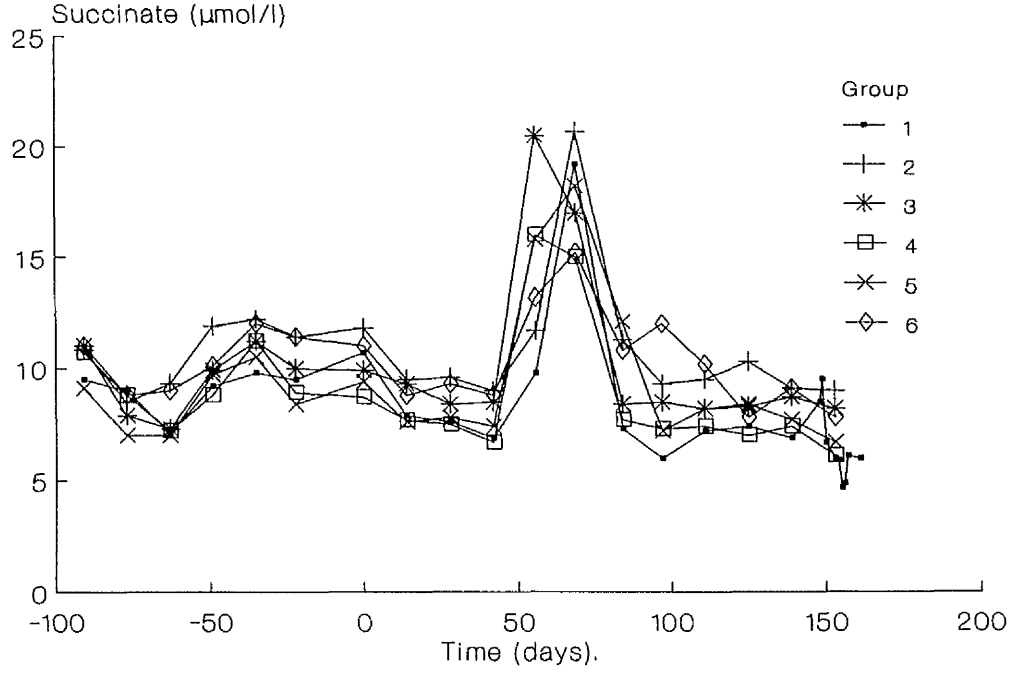


Fig 3.12 Serum Succinate Concentrations of Goats, Expt 2

The correlation between succinate concentration and vitamin B_{12} concentration is even lower than that for MMA (-0.017) and indeed the correlation coefficient for succinate concentration versus MMA concentration is also extremely low (-0.037).

3.4.4. Serum GOT Activity.

Serum GOT activities of goats, seen in Fig.3.13 and Table 3.19 Appendix I, show no particular trend in relation to cobalt intake. Group 1 was significantly higher than 2 other groups on day 0, group 6 was significantly higher than group 4 on day 42, and was significantly higher than groups 3,4 and 5 on day 56. Group 1 was significantly higher than group 4 on day 69, while group 5 was significantly higher than groups 3 and 4 on day 97. However if you count the number of occasions on which each group mean has exceeded 100 SF units, group 1 has 7, group 2 has 4, group 5 has 1 and group 6 has 4. This may suggest group 1 had the most liver damage but the evidence is not very convincing. Vitamin E treatment had no significant effect on serum GOT.

3.4.5. Liveweight.

All groups gained steadily in weight throughout the trial, see Fig.3.14. and Table 3.20 Appendix I. When liveweight on day 0 was used as a covariate in the analysis, group 1 gained significantly less weight during the trial than groups 2, 3 and 6. Group 2 was the first group to show a significant improvement over the other groups. On regressing the liveweights for each goat individually and comparing the derived constants there were no significant effects of either cobalt or vitamin E supplementation on the intercept, but cobalt supplementation group 3 had a significantly greater slope than group 1, all other groups being intermediate. Vitamin E treatment had no effect on liveweight.

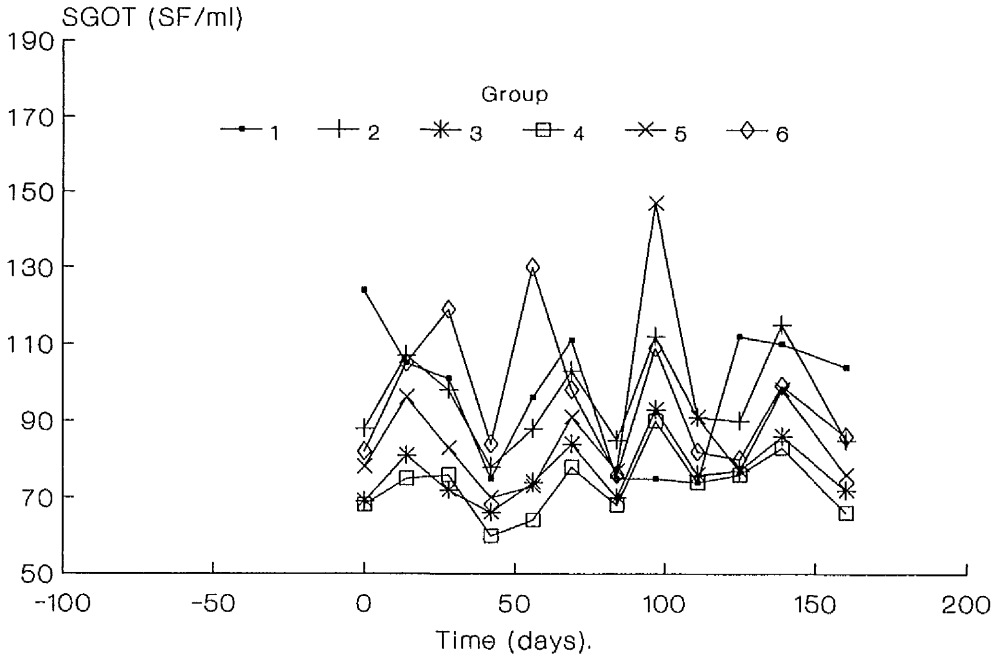


Fig 3.13 Serum GOT Activity of Goats, Expt 2

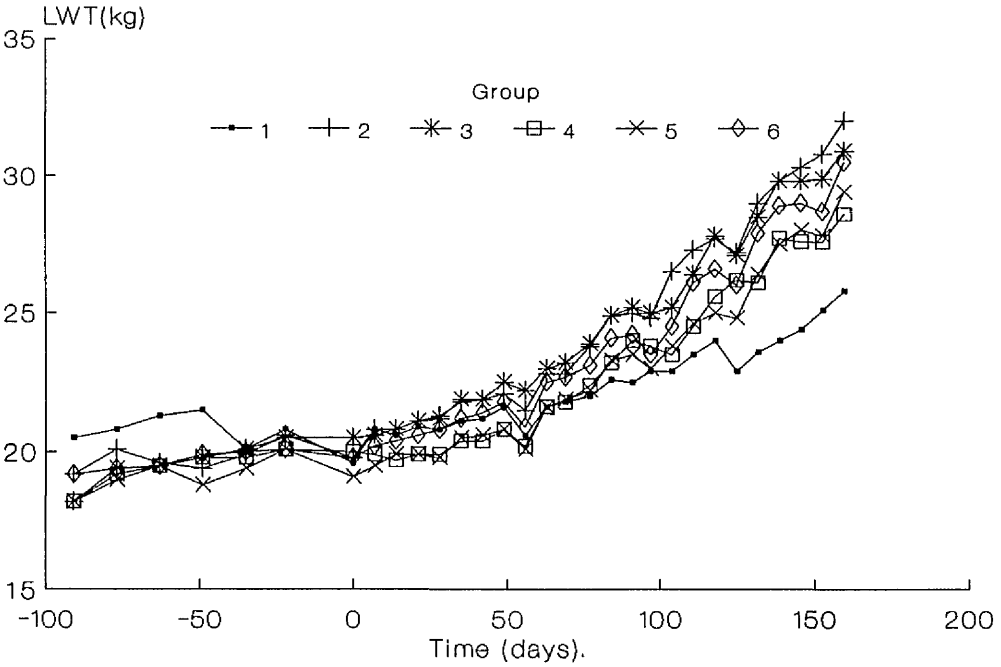


Fig 3.14 Liveweights of Goats, Expt 2

3.4.6. Plasma Vitamin E Concentration.

Vitamin E injection had a highly significant effect on plasma vitamin E concentrations which peaked at over 50 μmol/l, the day after injection, but fell rapidly to 7 μmol/l within 7 days, see Fig.3.15 and Table 3.21 Appendix I. Cobalt treatment had significant effects on vitamin E status on day 69 when group 1 was significantly lower than groups 3 and 6, on day 97 when group 1 was significantly lower than groups 2 and 3, on days 111, 125, 139, and 153 when group 2 was significantly greater than all other groups. This last effect was due however to an uneven distribution of goats between treatments as all goats in group 2 received vitamin E supplementation. This was because vitamin E supplementation was given to one pen of goats but the cobalt treatment groups were not equally distributed between the pens. Hence two animals from groups 1, 3, 5 and 6, and all 3 remaining animals from group 2 recieved the vitamin E supplement, while no animals from group 4 were supplemented.

3.4.7. Whole Blood GSH-Px Activity.

Group 3 GSH-Px activity tended to be significantly greater than that of group 4, but all groups were adequate until day 28, see Table 3.22 Appendix I. On day 35 all animals were supplemented and from this date all values rapidly returned to adequacy. Vitamin E treatment had no significant effects on GSH-Px activity.

3.4.8. Plasma CK Activity.

High CK activity was found in animals from each group, see Fig.3.16 and Table 3.23 Appendix I. Group 2 CK activity was significantly greater on day 56, group 5 on day 97, but group 1 was significantly greater on days 125 and 153 suggesting that

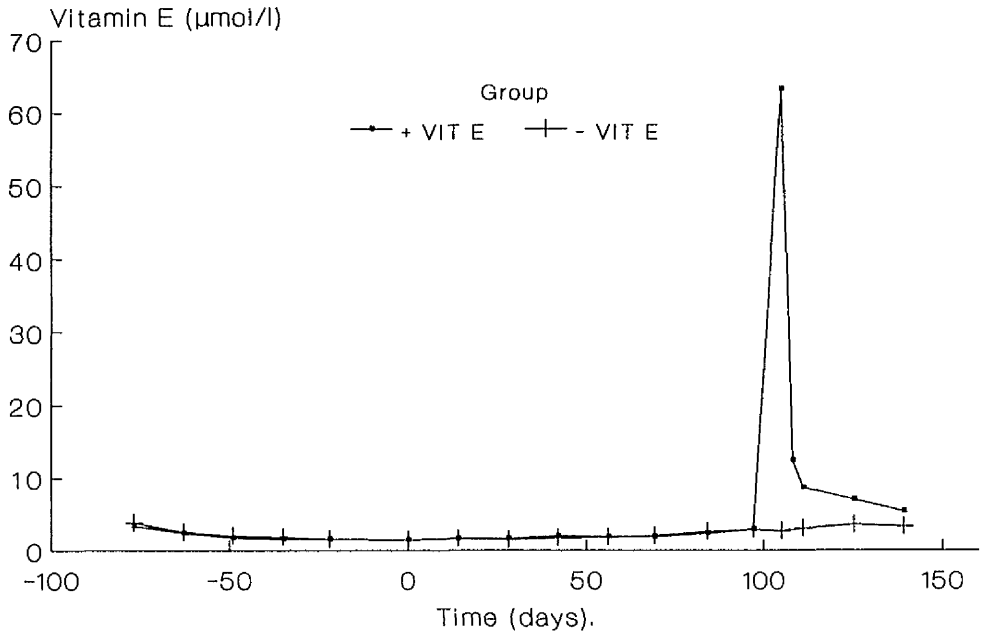


Fig 3.15 Plasma Vitamin E Concentrations of Goats, Expt 2

Vitamin E injection on day 104

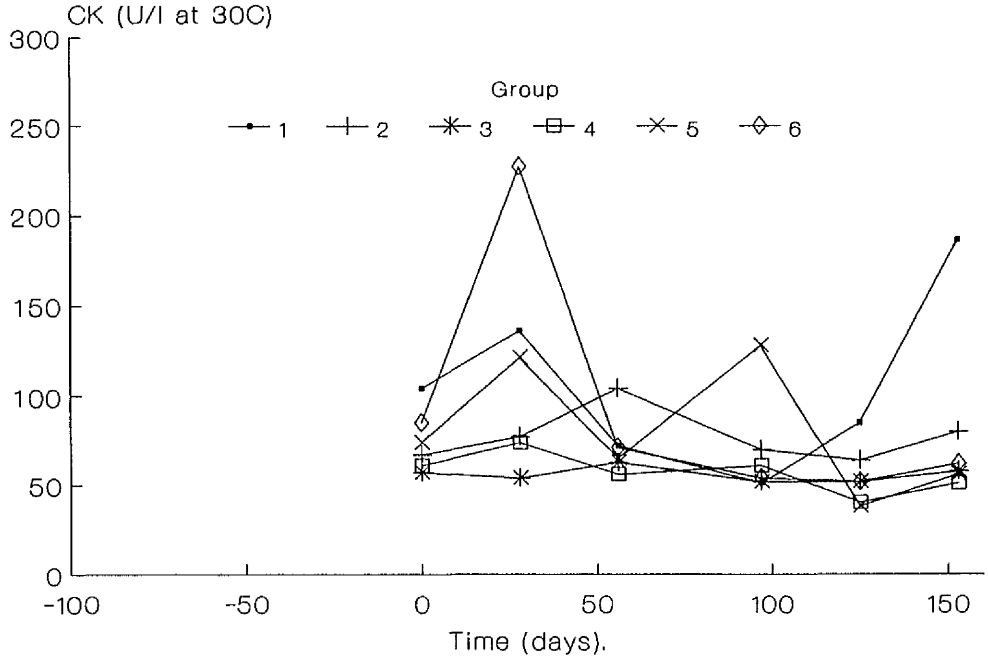


Fig 3.16 Plasma CK Activity of Goats, Expt 2

this group may have experienced significantly more muscle damage as the experiment progressed. Vitamin E treatment had no significant effect on CK.

3.4.9. Serum Copper, Calcium and Magnesium Concentrations.

There were some small but significant differences in copper, magnesium and calcium concentrations, see Table 3.24. Appendix I. However as all groups were always within the normal range for these parameters these differences are unlikely to have had any confounding effects.

3.4.10. Whole Blood Differential Cell Counts.

Group 1 had a significantly higher proportion of neutrophils than groups 3,5 and 6 on day 153, and a significantly lower proportion of lymphocytes than group 2 on day 125 and groups 3 and 6 on day 153, see Table 3.25 Appendix I. Group 1 had a significantly lower proportion of monocytes than groups 3,4 and 5 on day 28 and lower than group 2 on day 56. Group 4 had a significantly higher proportion of basophils on day 125 than any other group.

3.4.11. Rumen Succinate Concentrations.

GOAT	Days Post Supplementation						
	-1	0	1	2	3	7	8
1	66	102	54	24	22	140	19
2		312	122	64	33		
3		312	42	20	15		
4		138	68	86	210		
Mean	-	211	72	46	95	-	-

Rumen succinate concentrations, measured for group 1 goats during supplementation (from day 154), were much greater than serum concentrations (ranging from 20 to 300 μmol/l as opposed to 1 to 100 μmol/l). Concentrations decreased after supplementation but this was not consistent.

3.5. Results:- Experiment 3

3.5.1. Serum Vitamin B_{12} Concentration.

Once again the basal diet failed to maintain concentrations above 100 ng/l, (Fig.3.17 and Table 3.26 Appendix I). Concentrations for group 1 fell as low as 30 ng/l. Despite this they were only significantly lower than those of group 4, and on 2 occasions of group 3, even when concentrations on day 0 were used as covariates.

3.5.2. Serum MMA Concentration.

Concentrations for groups 1,2 and 3 rose even after day 0 with group 1 peaking at 136 μmol/l group 2 at 57 μmol/l and group 3 at 79 μmol/l, group 4 concentrations were significantly lower remaining below 20 μmol/l, see Fig.3.18 and Table 3.27 Appendix I. When concentrations on day 0 were used as covariates there were some significant differences between groups 1,2 and 3 with group 1 concentrations being the highest.

The relationship between vitamin B_{12} concentrations and MMA concentrations (Fig.3.19) was similar to the previous results with MMA concentrations >10 μmol/l corresponding to vitamin B_{12} concentrations <100 ng/l and vitamin B_{12} concentrations >100ng/l corresponding to MMA concentrations <10 μmol/l. The correlation was again low (-0.296).

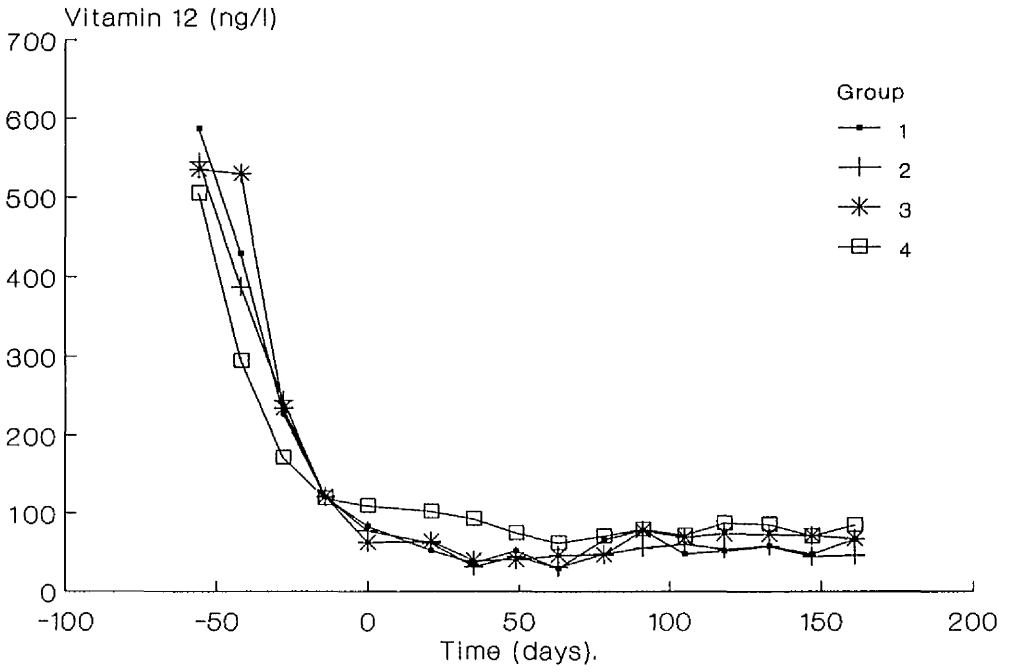


Fig 3.17 Serum Vitamin B12 Concentrations of Goats, Expt 3

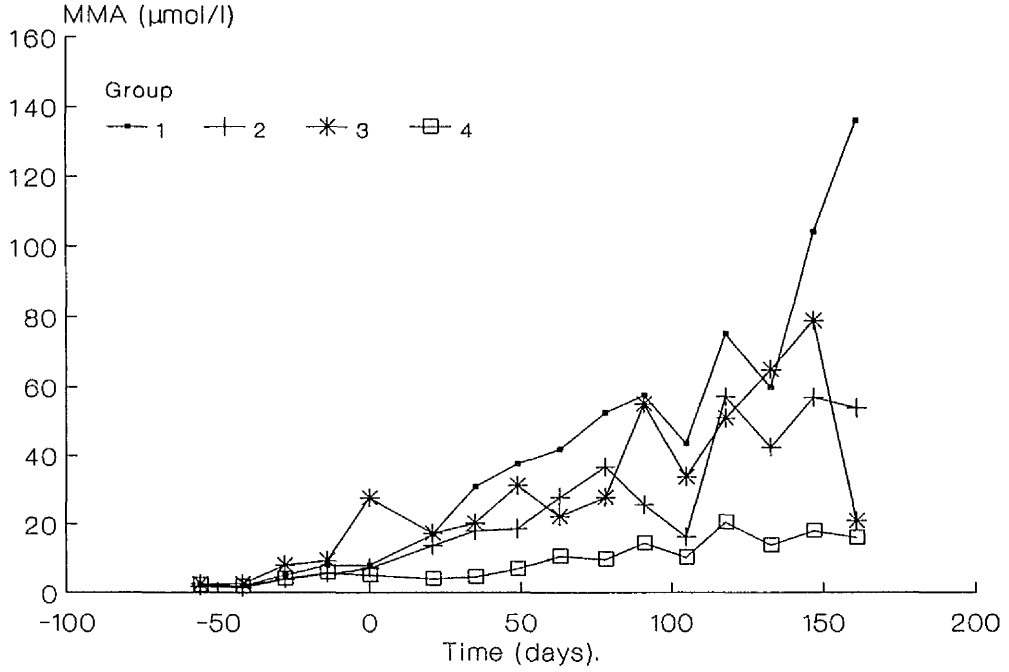


Fig 3.18 Serum MMA Concentrations of Goats, Expt 3

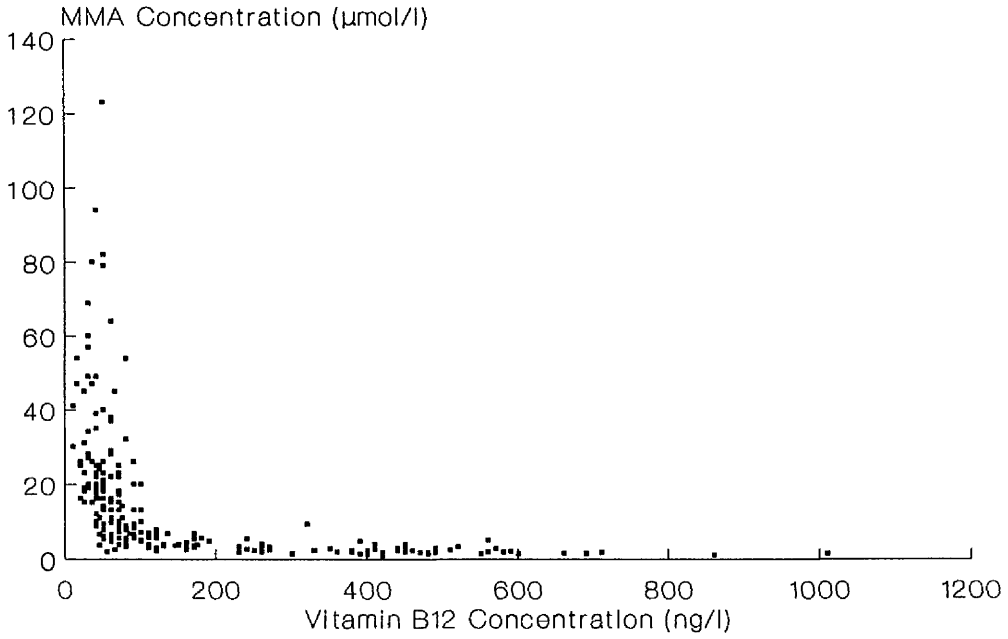


Fig 3.19 Serum Vitamin B12 versus MMA Concentrations

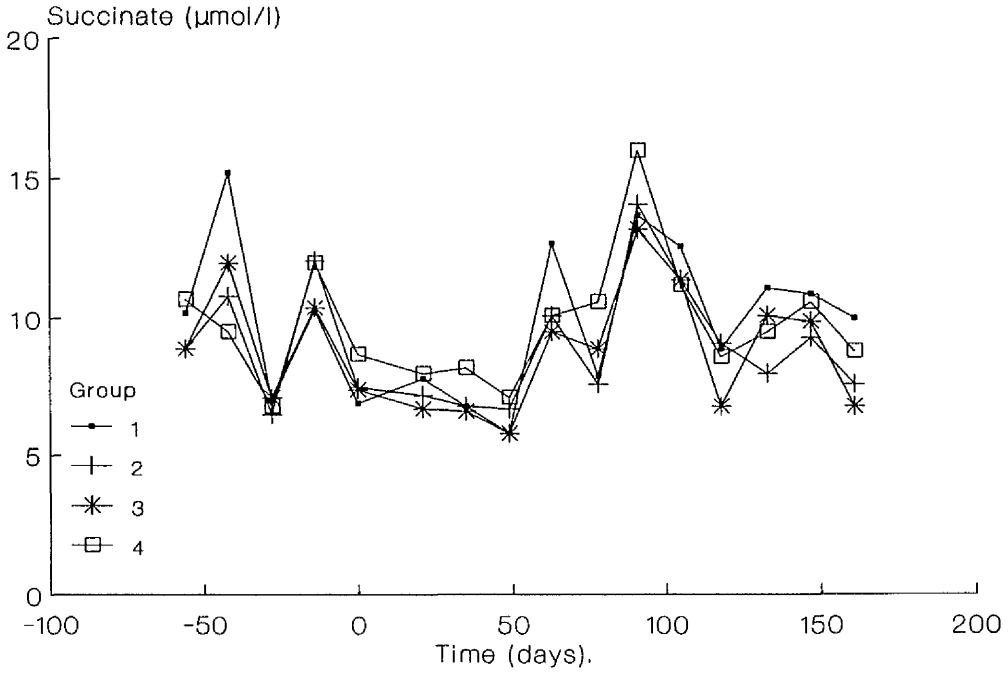


Fig 3.20 Serum Succinate Concentrations of Goats, Expt 3

3.5.3. Serum Succinate Concentration.

Generally trends for all groups follow the same pattern although group 3 concentrations were significantly lower than all other groups (when concentrations on day 0 were used as covariates) on day 118, see Fig.3.20 and Table 3.28 Appendix I. The correlation between succinate concentration and vitamin B_{12} concentration was very low (0.124) and that between succinate concentration and MMA concentration was even lower (0.022), suggesting that there was no strong relationship with either.

3.5.4. Serum GOT Activity.

The elevated serum GOT activities seen for group 1 in Fig.3.21 and Table 3.29 Appendix I, were only significantly greater than those of group 3 on day 133 as they are the result of very high values in one or two animals. In practical terms 2 out of 6 goats in group 1 were showing signs of severe liver damage.

3.5.5. Liveweight.

Group 1 goats did not show steady growth at all and by day 84 they began to lose weight, see Fig.3.22. and Table 3.30 Appendix I. Groups 2 and 3 grew significantly more than group 1 (using liveweight on day 0 as a covariate) while group 4 grew significantly better than groups 1, 2 and 3, although on the very last day of the experiment the differences in liveweight between groups 2,3 and 4 were just below significance. The difference in size between group 1 goats and group 4 goats at the end of the experiment can be seen in plates 3 and 4.

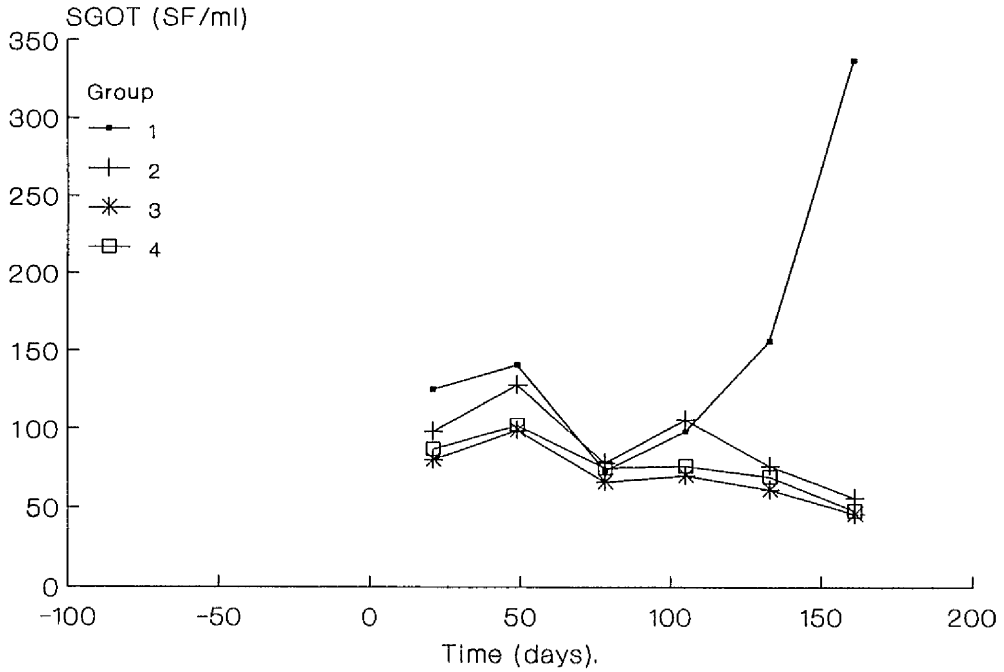


Fig 3.21 Serum GOT Activity of Goats, Expt 3

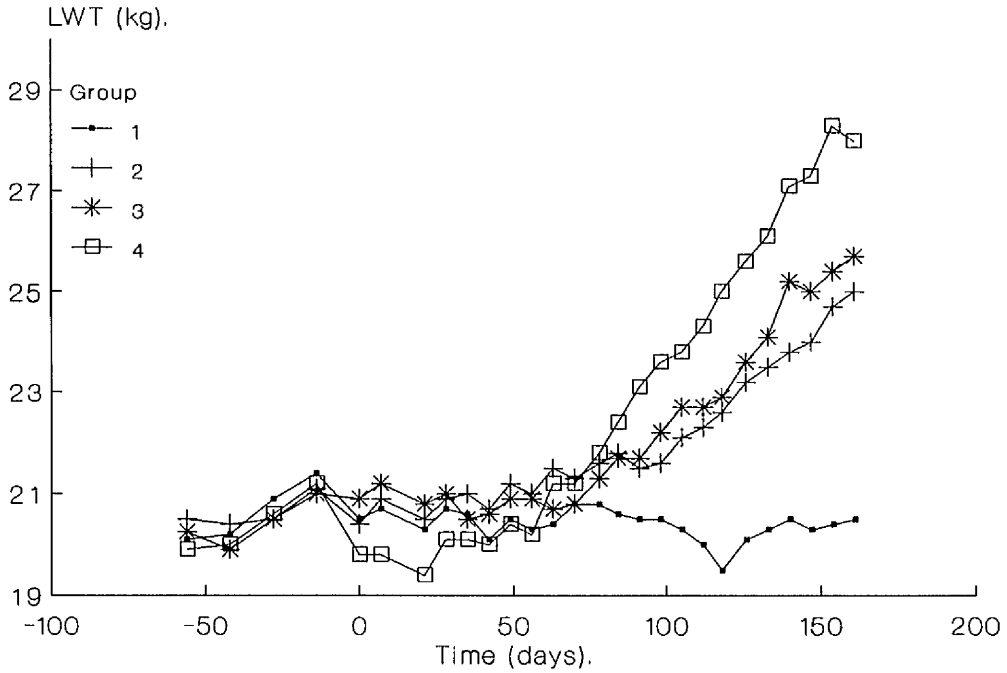


Fig 3.22 Liveweights of Goats, Expt 3

PLATE 3

Group 1 goat (left) compared to a Group 4 goat (right)

PLATE 4

Group 1 goat (right) compared to a Group 4 goat (left)

On regressing the individual results for each goat and comparing the derived constants there were no significant differences between groups. This may be due to the poor fit of the lines for group 1 due to the loss of weight.

3.5.6. Plasma CK Activity.

Group one had elevated CK activity on days 133 and 161, see Fig.3.23 and Table 3.31 Appendix I, but this was only significant on day 133 as 1 or 2 very high values influenced the group 1 mean. In effect 2 of the 6 goats had moderate muscle damage (CK > 100μl at 30°C) and 2 others had severe muscle damage (CK > 1000μl at 30°C).

3.5.7. Whole Blood GSH-Px Activity.

GSH-Px activity was normal for all groups, see Table 3.32 Appendix I. Nevertheless supplementation was carried out on day 154 as 2 goats had below adequate activity.

3.5.8. Serum Copper, Magnesium and Calcium Concentrations.

Plasma copper concentrations fell considerably during the trial but remained at the lower limit of normality. Magnesium and calcium concentrations remained adequate, see Table 3.33 Appendix I.

3.5.9. Plasma IgG Concentration.

No significant differences in IgG concentrations between groups, as measured by SRID, were observed, see Table 3.34 Appendix I.

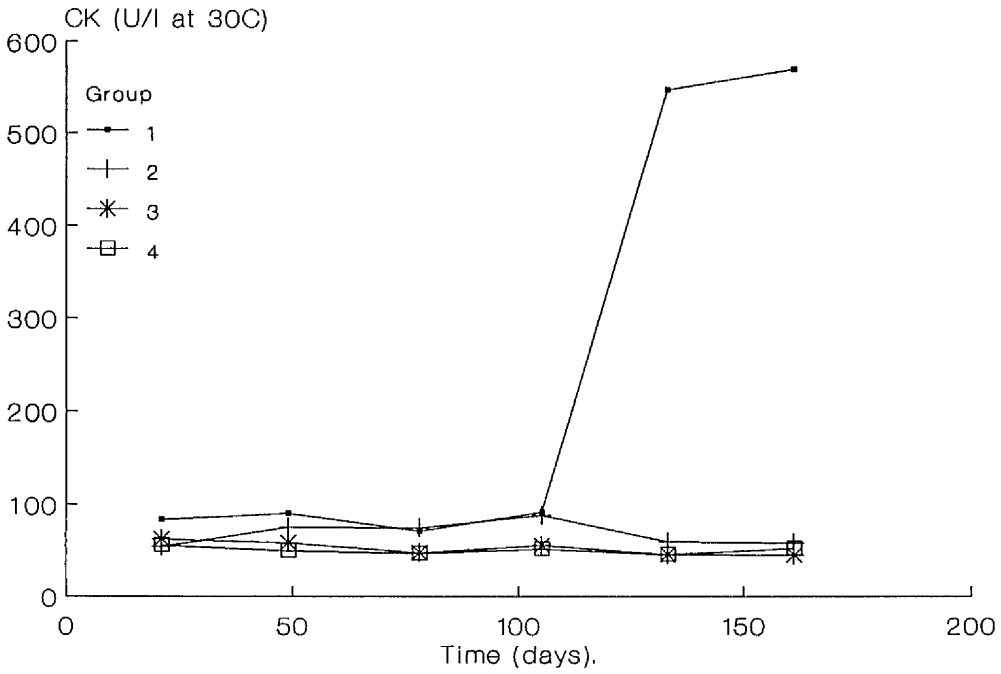


Fig 3.23 Plasma CK Activity of Goats, Expt 3

3.6. Discussion

From experiment 1 it was clear that group 1 were cobalt/vitamin B_{12} deficient, as they had very low serum vitamin B_{12} concentrations (30ng/l), elevated MMA concentrations (120μmol/l), some elevated serum GOT activity ($>$140 SF/ml) and they lost weight over the trial. All of the other three groups had vitamin B_{12} concentrations above 100ng/l, MMA concentrations below 10 μmol/l, serum GOT activities $<$100 SF/ml and they all gained weight. Some animals in group 1 also had watery eyes and pale mucous membranes. From this it was concluded that the requirement for dietary cobalt lay between 0.04 and 0.10 mg/kgDM.

Experiment 2 aimed to define the requirement more precisely, although this was complicated slightly as the basal diet contained more cobalt. Again group 1 had low vitamin B_{12} concentrations (30ng/l) and elevated MMA concentrations (80 μmol/l), although vitamin B_{12} concentrations for the other 5 groups were not much greater (40-150 ng/l) their MMA concentrations remained below 20 μmol/l. Serum GOT activities were not significantly greater for group 1 than for the cobalt supplemented groups and liveweight differences were not significant. However, at the end of this trial it was noticed that 3 of the 4 goats in group 1 were eating hay in preference to concentrates at feeding times. This symptom of cobalt deficiency had been recognised by MacPherson (1982) in sheep and cattle. From these results it appeared that cobalt concentrations of $<$0.06 mg/kgDM were inadequate.

The results of experiment 2 were not as clear cut as we had hoped because of the change in concentration of cobalt in the basal diet and because of the small group sizes. For these reasons the third experiment was carried out and straw was used to decrease the concentration of cobalt in the basal diet. Once again group 1 were deficient, showing low vitamin B_{12} concentrations (30 ng/l), elevated MMA concentrations ($>$130 μmol/l), elevated serum GOT activity and liveweight loss. In

this experiment groups 2 and 3 also had elevated MMA concentrations (>50 μmol/l) although their vitamin B_{12} concentrations rose to 90ng/l. Once more it appeared that 0.06 mgCo/kgDM was insufficient to prevent elevated MMA concentrations.

Figures 3.24 to 3.27 summarise the data for the marginal groups from the three experiments. Clearly 0.04 mgCo/kgDM was insufficient to prevent liveweight loss, and these two groups also had elevated serum GOT activity. Concentrations up to 0.06 mgCo/kgDM maintained liveweight gain although possibly not at maximal rates, but did not prevent elevation of MMA concentrations. Concentrations above 0.06mgCo/kgDM maintained MMA concentrations below 20μmol/l, but only supplementation at 0.10mgCo/kgDM caused vitamin B_{12} concentrations to rise above 100 ng/l.

Overall 0.06 mgCo/kgDM is probably insufficient while 0.07 mgCo/kgDM would probably prevent deficiency. This is higher than the minimum of 0.04 mg/kgDM quoted by Andrews (1970a) for cows, but very similar to the minimum of 0.06 to 0.07 mg/kgDM quoted by MacPherson *et al* (1973). It is also similar to the 0.07 mg/kgDM (Underwood 1981) and the 0.08 mg/kgDM (Andrews 1970a) minimums quoted for sheep.

These results contradict Platten (1951) who considered that goats required more cobalt than sheep, however this opinion was not based on scientific trials merely on observations of one goat herd. These results confirm the findings of Mgongo (1984) who reported 0.01 mg/kgDM to be insufficient, and suggest that had Clark *et al* (1987) continued their trial beyond 4 months they would have found 0.035 mgCo/kgDM to be insufficient.

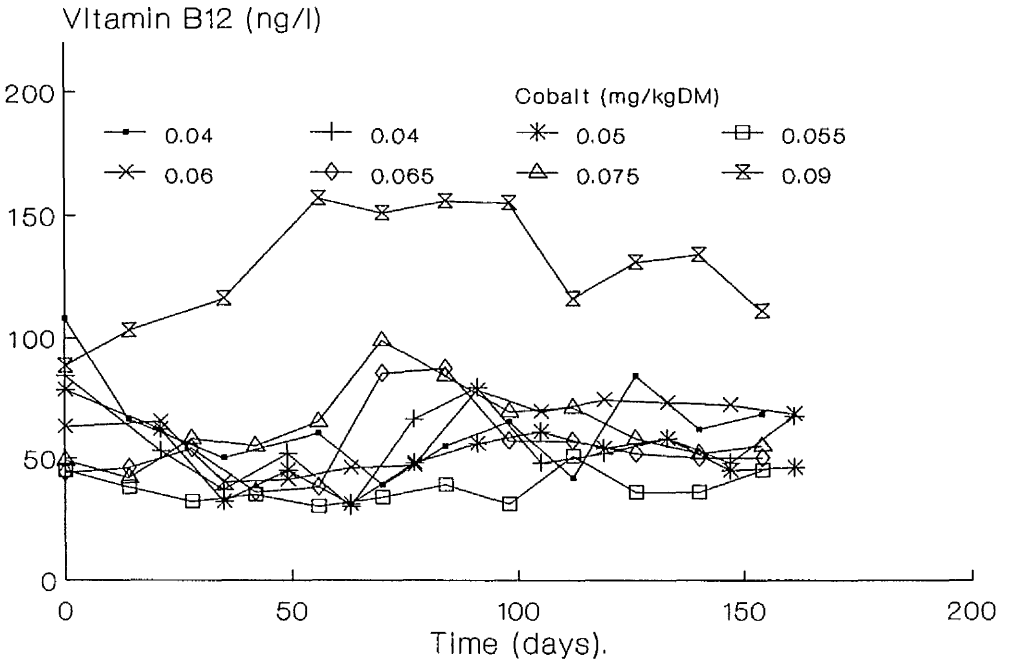


Fig 3.24 Serum Vitamin B12 Concentrations of Goats, Overall

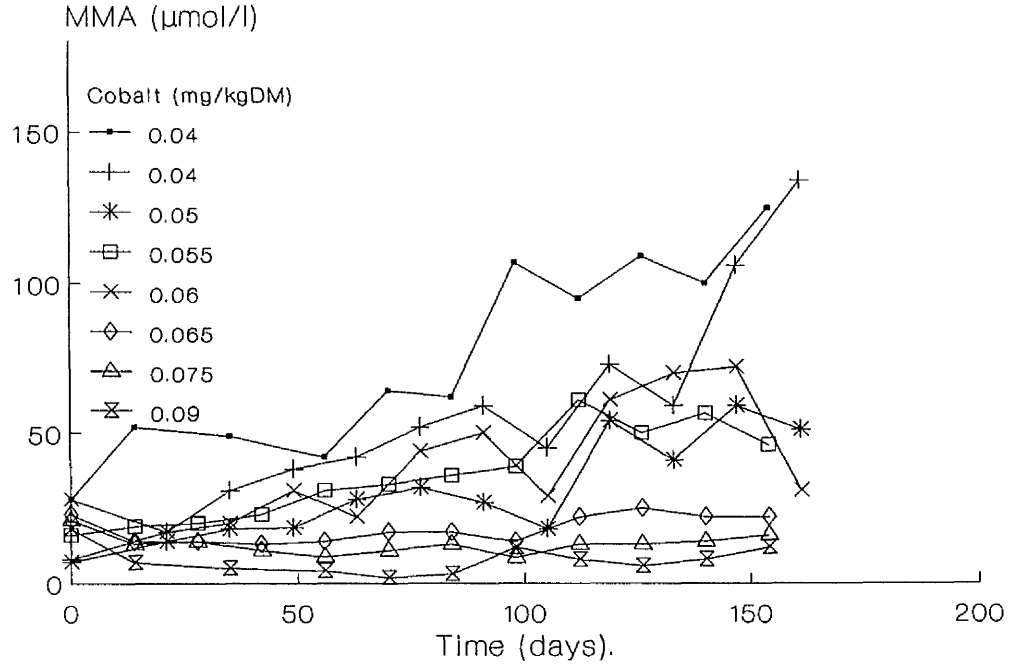


Fig 3.25 Serum MMA Concentrations of Goats, Overall

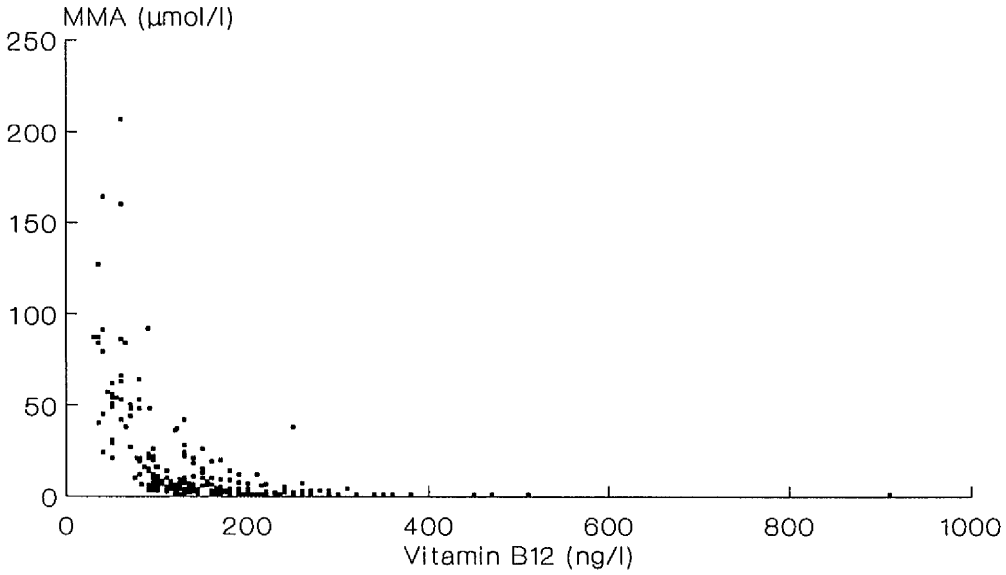


Fig 3.26 Serum Vitamin B12 versus MMA Concentrations of Goats, Over All Three Experiments

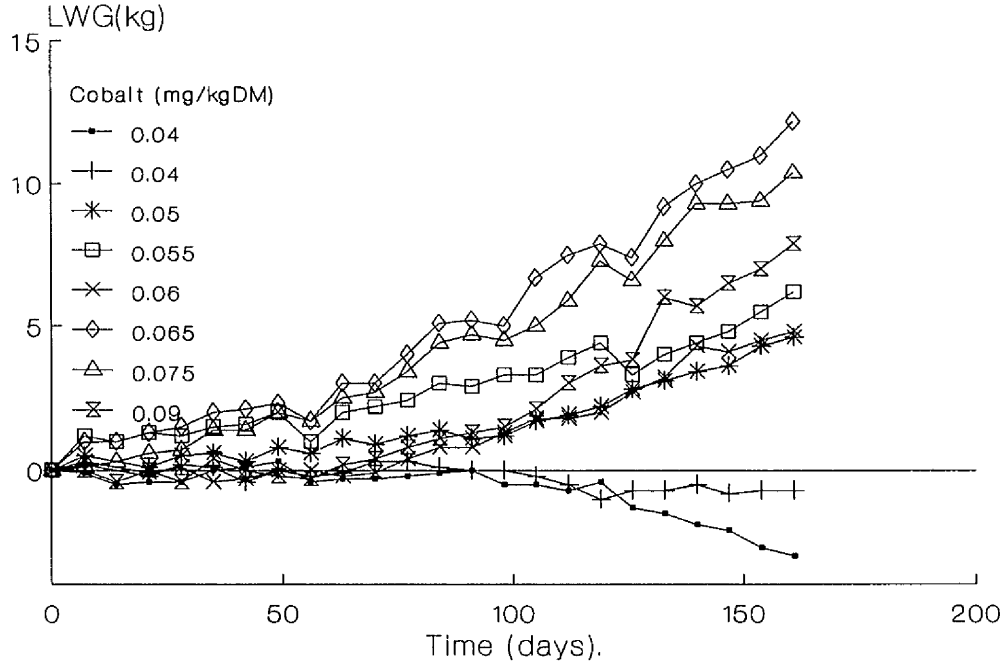


Fig 3.27 Liveweight Gains of Goats During Supplementation

MMA concentrations of up to 15 μmol/l occurred when no other symptoms of deficiency were apparent. In the deficient groups concentrations in excess of 100 μmol/l were recorded. Thus it is suggested that for diagnostic purposes serum MMA concentrations between 15 and 20 μmol/l are indicative of marginal cobalt/vitamin B_{12} status. This is higher than comparable values for sheep (10-15 μmol/l) and cattle (2-4 μmol/l).

Vitamin B_{12} concentrations as low as 30 ng/l were measured in all three experiments and concentrations between 50 and 100 ng/l were common in groups experiencing only slightly decreased liveweight gain, thus it is suggested that this probably represents marginal deficiency. This is much lower than the concentrations regarded as indicative of marginal vitamin B_{12} status in cattle (150-200ng/l) and sheep (200-400ng/l).

As far as diagnostic parameters are concerned, a combination of serum vitamin B_{12} and MMA measurement seems to offer the best solution as in cattle and sheep. However MMA concentrations seem to be a more reliable indicator than serum vitamin B_{12} values alone. There are some animals which had low (<100 ng/l) vitamin B_{12} concentrations and low (<10 μmol/l) MMA concentrations. There are two possible explanations for this:-

a) the animal is suffering marginal deficiency and blood vitamin B_{12} concentrations are low but there is still sufficient vitamin B_{12} in the liver to prevent functional deficiency and the build up of MMA.

b) Alternatively it could be explained by adequate circulating concentrations of vitamin B_{12}, some of which is bound to Transcobalamin 1. If Tc1 in goats is resistant to denaturation in current assay systems, as it is in cattle, vitamin B_{12} associated with this binder may not be detected, leading to underestimation of total

vitamin B_{12} in goat serum. Tc1 has been found in goat serum (Price personal communication 1991) but the suitability of current vitamin B_{12} assay systems has not been validated for application to goat plasma.

Contrary to the results of Kennedy *et al* (1991) and Young *et al* (1991) serum succinate did not increase during cobalt deficiency nor did serum concentrations of succinate appear to be a good indicator of cobalt status, being poorly correlated to both MMA and vitamin B_{12} concentrations. Rumen succinate results on the other hand showed some suggestion of decreasing during supplementation, however due to the small number of samples analysed (20) this was not conclusive. In addition to the expected elevated serum GOT activity in deficient goats, plasma CK activity was also elevated. There was no evidence that this was correlated to plasma vitamin E concentrations or to GSH-Px activity. CK activity in plasma is seen as a sign of muscle damage (as in white muscle disease). However there is frequently some muscle wasting during cobalt deficiency and this could be the cause of the elevated activity.

Overall the requirement of goats for cobalt is between that of sheep and cattle but more similar to that of sheep. Their marginal vitamin B_{12} concentrations are lower than either but more similar to cattle, while their marginal MMA concentrations are higher than either but more similar to sheep. Thus goats should be classified neither as cattle, nor as sheep for diagnosis of cobalt deficiency, but should be considered separately in their own right.

Vitamin E supplementation in either oral or injectable forms led to increased plasma vitamin E concentrations. The injection caused an immediate massive increase (to >50 μmol/l) which dropped (to <7 μmol/l) within a week, this concentration still indicates sufficiency however. The oral supplement resulted in a slower increase, but

still resulted in concentrations above adequacy. There was no evidence of a GSH-Px (or selenium) interaction as all animals were adequate in this respect. Elevated CK activity was associated with cobalt status not vitamin E treatment. There was some sign that erythrocyte stability was increased by vitamin E supplementation, but the results were not conclusive. However as supplementation only commenced on day 169, this result suggests that vitamin E supplementation of cobalt/vitamin B_{12} deficient-goats may be worthy of further investigation. Some apparently significant effects of cobalt treatment seem to indicate that erythrocytes from group 2 animals were more unstable. Of the 6 goats in group 2, only 1 received vitamin E supplementation, thus this is probably a vitamin E treatment effect and not a cobalt treatment effect. There was a great deal of day to day variation between results which needs to be decreased if this method is to be used as an estimate of vitamin E status as suggested by Stevenson and Jones (1989).

The immune function tests used showed no significant effects of cobalt supplementation on neutrophil function, antibody response to *Clostridium tetani* vaccination, or total IgG. There was a slight effect of cobalt treatment on differential cell count (increased neutrophils, decreased lymphocytes and decreased monocytes) however the changes involved were very small and not consistent, thus with this number of data points being tested, some are bound to show up as significant. Overall despite highly significant differences in cobalt status no effect on immune function was observed. This might be due to using incorrect measures of immune function, although, the neutrophil function test has shown differences related to cobalt/vitamin B_{12} status in both cattle and sheep. Alternatively it could be due to difficulties inherent in the techniques, since the neutrophil function test is highly subjective, or there may simply be no effect of cobalt deficiency on these immune parameters in goats. It is suggested that further work in this area would be justified using different immunological tests.

CHAPTER 4

THE INTERACTION BETWEEN VITAMIN E AND VITAMIN B_{12} SUPPLEMENTATION IN CALVES.

4.1. Introduction.

Until recently there has been no commonly available form of injectable vitamin E. In guinea-pigs (Barber *et al* 1977) an injection of an oil emulsion of vitamin E produced a severe local reaction whereas a free aqueous emulsion had no such effect. Experimentally it has been found that vitamin E given by intravenous injection has the greatest availability, followed by intramuscular injection; however elimination of vitamin E from tissues after an intramuscular injection is slower (Hidiroglou & Karpinski 1987). Much work has also been done on the availability of different forms of vitamin E and it is generally agreed that the free D-alpha tocopherol form is more available than either the DL mixture or the acetate form. (Hidiroglou & Karpinski 1988, Hidiroglou *et al* 1988, Hidiroglou *et al* 1989 and Reddy *et al* 1985).

It has long been known that vitamin E supplementation may affect erythrocyte stability and recently Stevenson and Jones (1989) proposed a simple functional test for vitamin E deficiency, based on the sensitivity of erythrocytes to the detergent Tween 20.

Vitamin E has been found to affect almost all aspects of immunity, including % kill by neutrophils (Prasad 1980), while some authors (Beisel 1982 & Prasad 1980) suggest that megadoses of vitamin E have an increased effect on other parameters. Cobalt deficiency has also been found to affect % kill by neutrophils. Thus the aims of this trial are threefold:-

1. To study the effects of two commercially available vitamin E supplements (formulated for administration by different routes) on plasma vitamin E concentrations, and plasma CK activity in cattle fed a low vitamin E diet .

2. To investigate the usefulness of the erythrocyte stability test as a functional indicator of vitamin E deficiency.

3. To study the effects of vitamin E supplementation on immune function and investigate the interaction between vitamin E and vitamin B_{12} status.

4.2. Experimental Design.

4.2.1. Animals.

For this trial we used 12 Freisian bull calves, aged approximately 5 months. Half the animals received clamoxyl treatment for bovine rhinotracheitis virus between days -65 and -56, and all animals recieved Griseofulvin for ringworm from day -35 to day -28.

4.2.2. Treatments.

The calves were maintained on a vitamin E deficient diet until their plasma vitamin E concentrations suggested that they were deficient whereupon they were randomly allocated into three vitamin E treatment groups each containing 4 animals:-

Group C, the controls received no vitamin E supplement.

Group O, received 2g of oral vitamin E supplement (Rovimix E50 Roche) on day zero, and thereafter received 1g/day in the feed (containing 500 mg of DL-α-tocopheryl acetate, 500 IU vit E); from day 57 this dose was halved, to see if the lower level could maintain an adequate plasma vitamin E concentration.

Group I, received a 5ml intramuscular injection of vitamin E (Stuart Products Inc.) providing 1500 IU of D-α-tocopherol on days 0, 21 and 70.

These groups were then further subdivided into 2 vitamin B_{12} treatment groups with two animals from each vitamin E treatment receiving injections of vitamin B_{12} (3ml of Neo-Cytamen, Glaxovet Ltd. containing 3mg of hydroxycobalamin BP) on days 14, 42 and 70, and also 7mg of cobalt orally on days 70, 77 and 85.

Treatment effects were monitored by weekly liveweight (Ampac electronic Livestock weigher zeroed before use) and blood analysis.

4.2.3. Diets.

All calves received the same basal diet which was formulated using the SAC rationing software to provide sufficient protein and energy to support liveweight gains of 1kg/day, but to be deficient in vitamin E. Calves were fed individually and generally finished their food, however refusals were not measured. Initially the diet consisted of hay, barley, soya bean meal and a calcium phosphate supplement. Fifteen days before supplementation was started this was changed to urea treated hay with moist (propcorn) barley and the mineral supplement, as vitamin E concentrations in the plasma were not as low as was desired. The propcorn barley was later (between days 54 and 80) partly replaced by sodium hydroxide treated barley, to try and further decrease vitamin E concentrations in the diet. The cobalt content of the diet fell from 0.055 mgCo/kgDM to 0.045mg/kgDM as sodium hydroxide treated barley replaced propcorn treated barley, thus although the diet was not formulated intentionally to be low in cobalt, it might be expected that cobalt/vitamin B_{12} deficiency would also result from prolonged feeding. Detailed analysis of all constituents of the diet are included in Tables 4.1 to 4.3 of Appendix II.

The sodium hydroxide treatment was carried out according to a modification of the method used by Rice *et al* (1985) and Rice and McMurray (1986). Whole barley was treated in batches of 25kg (wet weight) in a cement mixer. Sodium hydroxide (30% soln.) was added to give a final concentration of 3%/kgDM of the barley. After mixing the treated barley was left on a cement floor for one week (to allow the sodium hydroxide to react and form harmless carbonates) before being slowly introduced into the diet.

SUMMARY OF TREATMENTS

Day No.	
-72	Vitamin E deficient diet introduced.
-15	Barley replaced with propcorn barley.
0	Start vitamin E treatments.
14	1st vitamin B_{12} treatment.
21	2nd vitamin E injection.
42	2nd vitamin B_{12} treatment.
54	Sodium hydroxide treated barley began to be introduced.
57	Oral vitamin E dose halved.
70	3rd vitamin E injection,
	3rd vitamin B_{12} treatment,
	Start weekly cobalt treatment.

4.2.4. Experimental Parameters.

Plasma vitamin E concentrations, whole blood erythrocyte stability, plasma CK and serum GOT were monitored to assess the effects of the vitamin E treatment on these parameters. Vitamin B_{12}, MMA and succinate concentrations were measured in

serum to monitor the changes in vitamin B_{12} status. Liveweight was monitored to determine whether vitamin E or vitamin B_{12} treatments influenced growth rate. Whole blood GSH-Px activity, and plasma calcium, magnesium and copper concentrations were monitored to check for other possible deficiencies that might affect the results. Neutrophil function tests were carried out on days 70 and 85 using *E. coli* and *S. aureus* and on days 77 and 91 using *C. albicans*.

4.2.5. Statistical analysis.

Statistics were carried out on the results using the Genstat V programme (Lawes Agricultural Trust 1988). Analysis of variance or covariance was used unless otherwise stated.

4.3. Results

4.3.1. Plasma Vitamin E Concentrations.

The basal diet did not deplete the animals of vitamin E as much as expected below normal values which are $>3\mu$mol/l. Marginal vitamin E deficiency in calves is associated with plasma vitamin E concentrations between 1μmol/l and 3 μmol/l, clinical vitamin E deficiency is associated with plasma vitamin E concentrations below 1μmol/l. The plasma vitamin E concentration in the unsupplemented group was 1.70 μmol/l on day 0 (Fig.4.1 and Table 4.4 Appendix II), and thereafter remained at or near this level despite the introduction of sodium hydroxide treated barley in place of some of the propcorn barley. This suggests that these unsupplemented calves were marginaly deficient in vitamin E. Sodium hydroxide treated barley was not a success when fed alone, as it was not very palatable; however a 75% mix with propcorn barley seemed to be fairly acceptable.

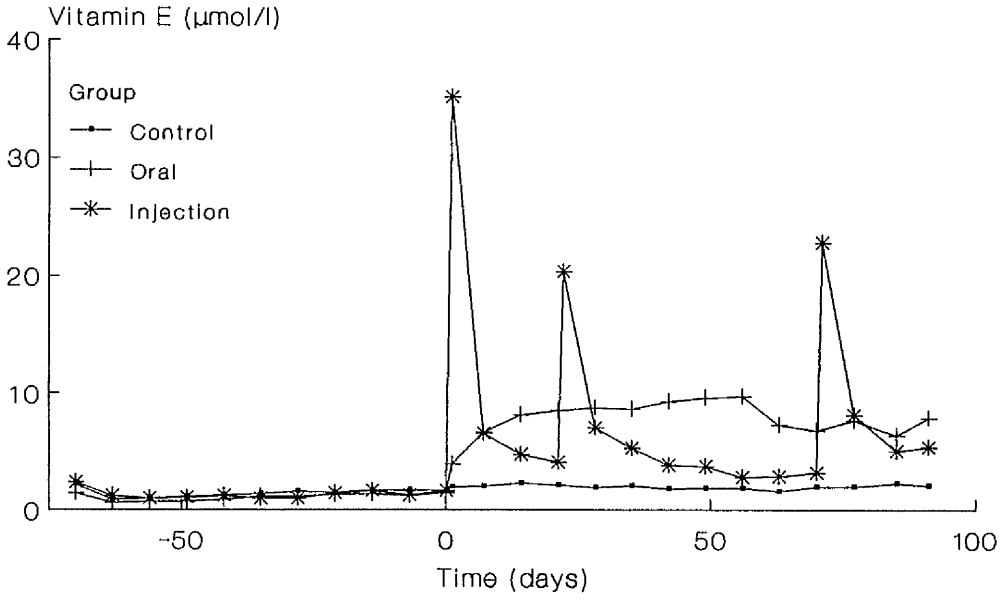


Fig 4.1 Plasma Vitamin E Concentrations of Calves

Day 0 for all figures was the day on which vitamin E supplementation was initiated

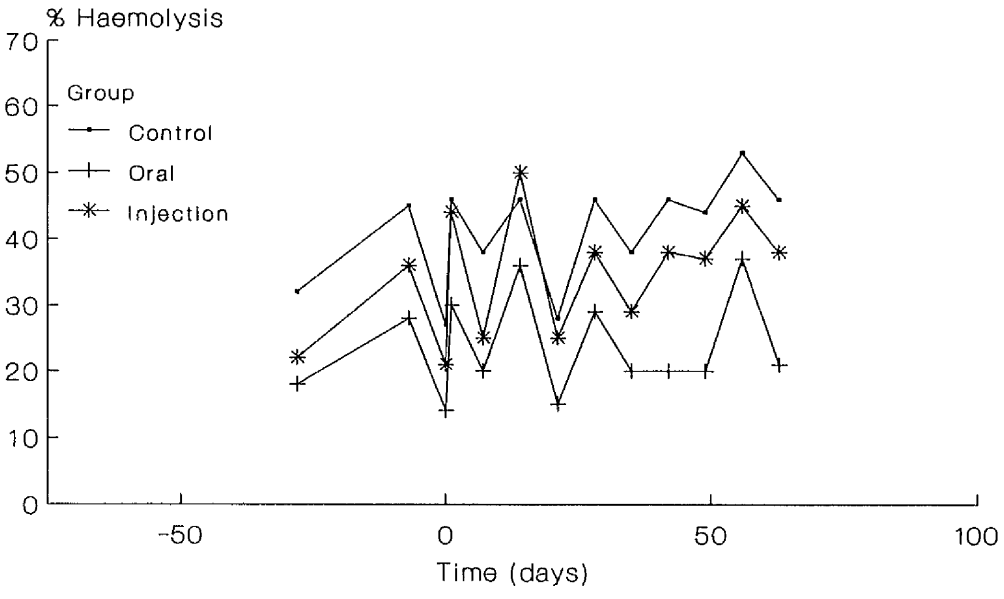


Fig 4.2 Erythrocyte Stability of Calves
(1% TWEEN)

The oral supplementation raised vitamin E concentrations steadily to between 8 and 9 μmol/l. When the dose was halved these concentrations fell to around 6 μmol/l, which is however still well above the lower limit of normality, 3μmol/l.

The vitamin E injections produced a very rapid increase in blood plasma vitamin E concentrations (to >30 μmol/l) but this dropped down to around 5.5 μmol/l within a week and within 4 weeks concentrations were bordering on deficient. The second injection, given 3 weeks after the first, produced some form of adverse anaphylactic reaction (loss of appetite, hyperventilation and sweating) on the day it was given, in some animals. The third injection 7 weeks later produced no such response.

Vitamin B_{12} treatment appeared to have a significant effect on plasma vitamin E on day -21, and the interaction between the two treatments was significant on days 28, 35, 77, 85, and 91 (see Table 4.5 Appendix II), with vitamin E concentrations apparently being greater for the vitamin B_{12} supplemented group on the oral vitamin E treatment, but being lower for the vitamin B_{12} supplemented group on the injectable vitamin E treatment. Because of the number of statistical tests carried out, some apparently significant results may arise due to chance. This is probably the case on day -21, however the interaction result is more consistent and so may be a true effect.

4.3.2. Erythrocyte Fragility.

The erythrocyte stability test (EST) did not show any significant differences between groups in the fragility of erythrocytes challenged (*in vitro* with detergent at 3 concentrations EST1, EST2 and EST3 (Figs.4.2 to 4.4 and Tables 4.6 to 4.8 Appendix II). The mean values for vitamin E supplemented groups were, however, consistently lower than those of the vitamin E deficient controls but between animal and between assay-batch variability was large, making it difficult to detect any

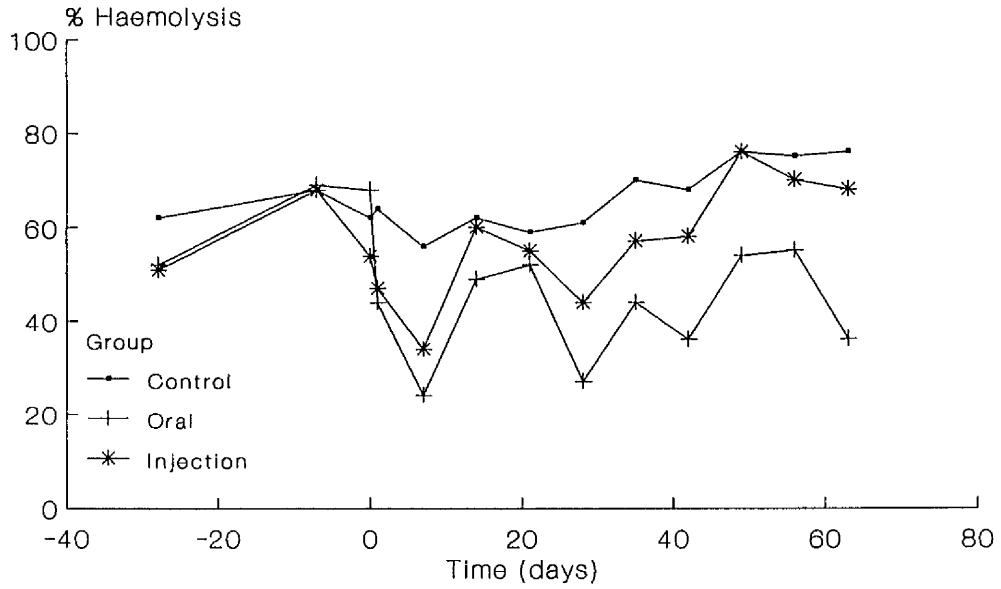


Fig 4.3 Erythrocyte Stability of Calves

(3% TWEEN)

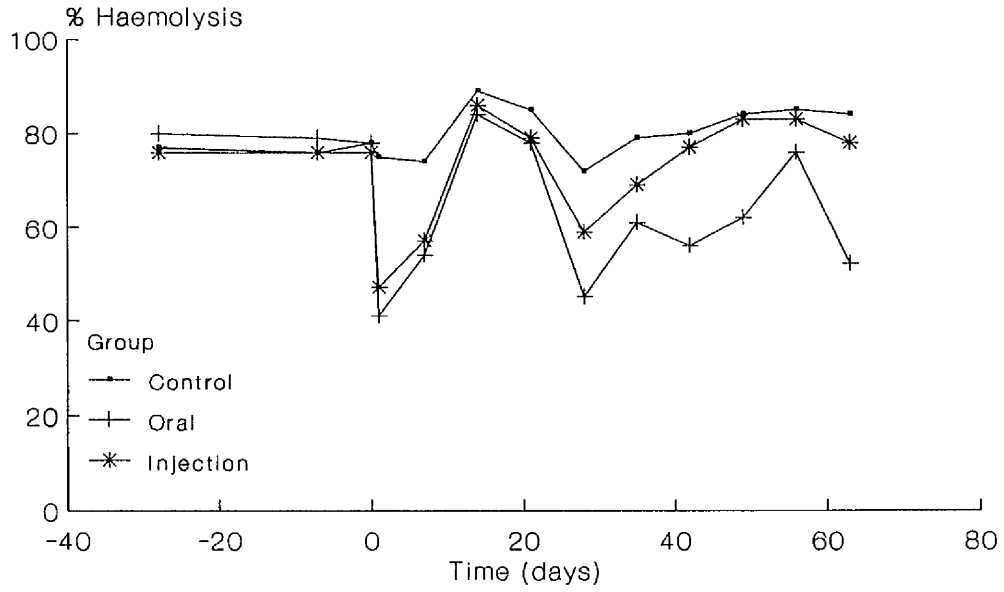


Fig 4.4 Erythrocyte Stability of Calves

(5% TWEEN)

treatment effects.

The correlation coefficients for the relationship between values obtained with the individual Tween concentrations and plasma vitamin E concentrations were:-
EST1 = -0.008, EST3 = -0.233 and EST5 = -0.340. Thus there were no strong relationships. Correlations between the results obtained with the different Tween concentrations were high:-
EST1 v EST3 = 0.81, EST1 v EST5 = 0.685 and EST3 v EST5 = 0.862.

4.3.3. Plasma CK Activity.

CK activity remained in the normal range throughout, apart from group I on day 1 when levels were significantly elevated (124 iu/l), see Fig.4.5. and Table 4.9 Appendix II. Samples were not assayed on the day following subsequent injections, so it is unclear whether this is a side effect of the injection. Vitamin B_{12} treatment apparently had a significant effect on day 28, but its biological significance is questionable.

4.3.4. Serum GOT Activity.

SGOT values were generally between 50 and 70 for all groups throughout the trial and thus close to or just above the upper limits of the normal range of 20-60 SF/ml, see Fig.4.6 and Table 4.10 Appendix II. However neither serum vitamin B_{12} nor plasma vitamin E status correlated with SGOT values. Only the interaction on day -14 was statistically significant and this is probably spurious. Group C had an elevated mean value on day 91 but this was in fact due to only one animal.

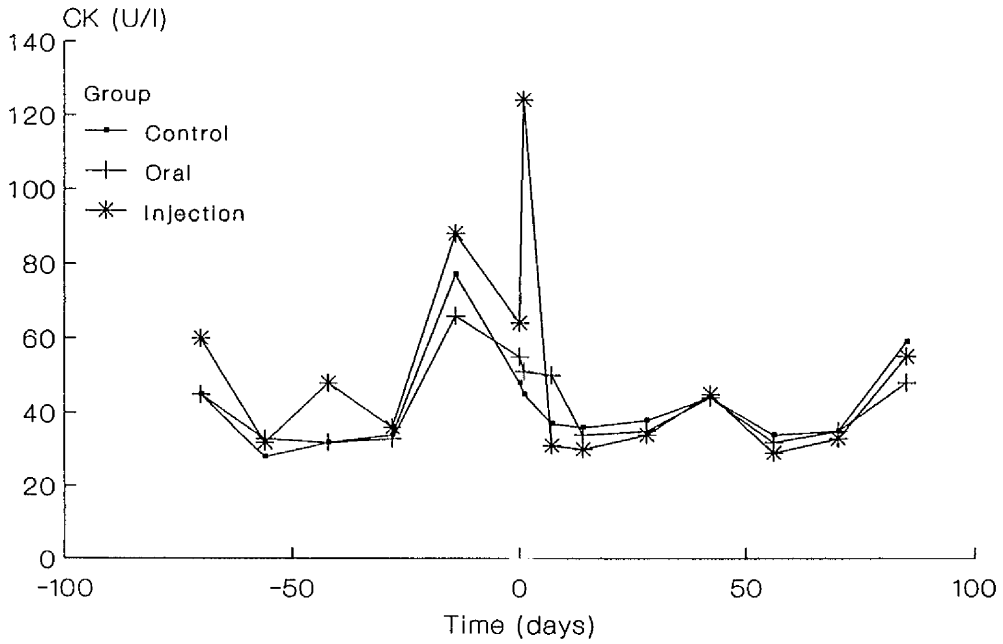


Fig 4.5 Plasma CK Activity of Calves

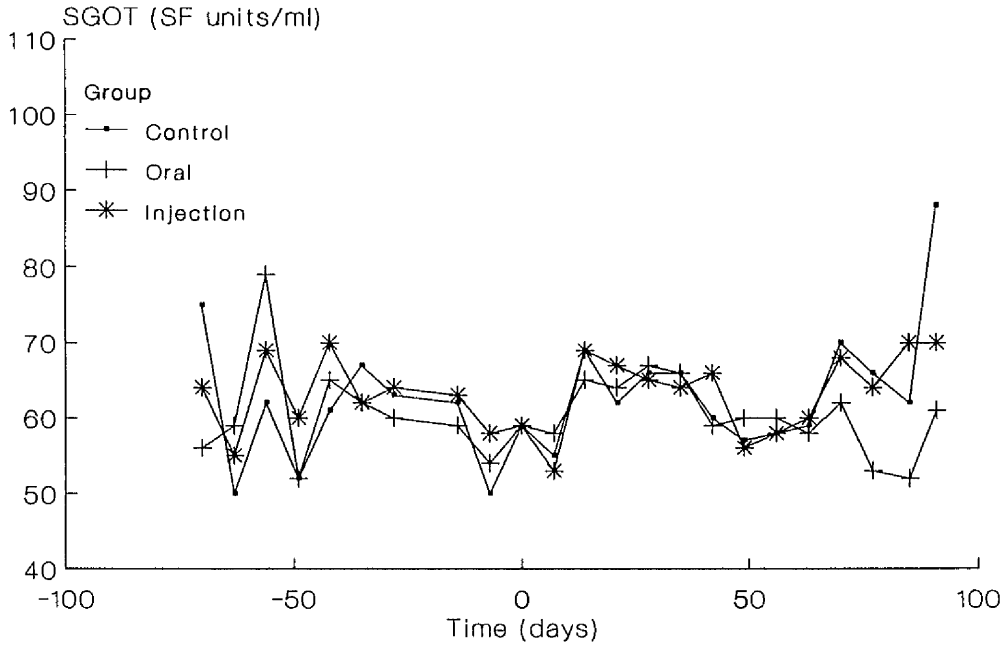


Fig 4.6 Serum GOT Activity of Calves

4.3.5. Serum Vitamin B_{12} Concentrations.

The three injections of vitamin B_{12} produced an immediate response in serum concentrations (>1250 ng/l by the afternoon on day 14, but not shown in Fig.4.7). Concentrations dropped back very quickly to around 200 ng/l by a week later although the values remained significantly greater than those of the corresponding control groups (Fig.4.7. and Table 4.11 Appendix II). Vitamin E treatment had no effect.

4.3.6. Serum MMA Concentration.

Vitamin B_{12} supplementation decreased MMA concentrations significantly, while MMA concentrations for the unsupplemented group exceeded the upper limit of normality (2 μmol/l) from day 49 (Fig.4.8. and Table 4.12 Appendix II). Vitamin E treatment had no effect on serum MMA concentration although the interaction was apparently significant on days -70 and 42; once more these are probably spurious results.

Although the correlation between vitamin B_{12} concentration and MMA concentration is not very high (-0.370) it is noticable from the graph (Fig.4.9) that for all vitamin B_{12} concentrations above 150 ng/l the MMA concentrations are below 2 μmol/l, while for all MMA concentrations greater than 2 μmol/l corresponding vitamin B_{12} concentrations are below 150 ng/l. However of the 168 results plotted 70 had vitamin B_{12} concentrations below 150 ng/l with MMA concentrations below 2 μmol/l. Of these most of those with MMA concentrations above 1 μmol/l were unsupplemented while most of those with MMA concentrations below 1 μmol/l were supplemented.

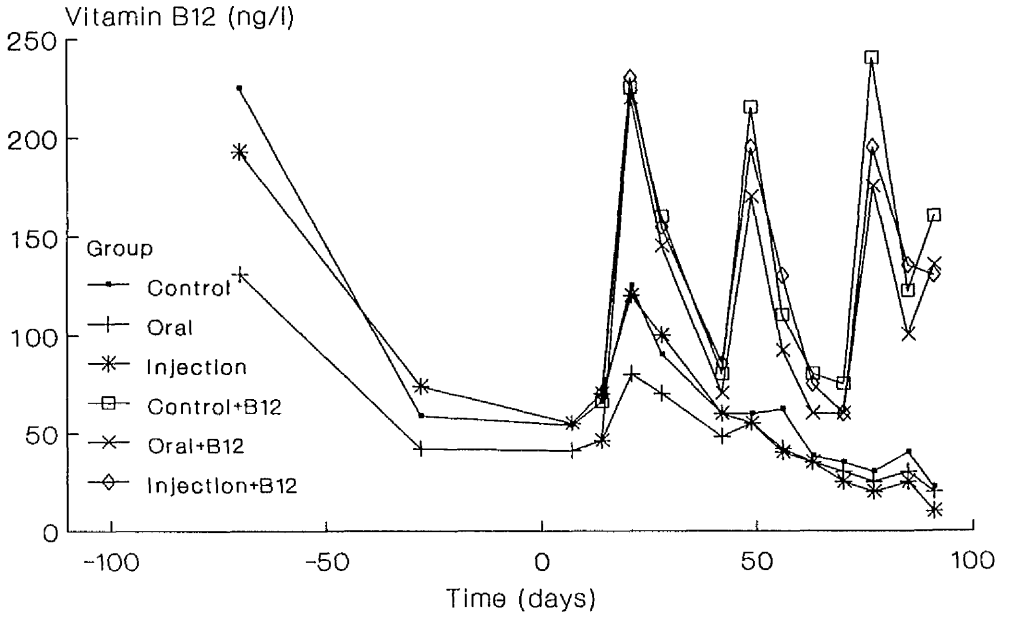


Fig 4.7 Serum Vitamin B12 Concentrations of Calves

Vitamin B12 injections days 14 42 and 70

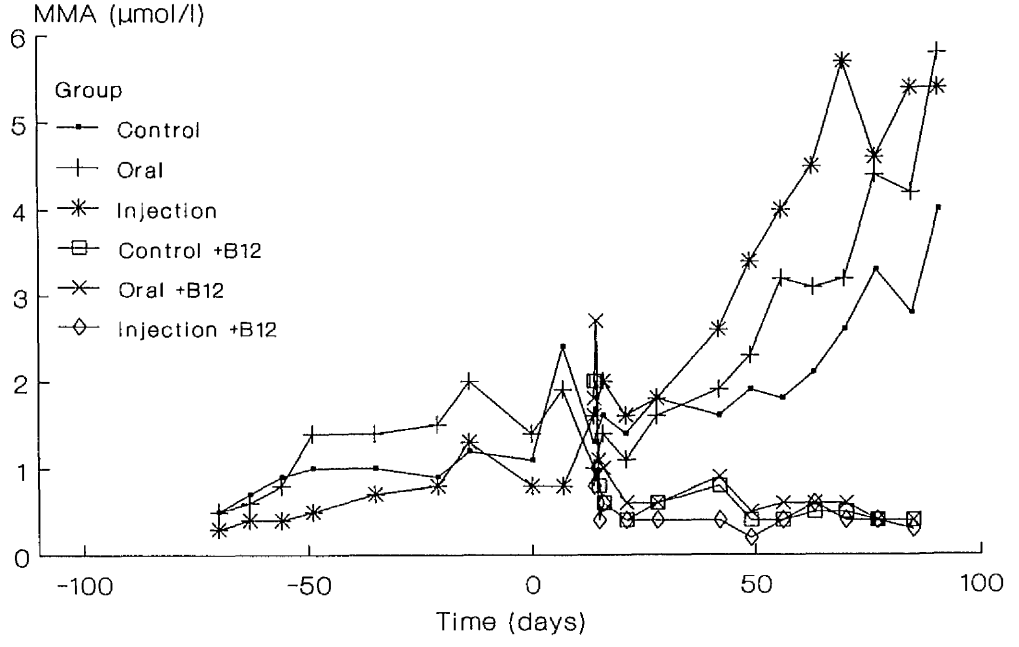


Fig 4.8 Serum MMA Concentrations of Calves

4.3.7. Serum Succinate Concentrations.

Vitamin B_{12} treatment only had a significant effect on succinate concentration on days 70 and 85 and in fact vitamin E treatment was significant on days -21 and 14 and the interaction was significant on days -70 and 14 all of which could be spurious (see Fig.4.10 and Table 4.13 Appendix II). However there does seem to be a slight trend for the vitamin B_{12} supplemented group to have elevated concentrations of succinate over the last 8 weeks. Succinate concentration was not highly correlated with either vitamin B_{12} concentrations (0.053) or MMA concentrations (-0.246).

4.3.8. Liveweight.

All three groups grew steadily, although the desired 1kg/day liveweight gain was not obtained (Fig.4.11 and Table 4.14 Appendix II). Group O seemed to grow slightly better towards the end of the trial and on days 49 and 77 the difference between groups was significant when liveweight on day zero was used as a covariate. When regression coefficients were calculated and these in turn were analysed, vitamin E treatment was found to have a small but significant effect on the slope of the line. Oral treatment gave a greater slope than injection, while the control group was not significantly different from either. Despite the large differences in vitamin B_{12} concentrations no effect on growth was observed.

4.3.9. Neutrophil Function Tests.

Vitamin B_{12} supplementation significantly increased % kill of *E. coli* on day 70, and of *C. albicans* on day 77, see Fig.4.12 and Table 4.15 Appendix II. Vitamin E injection decreased % kill of *E. coli* on day 70, compared to the control, and oral supplemented groups. However the differences were small and not consistent.

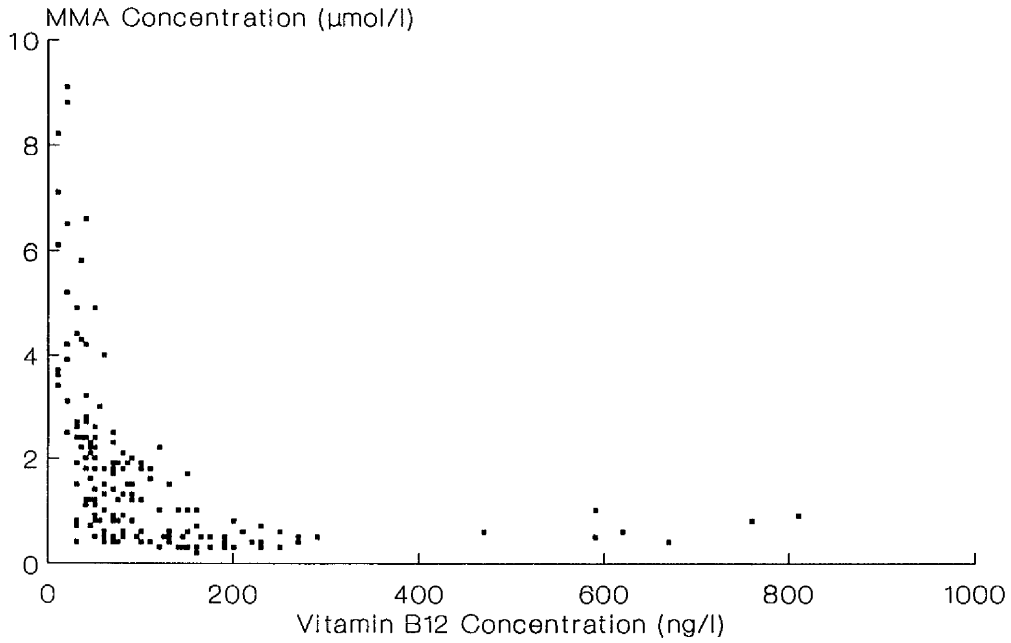


Fig 4.9 Serum Vitamin B12 versus MMA Concentrations

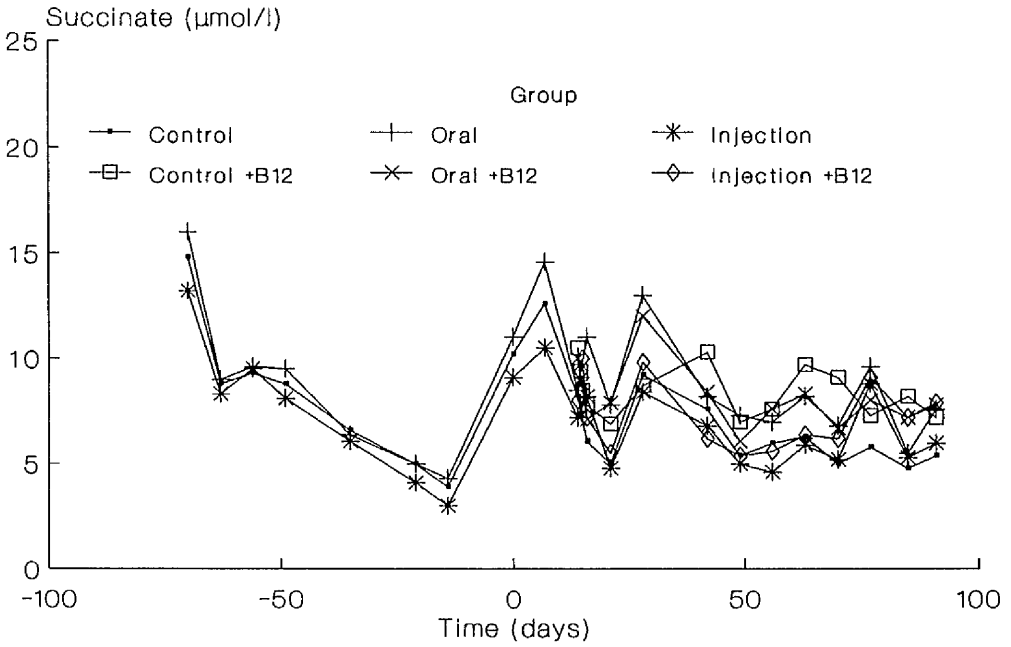


Fig 4.10 Serum Succinate Concentrations of Calves

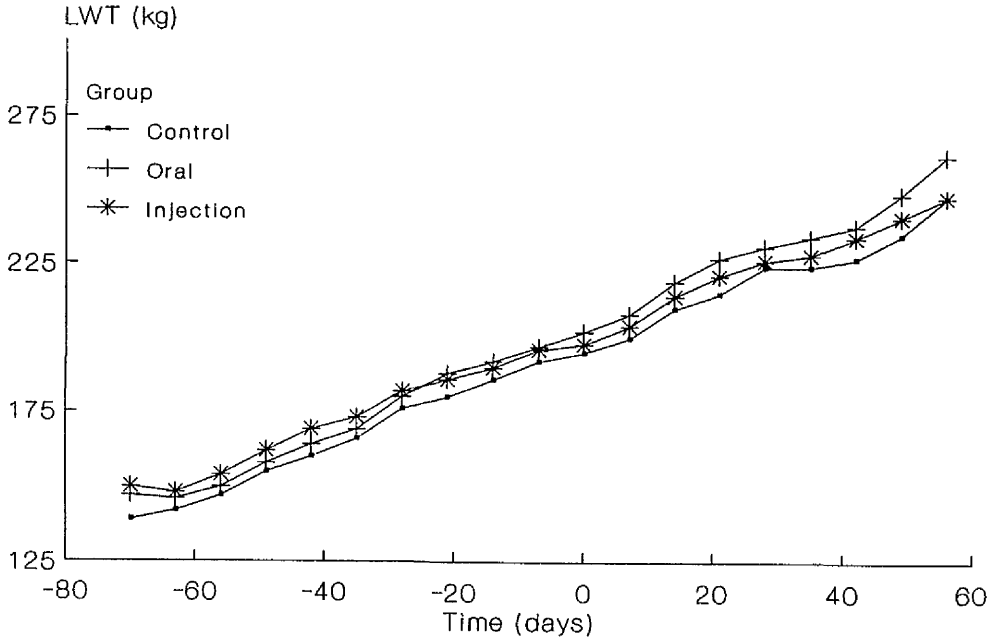


Fig 4.11 Liveweights of Calves

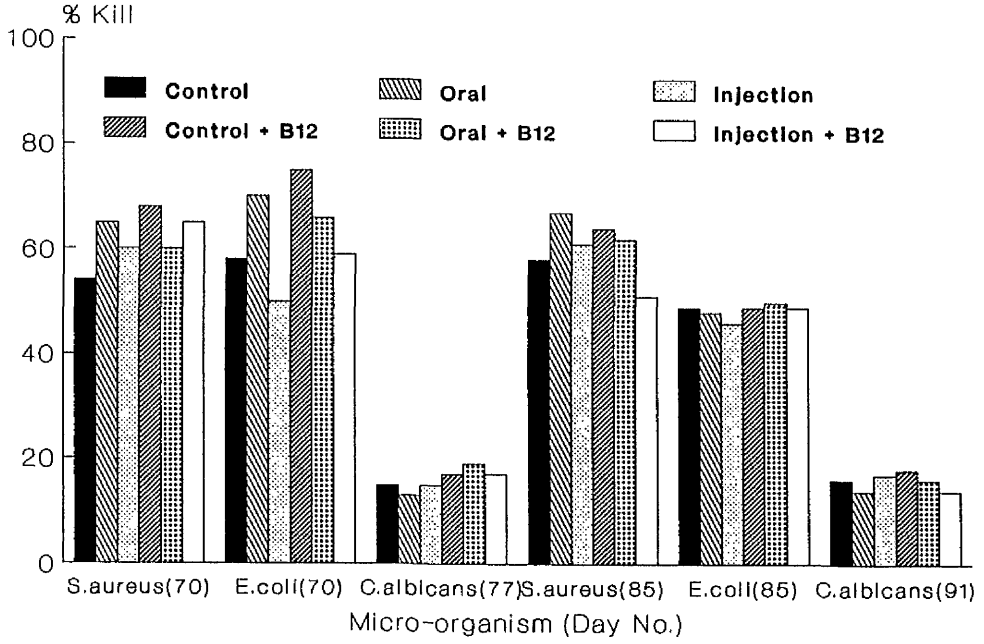


Fig 4.12 Neutrophil Function of Calves

4.3.10. Whole Blood GSH-Px Activity.

GSH-Px values remained normal (> 15u/ml) apart from one animal which was dosed with 5mg of selenium on day 7, see Table 4.16 Appendix II.

4.3.11. Serum Copper, Magnesium and Calcium Concentrations.

Calcium, magnesium and copper levels were within the normal ranges, see Table 4.17 Appendix II.

4.4. Discussion.

Due to the failure of the basal diet to deplete the animals of vitamin E the results of this experiment are less informative than had been hoped. This was probably due to the propcorn barley being too recently treated, and the fairly high concentration of vitamin E found in the hay. The sodium hydroxide treated barley was found to be low in vitamin E, but it was only fed for 40 days.

One possibility for overcoming these problems in future would be to feed hay and propcorn barley that was a year old, as vitamin E concentrations should drop during storage. Treating large quantities of barley with sodium hydroxide would be difficult, and it is doubtful whether the animals would eat much of it on its own. Mouth ulcers were found in some calves after only 24 days on a 75% ration, and this might be a serious problem if long term feeding was required.

Both levels of oral vitamin E maintained blood concentrations significantly higher than those of the controls, and also above deficiency levels (3μmol/l). Injectable vitamin E produced a rapid response ideal for treating severe deficiency symptoms,

but not suitable as a long term measure. Repeating the injection cannot be relied upon as problems seem to arise if the interval between administration is short, and yet concentrations dropped very quickly suggesting that frequent injections would be required if concentrations were to be maintained by this method alone. The elevated CK values for group I on day 1 might indicate local muscle damage due to the injection, some signs of local damage were also apparent at the injection site. Although SGOT values tended to be slightly elevated, concentrations were unaffected by treatments.

The lack of significant treatment effects on erythrocyte stability was unexpected. The calves were not as vitamin E deficient as the sheep used by Stevenson and Jones (1989) and the test may be more informative in severe vitamin E deficiency. However as a test for marginal deficiency in cattle it proved insufficiently sensitive. Despite improvements made to the method there was still a lot of variation between individuals and between sampling dates, and this was particularly noticable when the 1% Tween solution was used. Further improvements might be made if each animal was compared to its own 100% haemolysed blood, or if packed cell volumes were used to correct for differences in red cell numbers. However there are many other factors involved in red cell fragility. Glutathione peroxidase might be thought to play a role, although Stevenson and Jones (1989) found no relationship between erythrocyte glutathione peroxidase and detergent sensitivity in marginally selenium deficient sheep. The calves in this trial were not deficient in selenium, although their GSH-Px activities decreasd to fall within the marginal range (15-25 μ/ml) by the end of the trial.

Although the erythrocyte stability test is simple in that it requires no complicated or expensive equipment except for a spectrophotometer, the results obtained in this trial suggest that it is not a very robust technique. Vitamin E analysis by HPLC is quicker, more samples can be analysed in a day, and the results are more reliable.

Therefore as the availability of HPLC equipment continues to increase it seems likely that direct analysis of vitamin E will become the method of choice.

Vitamin B_{12} supplementation significantly elevated serum vitamin B_{12} concentrations, above those of the unsupplemented animals which were deficient (<150 ng/l in cattle). MMA concentrations in the unsupplemented animals were also elevated above the upper limit of normality, (>2 μmol/l Paterson and MacPherson 1990); supplementation rectified this. Vitamin B_{12} deficiency was not severe enough to affect liveweight, or at least no significant differences could be detected between the small numbers of animals in each group (2) and this may also explain why serum succinate concentrations were not significantly increased. However serum MMA was affected, and as it is the breakdown of the same biochemical pathway which is involved in each case it might have been expected that serum succinate would also be affected. However vitamin B_{12} may be available in the rumen for conversion of succinate to methylmalonyl coenzyme A in greater quantities than it is in the blood for conversion of methylmalonyl coenzyme A to succinate, because it is initially formed in the rumen and there may be little available for absorption in the ileum. Thus the trend for vitamin B_{12} supplementation to increase succinate concentrations in the final weeks of experimentation might have been significant under more severe deficiency conditions.

The results of the neutrophil function tests were inconclusive. They gave some suggestion that the vitamin E injection decreased % kill of *E. coli* compared to oral vitamin E or no treatment, while vitamin B_{12} injection stimulated % kill of both *E. coli* and *C. albicans*, compared to animals not supplemented with vitamin B_{12}, however these results were not consistent and the % changes involved were very small. Paterson and MacPherson (1990) found vitamin B_{12} deficiency affected neutrophil function adversely in calves, before serum concentrations of vitamin B_{12}

or MMA were affected, thus if more tests had been carried out it is possible that a positive effect of supplementation with vitamin B_{12} would have been observed. The unexpected effect of vitamin E injection, reducing % kill of *E. coli* on day 70, compared to the oral supplemented animals, could be because vitamin E concentrations in this group had dropped to nearly as low as the unsupplemented animals, although this does not explain why % kill should be lower in these animals than in the unsupplemented ones.

CHAPTER 5

AN INVESTIGATION OF THE EFFECTS OF COBALT DEFICIENCY ON THE APPETITE PREFERENCE OF SHEEP.

5.1. Introduction.

During the second goat experiment (described in chapter 3) it was observed that some of the cobalt deficient goats developed a preference for forage over concentrate. This symptom of cobalt deficiency has previously been observed in other ruminants (MacPherson 1982), however no data was published to confirm or quantify the observation. This experiment was designed to investigate this phenomenon, and attempt to quantify the preference for forage, and to try to clarify the possible mechanisms responsible. Animals were to be maintained on a diet low in cobalt until they were deficient in serum vitamin B_{12}, and such behaviour occurred, wherupon a variety of treatments would be given to attempt to alleviate the symptoms. It was decided to use sheep, as they demonstrate symptoms of cobalt/vitamin B_{12} deficiency more easily than cattle, are more economical to keep, and were also more readily available. There are many possible explanations for the decrease in appetite seen in this deficiency and for the preference for forage:-

a) an ability to detect and correct for the low cobalt concentration in the maize, by increasing intake of roughage which tends to have, and in the case of our goats, did have a higher cobalt content.

b) small increases in propionate concentration in the blood due to impaired conversion of propionate to succinate. The decreased activity of the methylmalonyl CoA mutase enzyme during cobalt/vitamin B_{12} deficiency is thought to lead to a build up of propionate in the tissues, as well as a build up of methylmalonic acid, and it is thought that this increase in propionate concentration may be the main cause of the decrease in appetite (Marston *et al* 1961 and 1972). As yet there is no evidence to suggest that increased propionate concentrations in the tissues leads to a preference for forage, only that it could cause a decrease in appetite. However concentrate diets favour a shift towards propionic acid production by the rumen

microbes, while forage diets stimulate acetic/butyric acid production in the rumen.

c) amino acid imbalance due to the failure of methionine recycling. The breakdown of methionine recycling due to lack of 5-methyltetrahydrofolate-homocysteine methyltransferase activity could result in an amino acid imbalance such as is found to cause loss of appetite in non-ruminants. Again there is as yet no evidence that amino acid imbalance leads to a preference for forage, only that it can lead to loss of appetite.

d) avoidance of the concentrate because eating it was associated with discomfort, possibly indigestion, while eating forage caused no discomfort.

e) a combination of b) with d). The discomfort resulting in the animal avoiding the concentrate could be caused by unusually high propionate concentrations. Eating more forage, and less concentrates would favour a shift towards acetate/butyrate production and away from propionate production in the rumen, leading to lower propionate concentrations in the tissues and so relieving discomfort. Overall this might result in a conscious shift towards eating more forage by the animal.

f) some other factor associated with the deficiency, acting either directly or indirectly.

Because there were a number of possibilities to explore there were two main parts to the experiment. The first aim was to determine whether in fact animals 'learn' to associate eating concentrate with discomfort during cobalt/vitamin B_{12} deficiency, and hence eat forage instead. To do this two cobalt deficient diets would be fed, the normal concentrate/forage diet permitting choice and a complete pelleted ration permitting no choice. Once the animals became depleted of vitamin B_{12} the animals

on the pelleted ration would be offered the normal diet to see if they demonstrated the same feeding behaviour as those which had been given a choice throughout.

The second part of the experiment aimed to establish whether supplementation with methionine injections, which would compensate for the failure of the 5-methyltetrahydrofolate-homocysteine methyltransferase enzyme, could reverse this symptom of cobalt/vitamin B_{12} deficiency. Additionally by comparing supplementation with oral cobalt or with vitamin B_{12} injections, or with oral 5,6-dimethylbenzimidazole (a precursor of vitamin B_{12}) to see which was more effective at restoring the appetite to normal it was hoped to confirm whether cobalt acted directly, or via increased vitamin B_{12} synthesis.

5.2. Experimental Design.

5.2.1. Animals.

13 mature castrate Suffolk cross sheep were used, their liveweights ranged from 64 to 112 kg at the start of the experiment. One animal received treatment for lice on 1:5:92.

5.2.2. Treatments.

All the sheep were offered a cobalt deficient ration designed to induce vitamin B_{12} deficiency and eventually it was hoped that the sheep would develop typical symptoms of vitamin B_{12} deficiency including the decrease in appetite and the preference for forage. Initially it was intended that six sheep would be fed a complete diet also deficeint in cobalt, until the remaining sheep on the conventional concentrate plus forage diet showed a marked change in appetite. The sheep on the complete diet would then be given the same choice of concentrate and forage and

their comparative intakes of the two components would be recorded, and compared to that of the 7 sheep which had been offered this diet throughout. This was to demonstrate whether any preference for the type of feed developed as a result of learning which feed caused discomfort. Unfortunately this part of the experiment was discontinued because the complete diet, although formulated with and pelleted from cobalt deficient feeds, was insufficiently low in cobalt to deplete the animals of vitamin B_{12}.

The second part of the experiment consisted of treating the animals once they had developed a change in appetite. Three treatments were initially chosen,
-supplementation with oral cobalt (20mg/week),
-supplementation with a vitamin B_{12} injection (2ml containing 250 μg/ml cyanocobalamin),
-supplementation with a methionine injection (2g of L-methionine in 40 ml sterile physiological saline every 2 days) as used by Smith *et al* (1974).

The animals did not all develop symptoms at the same time, and as one animal in particular became very ill it was decided to treat the animals in two batches. 6 animals were treated in the first batch, including the seriously ill animal which died on the following day, leaving 5 animals in batch 1, death was confirmed as being due to cobalt deficiency at post mortem (VIC Auchincruive) by liver vitamin B_{12} concentrations and visual examination of the liver (plate 6) which revealed that it was exceedingly fatty, compared to a normal sheep liver (plate 5).

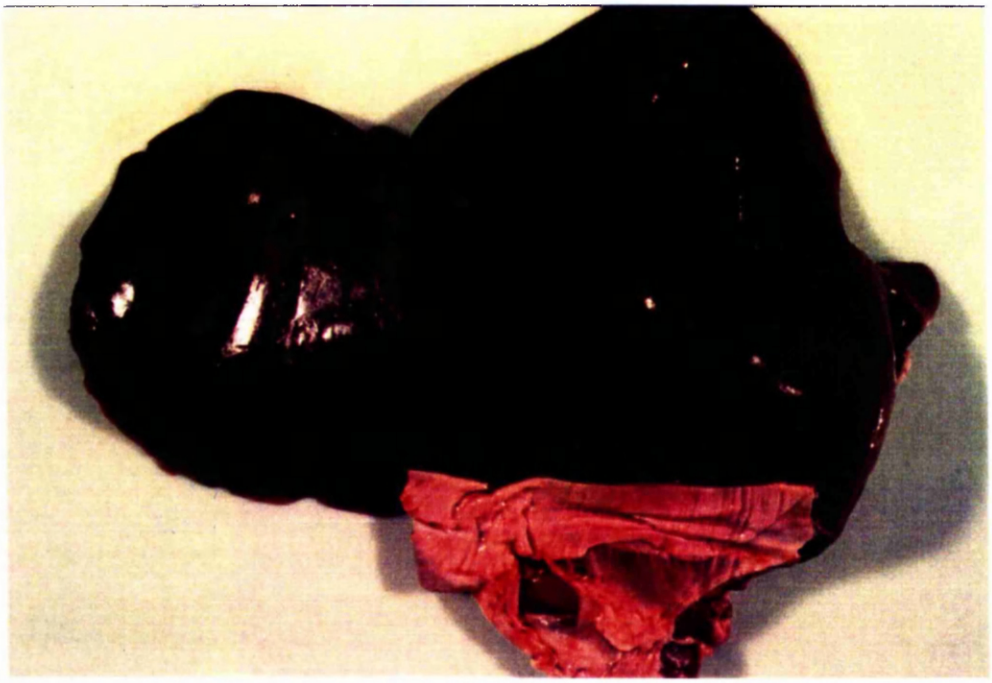
The remaining 7 animals were treated 5 weeks later and will be referred to as batch 2. With the second batch a fourth treatment was given to the 2 animals allocated to the methionine treatment. During the second two weeks of treatment they received 5,6-dimethylbenzimidazole (200mg per day, twice the dose used by Rickard *et al*

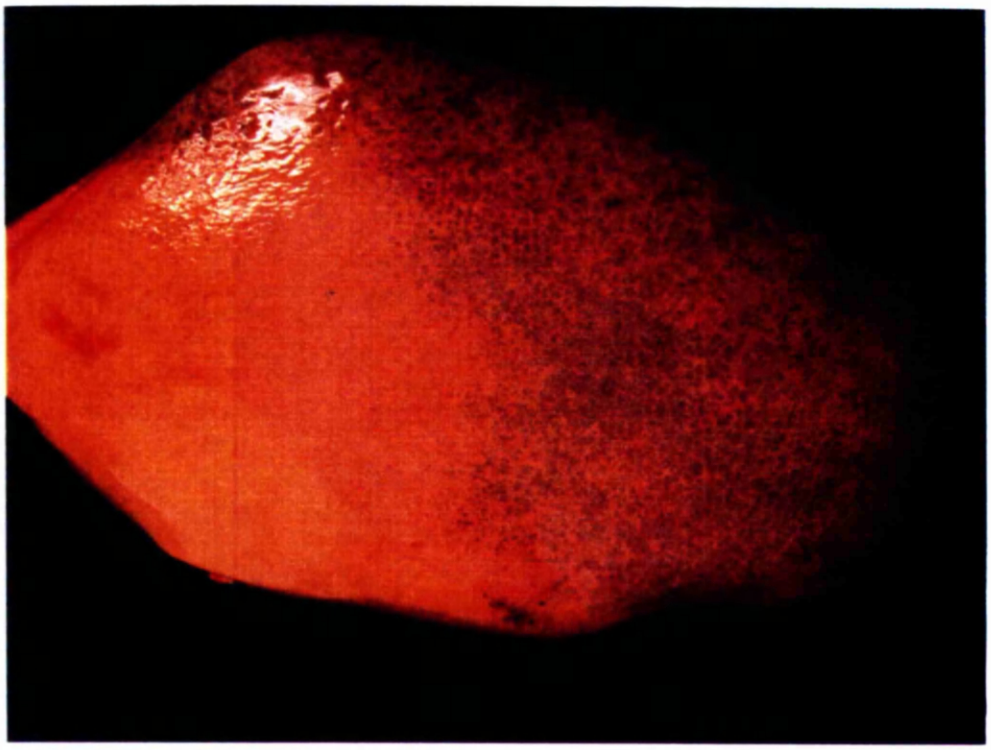
PLATE 5

Liver from a healthy sheep

PLATE 6

Fatty liver from a cobalt deficient sheep





1975) in place of methionine. By this time it was apparent that the methionine treatment was having little or no effect. By supplementing with 5,6-dimethylbenzimidazole, a precursor of vitamin B_{12}, we hoped to improve the efficiency of utilization of cobalt in the rumen, promoting vitamin B_{12} formation and reducing formation of α-analogues; this would have a sparing effect on the cobalt present in the diet, and hence should partially reverse the effects of cobalt deficiency.

Summary of Treatment groups

	Batch	
	1 (n)	2 (n)
Treatment		
Vitamin B_{12}	B1 (2/1)*	B2 (3)
Cobalt	C1 (2)	C2 (2)
Methionine	M1 (2)	M2 (2)**

* one animal from this group died the day after the first treatment
** received 5,6-dimethylbenzimidazole during the second two weeks of treatment in place of methionine.

5.2.3. Diet.

The diet used consisted of 60% forage (a 1:1 mix of hay and straw by weight) and 40% concentrate (maize) with urea and minerals balanced to provide maintenance rations for the sheep. The diet which was fed unpelleted was basically the same as that given to the goats in the third experiment and was deficient in cobalt (supplying only 0.04 mg/kgDM); for detailed analysis see tables 3.5 and 3.6 in Appendix I. Once the animals had become depleted (mean serum vitamin B_{12} concentrations below 150ng/l) they were divided randomlly into two groups, and one group, of six sheep, was fed a complete pelleted ration. Pellets contained 40% flaked maize, 60%

alkali treated straw with added urea and minerals as for the original diet, and contained <0.04 mgCo/kgDM as calculated based on the raw ingredients (for detailed analysis of the straw pellet see Table 5.1. Appendix III). Use of the pelleted ration was discontinued after 5 weeks, and replaced by the original forage/maize diet, because serum vitamin B_{12} concentrations in these six sheep became elevated to above 1000 ng/l. Subsequent analysis (also in table 5.1 Appendix III) revealed the true cobalt content of the pelleted ration to be 0.13 mg/kgDM. The source of the cobalt in this diet is unknown, but is presumed to have come from the pelleting equipment. The remaining seven sheep continued to receive the original ration throughout. Treatments were not started until the animals were showing symptoms of cobalt deficiency, including loss of appetite. Batch 1 were treated 11 weeks after feeding with the pelleted diet was discontinued, and batch 2, 5 weeks later. Only one of the six sheep which recieved the pelleted ration was in batch 1.

5.2.4. Experimental Parameters.

After introducing the low cobalt diet, blood samples were taken for the determination of vitamin B_{12}, MMA and succinate in serum. Feed intake and liveweight were monitored and succinate and volatile fatty acids determined in rumen fluid. Serum GOT, whole blood GSH-Px ans plasma copper, magnesium and calcium concentrations were also analysed periodically.

Feed preference was assessed, for one week periods at 4 weekly intervals and continually after treatments commenced. This was carried out by giving 100g of chopped hay/straw mix and 265.5g maize simultaneously and weighing refusals of each after 5 minutes. The feed refused and the remainder of the forage were then returned to the animal until the next feeding.

5.2.5. Statistical analysis.

Statistics were carried out on the results using the Genstat V programme (Lawes Agricultural Trust 1988). Analysis of variance or covariance was used unless otherwise stated.

5.3. Results.

5.3.1. Feed Preference.

There were no significant differences between maize refusals for the different treatment groups prior to treatment, although refusals rose from very low (5% or less) to high (>50%) for all groups (see Figs.5.1 & 5.2 and Tables 5.2 & 5.3 Appendix III). The 6 animals in batch 1 did have significantly greater concentrate refusals (78.2 $\pm$ 13.1 % of total concentrate offered), than the 7 animals in batch 2 (16.3 $\pm$ 10.9 % of the total concentrate offered), during the month prior to treatment of batch 1.

After treatment the 4 sheep receiving vitamin B_{12} (group B=B1+B2 in tables and graphs) ate significantly more concentrate than the 4 sheep receiving methionine (group M=M1+M2) in week 1. During weeks 2,3 and 4 the 4 sheep receiving cobalt (group C=C1+C2) also ate significantly more concentrates than those receiving methionine. Examples of the feed residues from a sheep which had eaten all its concentrate versus one which had not can be seen in plates 7 and 8 respectively. There was little difference between groups in the amount of hay eaten during this first 5 minute period. On returning the uneaten feed to the animal it could be seen that during deficiency some animals left a considerable amount of maize despite eating quite a lot of hay (plate 9).

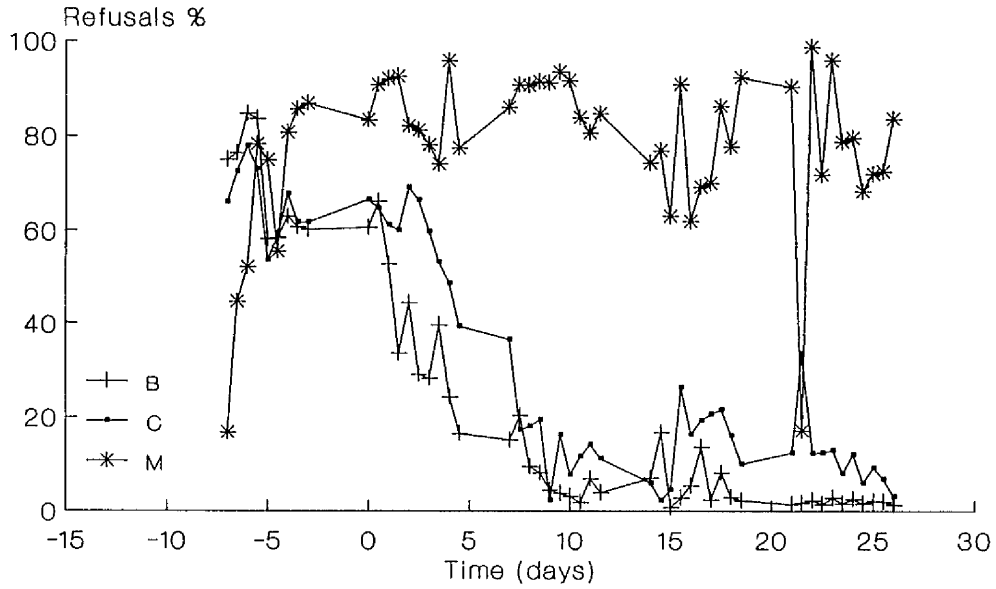


Fig 5.1 Concentrate Refusals of Sheep

Day 0 for all figures was the day on which supplementation was initiated

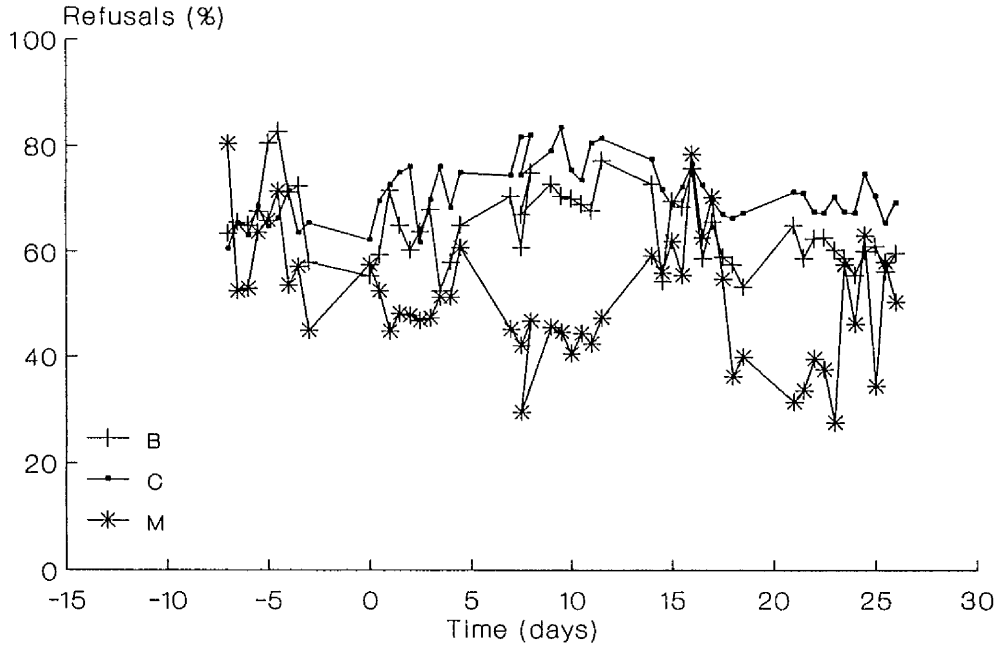


Fig 5.2 Forage Refusals of Sheep

PLATE 7

Feed residues from a cobalt sufficient sheep

PLATE 8

Feed residues from a cobalt deficient sheep

Hay intake, during the first 5 minutes, was not significantly different between the two batches, nor was it affected by treatment, although an apparent decrease in refusals by group B occured in February during the period when some of the sheep were offered the pelleted diet and could not have seperate concentrate and forage intake assessed. Overall hay intake was not measured so this can only be used as an indicator of preference not of total intake.

5.3.2. Serum Vitamin B_{12} Concentration.

Prior to treatment serum vitamin B_{12} concentrations fell in all groups, see Fig.5.3. One animal, later assigned to group M had elevated vitamin B_{12} concentrations due to being moved into a different pen which had not been mucked out. When concentrations were regressed for each sheep and the regression coefficients were compared there were no significant differences between treatment groups.

After treatment, group B and C serum vitamin B_{12} concentrations were significantly elevated compared to those of group M by day 1, but group B concentrations were also significantly greater than those of group C (Fig.5.4 and Table 5.4 Appendix III). However the vitamin B_{12}injection produced a rapid increase in serum vitamin B_{12} which quickly declined toward pretreatment levels, while the oral cobalt dose produced a steady rise in serum vitamin B_{12} concentrations. This pattern was repeated after each weekly treatment although basal concentrations on the injectable treatment rose gradually too. Concentrations of vitamin B_{12} in group M animals were slightly higher on days 21 and 28 due to the two animals which received 5,6-dimethylbenzimidazole during the second treatment period. However it was not a significant effect possibly due to the short treatment period (2 weeks) and the small number of animals involved (2).

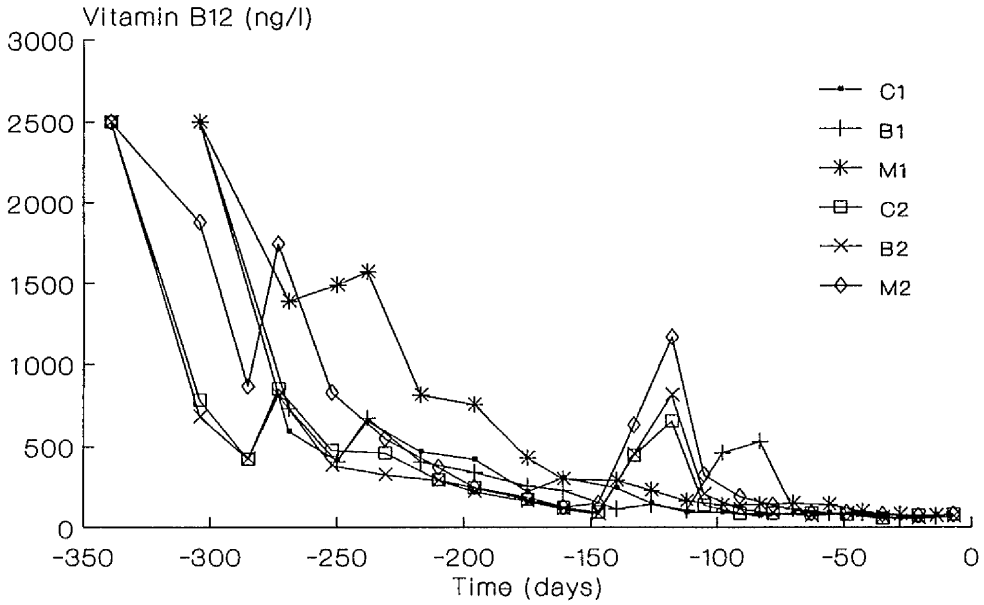


Fig 5.3 Serum Vitamin B12 Concentrations of Sheep Pre Treatment

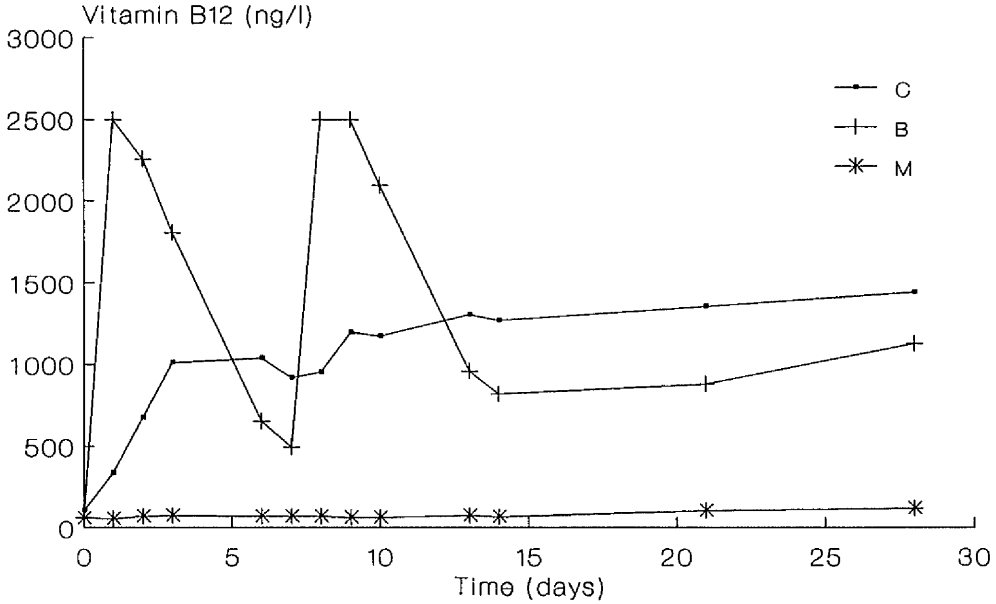


Fig 5.4 Serum Vitamin B12 Concentrations of Sheep Post Treatment

5.3.3. Serum MMA Concentration.

Prior to treatment serum MMA concentrations rose in all groups, see Fig.5.5, and when an exponential curve was fitted to the data for each sheep and the coefficients were compared there were no significant differences among treatment groups. After treatment serum MMA concentrations for groups C and B fell gradually while those for group M continued to rise. The difference became significant after 9 days of treatment, see Fig.5.6 and Table 5.5 Appendix III. There was some evidence of a drop in MMA concentrations for group M during the final 2 weeks, again this was due to the 2 sheep receiving 5,6-dimethylbenzimidazole (M2) but was not significant.

5.3.4. Serum Succinate Concentration.

Prior to treatment there were 4 occasions when there were significant differences in serum succinate concentrations between 2 groups, see Fig.5.7; however the groups involved and the differences between them varied, (C > B&M, M > C&B, M < C&B and M < C). No significant differences were found on regression since the regression equation did not account for a great deal of the variation in the data (R^2 = 20%).

After treatment mean succinate concentrations for group C were consistently greater than those of the other two groups but the only significant difference found was between groups C and M on day 14, see Fig.5.8 and Table 5.6 Appendix III.

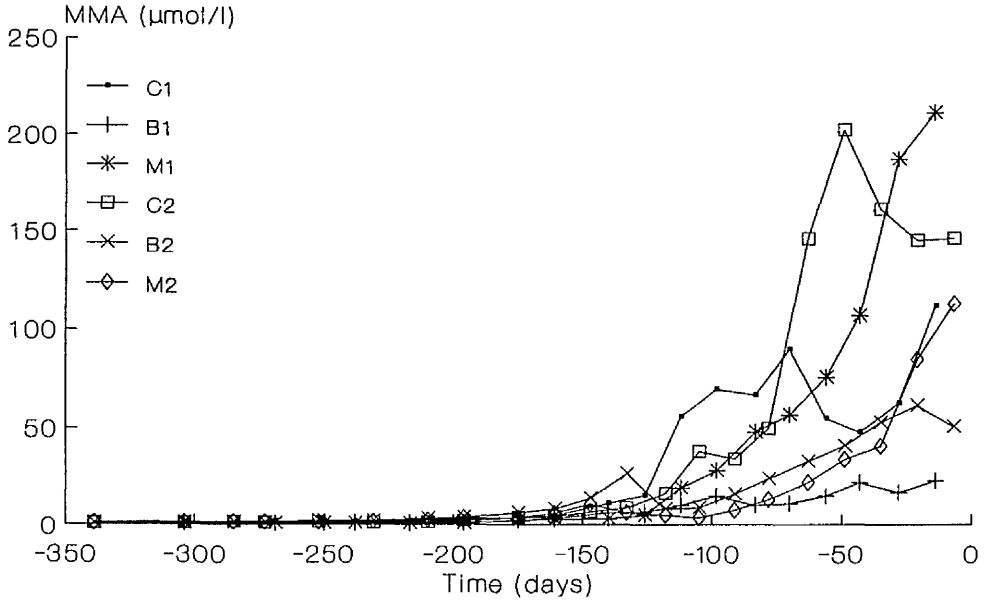


Fig 5.5 Serum MMA Concentrations of Sheep Pre Treatment

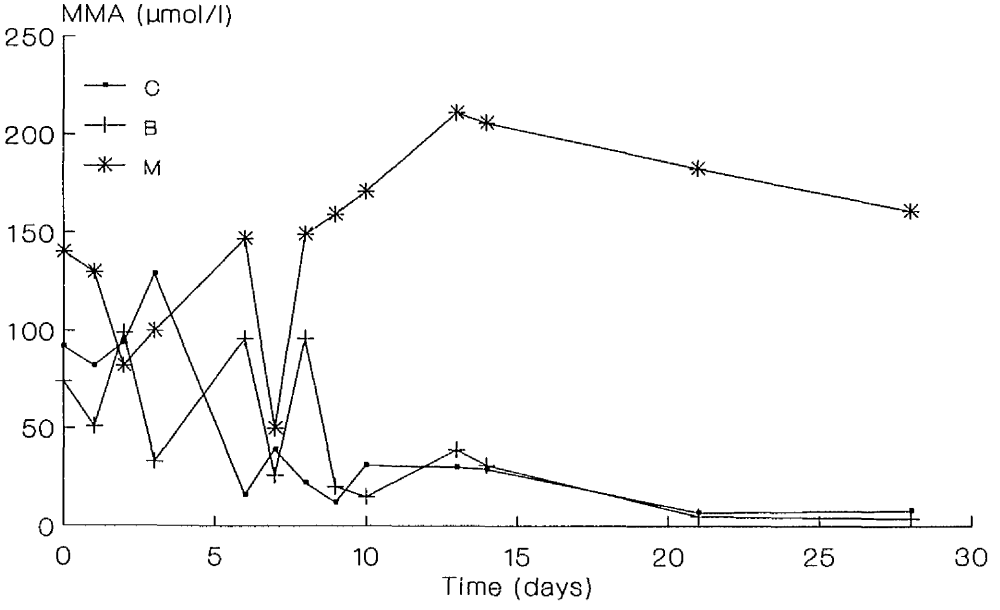


Fig 5.6 Serum MMA Concentrations of Sheep Post Treatment

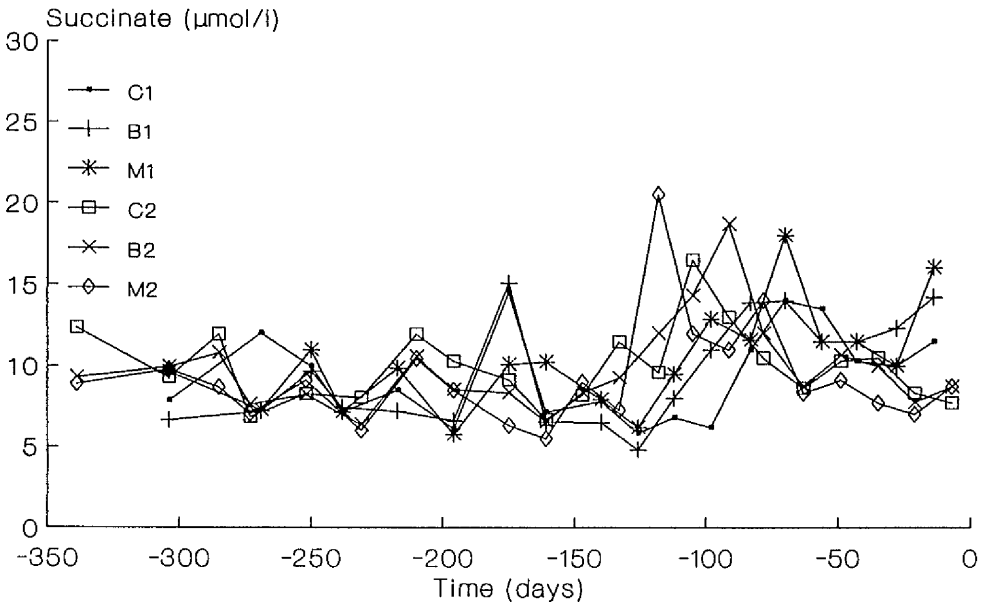


Fig 5.7 Serum Succinate Concentrations of Sheep Pre Treatment

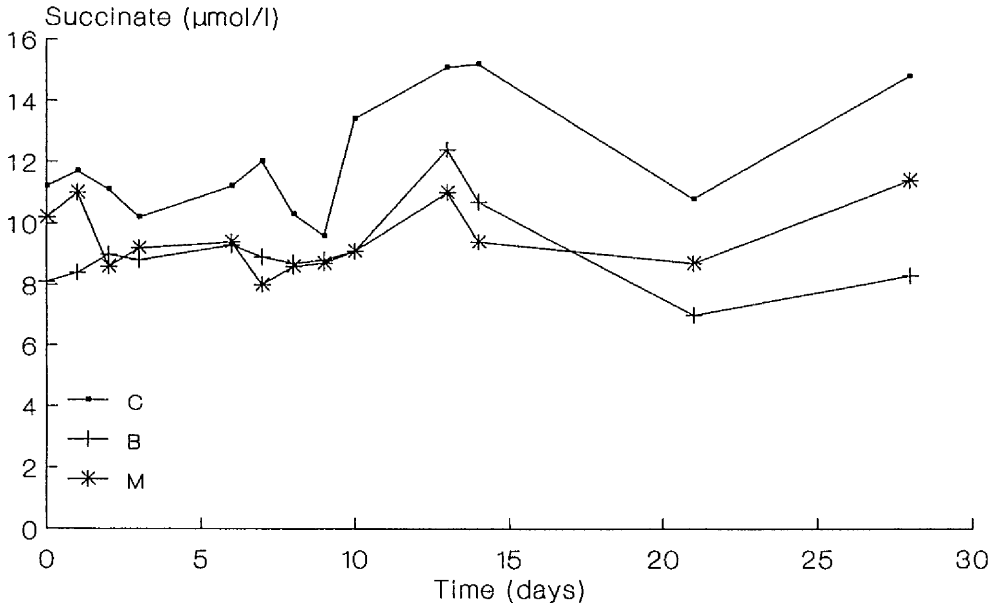


Fig 5.8 Serum Succinate Concentrations of Sheep Post Treatment

5.3.5. Rumen Succinate Concentration.

Samples of rumen contents were only taken after treatment commenced, and no significant differences between treatments in succinate concentrations in the rumen were recorded, see Fig.5.9 and Table 5.7 Appendix III. From the graph it can be seen that there was a large but short-lived increase in mean rumen succinate in group C, and an earlier but smaller peak in group B, followed by a consistently elevated level. The peak for group C is due to 2 animals with exceptionally high concentrations on consecutive days, both of which were in the first group to be treated. The rise in group B concentrations involved all 4 sheep but each reacted differently. One sheep peaked in the first week and then returned to normal while another sheep had elevated concentrations throughout the second and third weeks. A third sheep peaked on days 9 and 10, while the last sheep peaked in the final week. Thus at any one time only one or at most two sheep had elevated rumen succinate concentrations.

5.3.6. Serum GOT Activity.

Serun GOT activity remained within the normal range except for a slight elevation at the end of the trial, see Fig.5.10 and Table 5.8 Appendix III. At the beginning of the experiment there was a significant difference in serum GOT activity between the first and second treatment groups (group 2 being higher) but as the animals were within the normal range this was not of practical importance.

5.3.7. Liveweight.

Liveweights fell at the start of the trial as the animals were becoming accustomed to the new diet, they were also shorn during this period. Once they levelled out they remained steady until towards the end of the trial when the effects of decreased

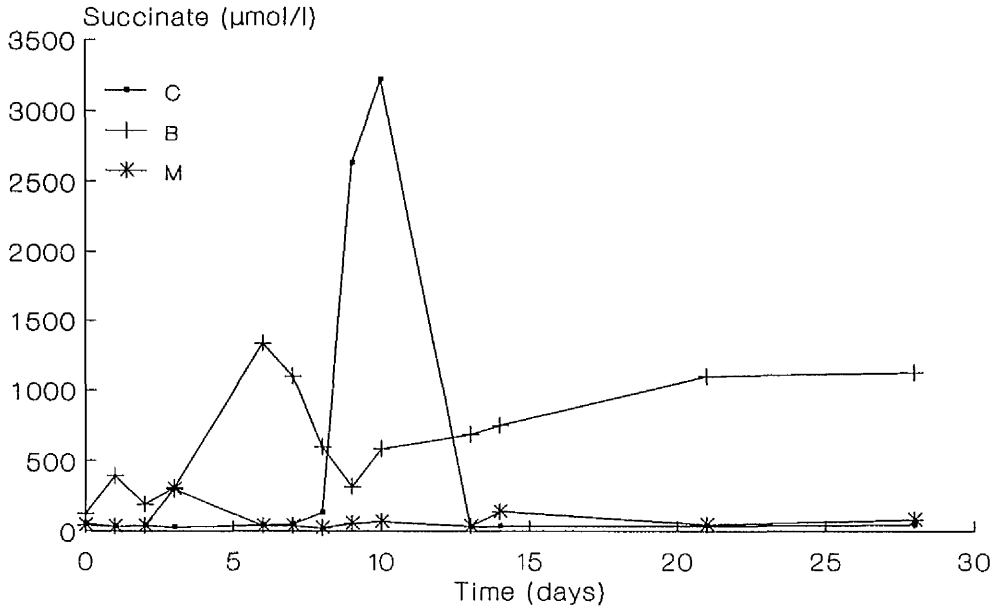


Fig 5.9 Rumen Succinate Concentrations of Sheep Post Treatment

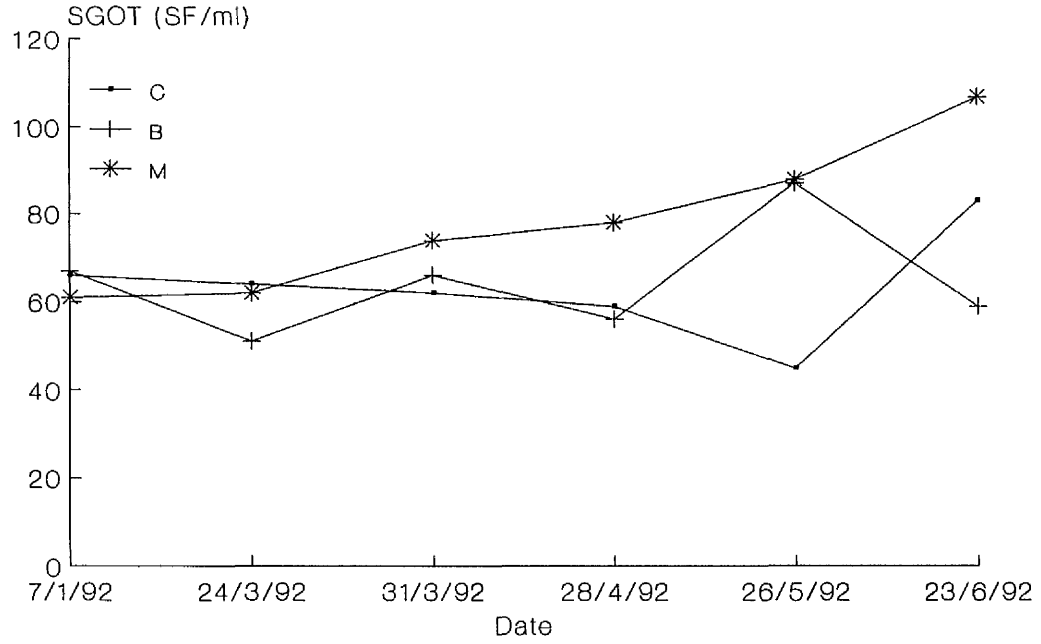


Fig 5.10 Serum GOT Activity of Sheep

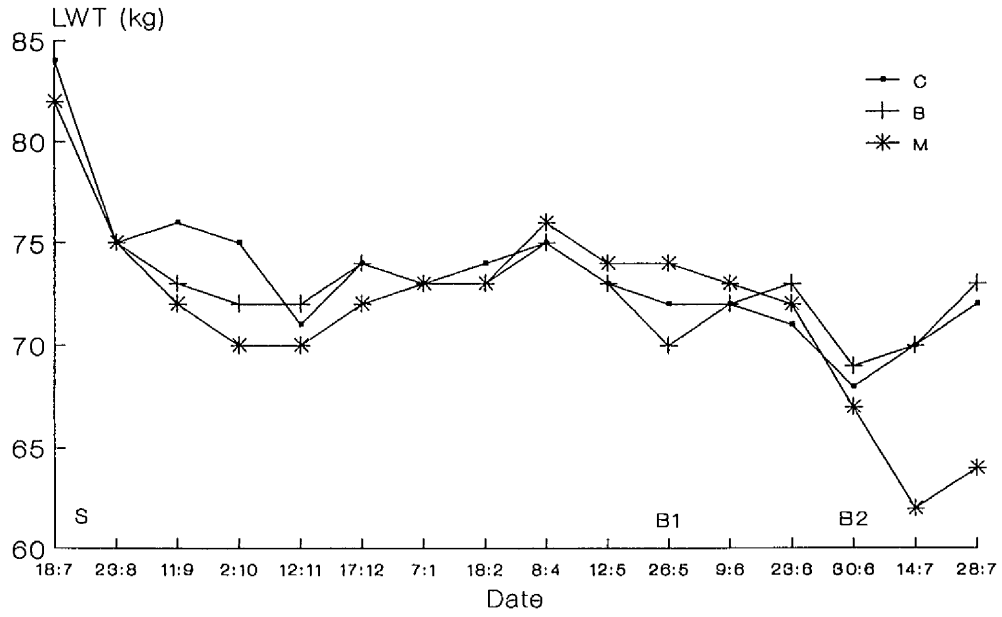


Fig 5.11 Liveweights of Sheep

S=shearing, B1=batch 1, B2=batch 2 treatment dates

appetite were noticed, see Fig.5.11 and Table 5.9 Appendix III. Group M liveweights continued to fall after treatment but groups C and B started to regain a little weight.

5.3.8. Whole Blood GSH-Px Activity.

There were no significant differences in GSH-Px activity, which remained adequate and fairly constant throughout the trial, an oral selenium supplement was administered slightly prior to treatments and this caused a rise in the last 3 months, see Table 5.10 Appendix III.

5.3.9. Serum Copper, Magnesium and Calcium Concentrations.

Copper concentrations dropped considerably during the trial, particularly for group B with the result that in the last month they fell to just below the 'normal' range, see Table 5.11 Appendix III. Magnesium concentrations remained stable, and calcium concentrations rose slightly during the trial.

5.3.10. Rumen Volatile Fatty Acids.

After treatment rumen acetate as a percentage of total volatile fatty acids decreased in group C relative to group M, from 65 to 60 %, and this was significant from the second week, acetate percentages for group B were only significantly lower, 63%, on the last day, see Table 5.12 Appendix III. Rumen propionate, as a percentage of total volatile fatty acids, behaved in an inverse fashion, increasing in groups C and B towards the end of the trial, from 20 to 22 and 26% respectively; propionate percentages for group C were also significantly higher than those for group B on one occasion, see Table 5.13 Appendix III. Rumen butyrate, as a percentage of total

volatile fatty acids, appeared to rise from 9 to 11 % in group M after the first week, but rapidly returned to normal, see Table 5.14 Appendix III. On comparison of the ratio of acetate to propionate in the rumen, see Table 5.15 Appendix III, this decreased on treatments C and B from >3 to <2.5. The ratio of acetate plus butyrate to propionate followed the same pattern, falling from >3.5 to <3, see Table 5.16 Appendix III.

5.4. Discussion.

There were many problems during the course of this trial which have complicated the interpretation of the results and made it difficult to draw conclusions, not least of which was the length, and particularly the difference in length, of time taken for the sheep to develop the decreased appetite. It had been hoped to complete a second round of treatments during the year, which would have answered more questions but unfortunately this proved to be impossible.

From the pre treatment data concentrate refusals began to increase from April which corresponds to day -50 for group 1. At this point vitamin B_{12} concentrations were below 100ng/l and MMA concentrations had risen to between 10 and 200μmol/l suggesting that the animals were very deficient. Liveweight loss was also apparent in some sheep from this time.

Treatment with vitamin B_{12} restored appetite within a week, and by the end of the month these sheep were eating all their concentrate within 5 minutes. Their serum vitamin B_{12} concentrations were very high and their MMA concentrations were below 5 μmol/l. Cobalt treatment had a very similar effect rapidly reducing concentrate refusals, although not quite to original levels within the month. Again vitamin B_{12} concentrations were restored to their original level, and MMA

concentrations fell to within the normal range (< 10μmol/l). Methionine treatment had no effect on concentrate refusals, serum vitamin B_{12} or MMA concentrations. Both vitamin B_{12} and cobalt treatments halted and to some extent restored liveweight loss. Treatment with 5,6-dimethylbenzimidazole showed hopeful preliminary signs of increasing vitamin B_{12} concentrations and reducing MMA concentrations.

These results are much as might have been expected, the findings that vitamin B_{12} and cobalt supplementation alleviate the symptoms of cobalt deficiency are not surprising, and these results appear to support the suggestion (Rickard *et al* 1975) that 5,6-dimethylbenzimidazole may be used to replace cobalt at least partially, presumably through its action of sparing cobalt by increasing the proportion of true vitamin B_{12} relative to α-analogues produced and so reducing wastage of what cobalt is available.

This trial did not suggest, as had been thought possible, that deficient animals develop a preference for forage. On the contrary the results suggest the development of a dislike of concentrates, as the percentage of hay eaten remained unchanged by deficiency and treatment. However as only the intake during the first five minutes was recorded, it is impossible to speculate on total forage intake.

Methionine supplementation (sufficient to restore elevated FIGLU concentrations to normal according to Smith *et al* 1974) failed to restore appetite for concentrates. This is not particularly surprising but it tends to confirm our original suggestion that lack of appetite is connected with the malfunction of the methylmalonyl CoA mutase enzyme, and not with failure of the methyltetrahydrofolate-homocysteine methyltransferase enzyme upsetting methionine recycling and causing an amino acid imbalance.

Because of the speed of appetite recovery after treatment it is considered unlikely to be a 'pain taught' response. However due to the failure of the experiment with the pelleted ration it was impossible to confirm this.

Serum succinate did not appear to be correlated with vitamin B_{12} status in this experiment although there was some evidence that supplementation with either cobalt or vitamin B_{12} increased rumen succinate concentrations, contrary to the work of Kennedy *et al* (1991c & d) and Young *et al* (1991) which suggested that rumen succinate concentrations increase during deficiency. Each sheep seemed to respond after a different time interval, and for a different period of time however. This variation could have been exacerbated by the short time interval between feeding (8.30am) and sampling (10.30am). However, these times were maintained constant throughout. No mention of feeding time relative to sampling time is made by Kennedy *et al* (1991c or d) or by Young *et al* (1991) but they did find that 60 minutes after oral administration of sodium succinate, a rise in portal vein succinate was observed (Kennedy *et al* 1991c & d, & Young *et al* 1991). This suggests that once formed rumen succinate would be absorbed fairly rapidly.

The rumen volatile fatty acid data suggests that treatment had some effect. As treatments C and B increased concentrate intake, the percentage of propionate increased, and the percentage of acetate decreased leading to a decreased acetate to propionate ratio. This is what would have been anticipated from the consumption of more concentrates as they favour a propionic type fermentation. Group M had a more acetic/butyric type fermentation than groups C and B by the end of the experimental period. It is not clear why group C has a more propionic type fermentation than group B and yet group Bs' appetite for concentrates was restored earlier and more successfully.

During cobalt deficiency Young *et al* (1991) associated decreased rumen propionate concentrations with increased rumen succinate and attributed it to decreased formation of propionate from succinate. Despite finding increased propionate concentrations on supplementation, no concomitant decrease in rumen succinate was observed, and so it is thought to be due to the change in diet and not to increased conversion of succinate to propionate.

CHAPTER 6

DISCUSSION

6.1. Defining Cobalt Deficiency in Goats.

The results of the three experiments reported in chapter 3 indicate that 0.04 mgCo/kgDM of the diet was insufficient to prevent liveweight loss in growing goats. Goats receiving less than 0.04 mgCo/kgDM showed an elevation in serum GOT activity indicating that some damage to the liver had occured. Concentrations up to 0.06 mgCo/kgDM maintained liveweight gain although possibly not at maximal rates, but did not prevent development of a preference for forage, pale mucous membranes or elevation of MMA concentrations. Concentrations above 0.06 mgCo/kgDM maintained MMA concentrations below 20 μmol/l, but only supplementation at 0.10 mgCo/kgDM caused vitamin B_{12} concentrations to rise above 100 ng/l.

Overall 0.06 mgCo/kgDM is probably insufficient while 0.07 mgCo/kgDM would probably prevent deficiency. This is higher than the minimum of 0.04 mg/kgDM quoted by Andrews (1970a) for cows, but very similar to the minimum of 0.06 to 0.07 mg/kgDM quoted by MacPherson *et al* (1973). It is also similar to the 0.07 mg/kgDM (Underwood 1981) and the 0.08 mg/kgDM (Andrews 1970a) minimums quoted for sheep.

These results contradict Platten (1951) who considered that goats required more cobalt than sheep, however this opinion was not based on scientific trials merely on observations of one goat herd. These results confirm the findings of Mgongo (1984) who reported 0.01 mg/kgDM to be insufficient, and suggest that had Clark *et al* (1987) continued their trial beyond 4 months they would have found 0.035 mgCo/kgDM to be insufficient.

MMA concentrations of up to 15 μmol/l occurred when no other symptoms of deficiency were apparent. In the deficient groups concentrations in excess of 100

μmol/l were recorded. Thus it is suggested that for diagnostic purposes serum MMA concentrations between 15 and 20 μmol/l are indicative of marginal cobalt/vitamin B_{12} status. This is higher than comparable values for sheep (10-15 μmol/l) and cattle (2-4 μmol/l). Concentrations below 15μmol/l are normal while concentrations above 20μmol/l are indicative of cobalt/vitamin B_{12} deficiency.

Vitamin B_{12} concentrations as low as 30 ng/l were measured in all three experiments and concentrations between 50 and 100 ng/l were common in groups experiencing only slightly decreased liveweight gain, thus it is suggested that this probably represents marginal deficiency. Concentrations below 50 ng/l indicate deficiency, while concentrations above 100ng/l suggest that the goat is not suffering from cobalt deficiency. This range is much lower than the concentrations regarded as indicative of marginal vitamin B_{12} status in cattle (150-200ng/l) and sheep (200-400ng/l).

Initial vitamin B_{12} concentrations in the goats were high (600-800 ng/l) and although they depleted to very low levels (30ng/l), supplementation with cobalt failed to restore them to anywhere near their original levels. Post treatment levels were 200ng/l maximum. There are several possible explanations for this. Prior to the experiment the goats were newly weaned, thus the initially high serum vitamin B_{12} concentrations may be unrepresentative of adult goats. Alternatively the availability of vitamin B_{12} directly from milk may be greater than the availability of vitamin B_{12} which must first be synthesised from cobalt in solid feed, particularly as synthesis of vitamin B_{12} from cobalt is known to be inefficient (Underwood 1981). A third possibility is that preruminant goats may have a smaller requirement for vitamin B_{12}. It has recently been found that before weaning, lambs with a plasma vitamin B_{12} concentration of 350 ng/l do not show reduced growth rates compared to those with higher plasma vitamin B_{12} concentrations, however after weaning a plasma concentration in excess of 650 ng/l is required for optimum growth (MacPherson *et*

al In press). Thus it seems reasonable that goats may be similar.

Alternatively in vitamin B_{12} deficient animals cobalt supplementation might possibly result in binding of absorbed vitamin B_{12} onto Transcobalamin 1 and it is not known whether the microbiological assay used is suitable for measuring vitamin B_{12} associated with this binder in goat plasma. Transcobalamins Tc0, Tc1 and Tc2 are known to be present in the serum of goats and cattle (Price, personal communication, 1991). Although vitamin B_{12} bound to Tc1 does not present any assay problems, current assay procedures do not measure all of the vitamin bound to cattle Tc0 and Tc1 because of the unique thermostability of these binder/B_{12} complexes (Price *et al*, 1992). Thus if the Tc0/B_{12} and Tc1/B_{12} complexes in goat serum are as thermostable as those of cattle, total vitamin B_{12} concentration in goat serum may be underestimated. This explanation is, however, highly speculative and requires further investigation.

Supplementation of cobalt deficient sheep with 0.7 mg oral cobalt /week resulted in serum vitamin B_{12} increasing from <200 ng/l to >700 ng/l (Fisher & MacPherson 1990), while supplementation of cobalt deficient cattle with a mixture of oral dosing (10 or 125 mg cobalt/week), Cosecure boluses and vitamin B_{12} injections resulted in an increase in serum vitamin B_{12} from <100 to >200 ng/l (Paterson & MacPherson 1990), both trials used diets similar to those used in the present study. These results suggest that it is easier to influence the serum vitamin B_{12} concentrations of sheep.

A third explanation could be that high energy diets favour synthesis of α-analogues of vitamin B_{12}. Thus Sutton and Elliot (1972) found that on a high energy diet 'true' vitamin B_{12} amounted to only 86% of total analogs, whereas on an all hay diet it was 112%, in which case more of the supplemented cobalt might be bound in this form and so be unavailable to the host.

Overall the requirement of goats for cobalt is between that of sheep and cattle but more similar to that of sheep. Their marginal vitamin B_{12} concentrations are lower than either but more similar to cattle, while their marginal MMA concentrations are higher than either but more similar to sheep. Thus goats should be classified neither as cattle, nor as sheep for diagnosis of cobalt deficiency, but should be considered separately in their own right.

6.2. The relationship between serum vitamin B_{12} and MMA concentrations with respect to diagnosing cobalt deficiency in ruminants, and the lack of a relationship with succinate concentrations.

In sheep it has been observed that some instances occur where both serum vitamin B_{12} and MMA concentrations are low, suggesting that the animal is vitamin B_{12} deficient but as yet does not have elevated concentrations of MMA. This appears contradictory, however Fisher & MacPherson (1990) thought this might be due to MMA being a functional marker of deficiency, while serum vitamin B_{12} is a marker of how much vitamin B_{12} is being transported around the body. Upon first introducing a cobalt deficient diet, the amount of vitamin B_{12} absorbed would drop markedly decreasing the amount found in the circulation; however initially there would still be sufficient vitamin B_{12} in the liver for conversion of propionate to succinate and hence MMA would not start to build up. Only when liver vitamin B_{12} stores had been decreased would propionate metabolism be affected and MMA concentrations begin to rise. This is likely to be the case in mature animals which have sufficient stores of vitamin B_{12} to withstand cobalt deficient diets for some months. Such was the case for the sheep on the appetite trial where serum vitamin B_{12} concentrations took 150 days to drop from over 2500 ng/l to below 400 ng/l but MMA concentrations only became elevated after 200 days on the cobalt deficient diet.

In cattle Paterson & MacPherson (1990) attributed the same phenomenon to the decreased feed intake resulting from the loss of appetite which would lower the metabolic rate and slow down MMA production. Loss of appetite, for the majority of our sheep, was not severe until they had been on the cobalt deficient diet for 300 days by which time their serum MMA concentrations were already elevated. Less severe loss of appetite might delay the rise in MMA concentrations slightly, particularly as some individual animals appetites were affected long before the majority. Selective refusal of concentrates by vitamin B_{12} deficient animals would further decrease propionate production, and hence MMA concentrations but like loss of appetite this symptom of deficiency is only apparent during clinical deficiency. However if marginal deficiency in goats is indeed signified by serum vitamin B_{12} concentrations between 50 and 100 ng/l and MMA concentrations between 15 and 20 μmol/l then this phenomenon was not apparent during any of our three trials.

Contrary to the results of Kennedy *et al* (1991a & b) and Young *et al* (1991) serum succinate was not found to increase during cobalt deficiency in calves, sheep or goats, and day to day variation was considerable. Rumen concentrations of succinate in the sheep were even more variable than serum succinate concentrations, with some suggestion that supplementation with cobalt or vitamin B_{12} led to rapid but very short lived increases. One major dissadvantage with rumen samples however is the lack of homogeneity of the rumen contents and the difficulty in obtaining a representative sample. These sheep were not fistulated which increases problems when trying to ensure that the sample is taken from the same portion of the rumen. Additionally the nature of the samples is very dependent on how much feed or water the sheep has consumed immediately prior to sampling although an attempt was made to reduce this variability by always sampling at the same time of day.

Overall it appears that in goats as in sheep and cattle, the use of serum vitamin B_{12} concentrations in conjunction with MMA concentrations would be the most reliable method of detecting marginal cobalt deficiency. All the goats exibiting severe symptoms of cobalt deficiency, liveweight loss, loss of appetite, and pale mucous membranes, could easily be identified as having elevated MMA concentration or low serum vitamin B_{12} concentrations. However to identify those goats with reduced growth rates but no clinical deficiency, serum vitamin B_{12} concentrations alone are difficult to interperet, as many of the supplemented goats showed no reduced growth but their serum vitamin B_{12} concentrations remained very low. Serum MMA concentrations were easier to interpret although as explained earlier there could be some delay in response such that serum vitamin B_{12} analysis might suggest a requirement for supplementation before MMA concentrations had been affected, but this was not the case for these goats. Additionally as feed intake decreases during the development of severe cobalt deficiency, the formation of MMA might slow down resulting in a decrease in serum concentrations during severe deficiency. This might result in some false negatives if MMA concentrations alone were used for diagnostic purposes.

Rumen samples are not as easy to obtain as blood samples and rumen succinate in this trial was not suitable for diagnostic purposes. Because of the lack of relationship found between serum succinate and vitamin B_{12} or MMA concentrations, or reduced growth rates or feed intakes, it would not appear to be a reliable marker of cobalt deficiency.

In future, research into the nature and role of the transcobalamins deserves closer attention because if a reliable and accurate method of measuring total serum vitamin B_{12} were to be developed it would improve the diagnosis of marginal cobalt deficiency in both cattle and goats. The serum vitamin B_{12} response of goats to cobalt supplementation on different types of diets is also worthy of further

investigation. These two aspects need to be considered together to determine whether diet does indeed affect the transport and metabolism of vitamin B_{12} in the goat and in cattle.

6.3. The effect of cobalt deficiency on feed intake in sheep and goats.

Our observations of the goats in experiment 2 led us to try to quantify the effects of cobalt/vitamin B_{12} deficiency on preference for forage or concentrates. A preference for forage over concentrate had previously been noted by MacPherson (1982) in ruminants and Ferguson (1990) in sheep.

In the present study in sheep, low cobalt intake resulted in a decrease in concentrate intake while forage intakes remained stable in the appetite assessment protocol used here. Supplementation with vitamin B_{12} produced a rapid response, markedly increasing concentrate intake within a week; cobalt supplementation produced a similar but slower response in concentrate intake. This supported our preliminary observations in goats where vitamin B_{12} injection was seen to produce a visible response in feed intake by the following day, whereas cobalt supplementation required a week to produce a similar effect. This suggests that it is absorbed vitamin B_{12} which is required to correct appetite failure as opposed to cobalt itself in the rumen. Methionine supplementation failed to increase concentrate intake suggesting that appetite failure in cobalt/vitamin B_{12} deficiency may be related to impaired conversion of succinate to propionate.

A build up of propionate in the tissues could cause this effect according to Marston *et al* (1961 and 1972), although Kennedy *et al* (1991b & c) and Young *et al* (1991) suggest that propionate would not build up in the tissues because succinate from the rumen would be absorbed directly and utilized by the liver bypassing the

requirement for conversion to propionate in the rumen and back to succinate in the liver, this bypass would prevent any ill effects due to increased propionate in the tissues. Kennedy *et al* (1991b & c) and Young *et al* (1991) claim that impaired propionate metabolism in the liver is not the primary metabolic defect in ovine cobalt deficiency, but suggest instead that the accumulation of branched chain and odd-numbered fatty acids affects the fluidity of membranes. Hence the defect in methionine synthase activity may be more important than the defect in propionate metabolism, because it is the failure of this pathway which results in increased concentrations of branched chain and odd-numbered fatty acids. From our work it would appear that as far as appetite is concerned the primary defect is not located in the methionine synthase pathway. This was also the conclusion of Price (1990) who reported elevated MMA concentrations in sheep well before any impairment in methyltransferase metabolism which occured only when the animals were severely deficient.

6.4. A comparison of vitamin E supplementation protocols in calves and goats.

In goats and calves supplementation with either oral vitamin E (100 IU/day for goats, or 500 IU/day, later reduced to 250 IU/day for calves as DL-tocopheryl acetate, Rovimix E50, Roche) or injectable vitamin E (900 IU for goats, 1500 IU for calves, as D-α-tocopherol, Stuart Products Inc.) raised plasma vitamin E concentrations from inadequate (<2 μmol/l) to sufficient (>3 μmol/l). Injection of vitamin E resulted in a rapid rise in plasma vitamin E concentrations by the following day (20-50 μmol/l), but this was short lived and within a week concentrations returned to <7 μmol/l. As a long term measure, injection alone would not be suitable as some adverse effects on calves temperatures and appetite were noted when vitamin E injections were repeated within 3 weeks and yet by this time plasma vitamin E concentrations were marginal. Also injections, particularly with the large doses administered to the calves caused abscesses, and farmers would

also find repeated injections unacceptable on a routine basis. However as a one-off treatment for a deficient animal together with a change to a vitamin E sufficient diet this could be very useful. Oral administration of vitamin E did not cause any adverse reaction.

6.5. The relationship between vitamin E status and erythrocyte stability.

The preliminary trial in goats suggested that the erythrocyte stability technique might be useful as an indicator of vitamin E status if it could be modified to reduce day to day variation between results for the same animal. Although supplementation with vitamin E was only introduced two weeks before the end of the goat experiment, the results using 6% Tween looked promising as there was a small decrease in % haemolysis in the supplemented goats during the second week of supplementation.

In the calf trial, although day to day variation was much lower, particularly with the 3 and 5 % Tween solutions, due to changes to the method, there was still considerable variation between animals. This variation could be due to differing concentrations of red cells in the blood samples in which case haematocrit reading could be used as a correction factor. However there are many other factors which could affect the results which would need to be considered such as preventing premature haemolysis of blood samples due to careless sampling, vigorous mixing, or being left around too long. Additionally an assessment of the within and between assay variation should be made. Within assay variation was not measured during this experiment as processing 12 samples in one day proved problematical without doing duplicates. Between assay variation is further complicated due to the fact that fresh blood samples are required, and that these cannot be stored or frozen without affecting the results. It would be neccessary to assume that provided no changes in diet or treatment were made the result from one animal would be the same on two

different days.

The variability encountered may have made it difficult to detect treatment effects if they occurred. The degree of vitamin E deficiency in the calves was not as severe as in the sheep used by Stevenson and Jones (1989) so differences between severely vitamin E-deficient animals and vitamin E-sufficient calves might be more readily detected. It is also possible that sheep are inherently less variable in this respect than cattle or goats.

From work reported in sheep (Stevenson & Jones 1989) it appeared that 2% Tween might be the optimum concentration for detecting erythrocyte fragility. However in the goats 6% Tween apparently showed up differences better than 2% Tween and in the calves there was little to choose between 3 and 5% Tween. Further work on the erythrocyte stability test, possibly comparing different Tween 20 concentrations might result in development of a more reliable measure of erythrocyte fragility and it may be possible to use this as an indicator of vitamin E status. However unless variability between animals of similar vitamin E status can be minimised, then this test is unlikely to replace plasma vitamin E concentrations as an indicator for vitamin E deficiency, especially as HPLC techniques for vitamin E analysis are more rapid and cheaper. To be of use as a diagnostic test reliable estimates would be required of the % haemolysis representing deficiency, marginal deficiency and adequacy, but until variation is decreased critical diagnostic values cannot be established for this parameter.

6.6. The relationship between cobalt status and immune function in ruminants.

Cobalt deficiency has been associated with impaired neutrophil function (particularly % kill) in humans (Kaplan and Basford 1976) calves (Wright, MacPherson and Taylor 1982, MacPherson *et al* 1987, Paterson and MacPherson 1986) and in ewes (Fisher and MacPherson 1986) and with decreased IgG concentration (Fisher & MacPherson 1991) and response to clostridial vaccination in lambs (Ferguson 1990).

In the calf experiment, chapter 4, vitamin B_{12} injections given to cobalt deficient calves caused a small but significant increase in % kill of *E. coli* on day 70 and of *C. albicans* on day 77, but not of *S. aureus*. These trends were not evident on the second date of sampling. While in the goat experiment, neutrophil function tests, antibody response to *Clostridium tetani* vaccination and measurements of total IgG failed to show any significant effects of cobalt deficiency or cobalt supplementation on immune function of goats.

The results from the calves do not provide convincing evidence that cobalt supplementation improved the killing ability of neutrophils. Repetition of the experiment with a larger number of animals might clarify the results, since the work of others (Wright *et al* 1987, Paterson & MacPherson 1986, Fisher & MacPherson 1986 & 1991 & Ferguson 1990) in both cattle and sheep suggests that there is likely to be an effect, if only a relatively small one.

In the goat experiments all three tests used indicated that immune function was not adversely affected by cobalt deficiency. Responses have been observed in sheep and/or cattle to all these tests suggesting that we did not choose the wrong tests. However other tests might still show up impaired immune responses. Very little work has been done on the immune response of goats although Aziz *et al* (1984) found depressed neutrophil function in selenium deficient goats as assessed by

polymorphonuclear leucocyte migration and phagocytosis of opsonised zymosan, and Aziz & Klesius (1986) found lower concentrations of leucotriene B_4 produced by selenium deficient goats which would lead to decreased leucotriene B_4 mediated neutrophil chemotaxis.

The mechanism of action of selenium on the immune system is much better understood than that of cobalt, and without specific knowledge of the mode of action it is difficult to speculate whether goats might indeed differ from calves and sheep in this respect. Ferguson (1990) speculated that the effect of cobalt deficiency on resistance to worms in sheep might be partly explained by a decreased protein intake due to loss of appetite. Such an indirect mode of action could be affected by many other factors such as the nature of the diet, for instance in this case urea was used as a supplementary nitrogen source and this was applied to the forage. During deficiency the decreased concentrate intake may have resulted in a greater proportional intake of nitrogen.

In future it would be useful to know whether cobalt deficiency affects goat kid viability and resistance of goats to worms or resistance to any other common diseases of goats eg. pneumonia. Experiments in sheep (Fisher and MacPherson 1991 & Ferguson 1990) found that lambs born to cobalt deficient ewes were more likely to die than lambs born to cobalt sufficient ewes, and that cobalt deficient sheep had less resistance to worms than cobalt sufficient sheep. Findings such as these help to persuade farmers that it will be financially sound to supplement their animals.

A more scientific approach would be to try and pinpoint the mode of action of cobalt in the other ruminants. Both selenium and vitamin E have been used successfully at a cellular level. Supplementation of neutrophils from selenium deficient goats at a cellular level stimulated neutrophil function similarly to supplementation of the

whole animal (Aziz *et al* 1984). Cobalt/vitamin B_{12} is thought to act indirectly on cell function and supplementation of cell cultures with vitamin B_{12} might clarify this. Thus indirect effects of cobalt deficiency such as decreased protein intake due to inappetance would be eliminated if supplementation was carried out on cell cultures.

Use of alternative immune function tests such as mitogen induced lymphocyte proliferation responses or T_h versus T_s differential cell counts might help to identify the mode of action, because if only certain parts of the immune system are affected then only specific tests involving these areas will demonstrate an effect.

Fisher and MacPherson (1991) attributed the decreased resistance of cobalt deficient lambs to bacterial infections to decreased intake of colostrum during the first few hours after birth, and this in turn was due to a lack of vigour in the lambs. Presumably the lack of vigour of the lambs was due to the inadequate supply of vitamin B_{12} in the ewe during pregnancy, however this could have been the consequence of an indirect action of cobalt deficiency on appetite. MacPherson *et al* (1989) proposed that the effects of deficiency on cell mediated immunity (neutrophil function) and humoral immunity (antibody response to clostridial vaccination) were primary effects and not dependent on reduced food intakes. Certainly the effects of vitamin B_{12} deficiency on cell mediated immunity in humans and mice (cited by Gershwin *et al* 1985) were not due to impaired propionate metabolism affecting food intake. Kaplan and Basford found a decrease in phagocytosis-associated hexose monophosphate shunt activity in vitamin B_{12} deficient humans and they speculated that impaired protein or DNA synthesis had resulted in decreased concentrations of some of the enzymes involved. Thus experiments to confirm changes in hexose monophosphate shunt activity, or changes in concentration of specific enzymes could clarify the role of vitamin B_{12} in immune function.

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APPENDIX I

Table 3.1. Experiment 1 Hay.

	Contents				
	1	2	3	Mean	(S.D.)
DM g/kg	806	846	876	843	20.3
Co mg/kg	0.03	0.06	0.04	0.04	$8.8*10^{-3}$
Se mg/kg	-	-	0.06	-	-
CP g/kg	68	79	68	72	3.67
OM g/kg	952	961	956	956	2.60
IVOMD %	54.3	53.8	56.2	54.8	0.73
In vitro D	57.5	51.8	53.7	54.3	1.68
M/D	8.6	7.9	8	8.2	0.22
Ca g/kg	4.4	3.6	3.0	3.7	0.41
P g/kg	1.3	1.4	1.4	1.4	0.03
Mg g/kg	1.1	0.9	1.2	1.1	0.09
K g/kg	12.3	12.1	11.5	12.0	0.24
Na g/kg	0.88	0.28	0.73	0.63	0.18
Cu mg/kg	3.46	3.14	3.26	3.29	0.09
Mo mg/kg	0.48	0.35	0.24	0.36	0.07
S g/kg	1.27	1.19	0.87	1.11	0.12
Zn mg/kg	20.4	19.9	21.5	20.6	0.47
Fe mg/kg	47.3	69.1	52.8	56.4	6.55
Mn mg/kg	83.4	36.0	74.4	64.6	14.53
Available Cu mg/kg	3.46	0.29	-	0.30	$5*10^{-3}$

N.B. Urea contains <0.01 mg/kgDM Cobalt.

Table 3.2. Experiment 1 Concentrates

	MAIZE	BARLEY			
	Content	1	2	Mean	(S.E.)
DM g/kg	856	766	808	787	21
Co mg/kg	0.04	0.03	0.06	0.04	0.015
Vitamin E IU/kg	0.56	1.68	1.47	1.58	0.105
Se mg/kg	-	-	0.02	0.02	-
CP g/kg	87	98	96	97	1
OM g/kg	998	976	973	974	1.5
IVOMD %	94.2	84.8	82.6	83.7	1.1
In vitro D	94.1	82.8	80.4	81.6	1.2
M/D	15.0	13.2	12.9	13.05	0.15
Ca g/kg	<0.2	0.5	0.6	0.55	0.05
P g/kg	1.3	3.6	3.5	3.55	0.05
Mg g/kg	0.4	1.2	1.1	1.15	0.05
K g/kg	2.13	4.79	4.7	4.74	0.045
Na g/kg	0.11	0.01	0.13	0.07	0.06
Cu mg/kg	1.37	6.05	5.6	5.82	0.225
Mo mg/kg	0.11	0.41	0.4	0.40	$5*10^{-3}$
S g/kg	0.23	1.11	1.7	1.40	0.295
Zn mg/kg	11.2	30.20	29.8	30	0.2
Fe mg/kg	35.8	89.00	138.00	113.5	24.5
Mn mg/kg	3.03	12.5	12.4	12.4	0.05
Available Cu mg/kg	0.08	0.61	-	-	-

Table 3.3. Experiment 2 Hay

	Contents				
	1	2	3	Mean	(S.E.)
DM g/kg	838	813	879	843	19.24
Co mg/kg	0.06	0.09	0.1	0.08	0.01
Vitamin E IU/kg	11.07	10.11	-	10.6	0.48
Se mg/kg	-	0.04	0.07	0.06	0.02
CP g/kg	88	130	61	93	20.1
OM g/kg	940	940	940	940	0
IVOMD %	51.8	52.3	52.6	52.2	0.23
In vitro D	48.7	49.2	49.4	49.1	0.21
M/D	7.4	7.5	7.5	7.5	0.03
Ca g/kg	3.8	2.7	2.8	3.1	0.35
P g/kg	2.0	1.7	1.8	1.8	0.09
Mg g/kg	1.0	0.8	0.9	0.9	0.06
K g/kg	17.0	14.2	14.5	15.2	0.89
Na g/kg	2.12	1.64	1.88	1.88	0.14
Cu mg/kg	3.94	3.17	3.38	3.50	0.23
Mo mg/kg	0.19	0.57	0.49	0.42	0.12
S g/kg	1.14	1.34	1.44	1.31	0.09
Zn mg/kg	22.7	20.0	21.1	21.3	0.78
Fe mg/kg	73.7	73.6	126.0	91.1	17.4
Mn mg/kg	244	204	169	205.7	21.7
Available Cu mg/kg	0.38	-	0.29	0.34	0.04

N.B. Urea contains <0.01 mg/kgDM Cobalt.

Table 3.4. Experiment 2 Maize

	Contents					
	1	2	3	4	Mean	(S.E.)
DM g/kg	875	878	884	885	880.5	2.40
Co mg/kg	0.01	0.01	0.00	0.02	0.01	$4.1*10^{-3}$
Vitamin E IU/kg	1.24	-	6.57	-	3.9	2.66
Se mg/kg	-	0.07	0.04	0.02	0.04	0.01
CP g/kg	96	90	99	91	94	2.12
OM g/kg	988	989	974	992	986	4.01
IVOMD %	90.2	94.6	90.1	94.8	92.4	0.31
In vitro D	89.1	93.6	87.8	94	91.1	1.57
M/D	14.3	15.0	14.0	15	14.6	0.25
Ca g/kg	0.8	0.5	3.8	0.2	1.32	0.83
P g/kg	2.2	1.3	2.8	1.7	2	0.32
Mg g/kg	0.8	0.5	1.1	0.5	0.7	0.14
K g/kg	2.91	2.42	3.65	2.48	2.86	0.28
Na g/kg	0.28	0.1	0.02	0.02	0.10	0.06
Cu mg/kg	1.82	1.64	2.20	1.37	1.76	0.17
Mo mg/kg	0.13	0.10	0.14	0.09	0.12	0.01
S g/kg	0.90	0.63	1.03	0.99	0.89	0.09
Zn mg/kg	17.5	13.1	22.1	12.0	16.2	2.30
Fe mg/kg	30.8	24.3	34.7	67.0	39.2	9.5
Mn mg/kg	4.63	3.6	7.08	3.94	4.81	0.79
Available Cu mg/kg	0.18	0.16	0.22	0.14	0.18	0.02

Table 3.5. Experiment 3 Hay:Straw Mix

	Contents				Mean	(S.E.)
	1	2	3	4		
DM g/kg	820	833	857	804	828	11.2
Co mg/kg	0.1	0.05	0.05	0.05	0.06	0.01
Se mg/kg	0.02	0.03	0.01	0.02	0.02	$4.1 * 10^{-3}$
CP g/kg	106	84	84	77	88	6.3
OM g/kg	949	946	948	951	948	1.04
IVOMD %	45	46.6	44.4	47.1	45.8	0.64
In vitro D	42.7	44.1	42.0	44.8	43.4	0.64
M/D	6.3	6.5	6.2	6.7	6.4	0.11
Ca g/kg	3.1	3.2	3.2	3.2	3.2	0.02
P g/kg	1.5	1.6	1.2	1.1	1.4	0.12
Mg g/kg	0.9	1.1	1.0	1.1	1.0	0.05
K g/kg	15.5	16.5	16.3	16.4	16.2	0.23
Na g/kg	1.34	1.05	0.97	1.20	1.14	0.08
Cu mg/kg	3.51	3.62	3.18	3.09	3.35	0.13
Mo mg/kg	0.44	0.23	0.22	0.45	0.34	0.06
S g/kg	1.22	1.25	1.09	1.11	1.17	0.04
Zn mg/kg	21.5	15.8	13.3	15.2	16.4	1.77
Fe mg/kg	121.0	58.8	68.0	74.6	80.6	13.8
Mn mg/kg	73.3	76.8	61.7	79.9	72.9	3.98
Available Cu mg/kg	0.31	-	-	-	-	-

N.B. Urea contains <0.01 mg/kgDM Cobalt

Table 3.6. Experiment 3 Maize

	Contents					Mean	(S.E.)
	1	2	3	4	5		
DM g/kg	885	881	884	886	888	885	1.16
Co mg/kg	<0.02	0	0.01	0.01	0.01	0.01	$3.2 * 10^{-3}$
Se mg/kg	0.02	0.02	0.01	0.05	0.08	0.04	0.01
CP g/kg	91	93	88	97	99	94	1.99
OM g/kg	992	992	992	992	989	991	0.6
IVOMD %	94.8	92.5	92.5	88.7	88.5	91.4	1.22
In vitro D	94	91.8	92.1	87.9	87.6	90.7	1.26
M/D	15.0	14.7	14.7	14.1	14.0	14.5	0.19
Ca g/kg	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	0
P g/kg	1.7	1.7	1.6	2.4	3.0	2.1	0.27
Mg g/kg	0.5	0.6	0.5	0.8	1.0	0.7	0.10
K g/kg	2.48	2.41	2.21	3.29	3.76	2.83	0.30
Na g/kg	0.02	-	0.01	0.05	0.02	0.025	$8.7 * 10^{-3}$
Cu mg/kg	1.37	1.37	1.39	1.81	1.82	1.55	0.11
Mo mg/kg	0.09	0.09	0.12	0.14	0.16	0.12	0.01
S g/kg	0.99	1.02	1.00	0.85	0.81	0.93	0.04
Zn mg/kg	12.0	12.2	13.4	17.5	21.1	15.2	1.77
Fe mg/kg	67.0	31.0	32.2	26.6	34.7	38.3	7.29
Mn mg/kg	3.94	3.85	3.56	5.57	5.57	4.50	0.44
Available Cu mg/kg	0.14	0.14	0.14	-	-	0.14	0

Table 3.7. Serum Vitamin B_{12} Concentration (ng/l)

Day No.	Cobalt Treatment Group Number				S.E.D	p
	1	2	3	4		
-63	281	365	344	285	90.4	0.775
-47	161^{a}	255^{c}	217^{bc}	191^{ab}	26.6	0.018
-27	98^{a}	137^{b}	124^{ab}	123^{ab}	18.5	0.234
-14	180	176	198	190	32.2	0.885
0	(108)	(88)	(78)	(104)	-	-
14	50^{a}	111^{b}	123^{b}	129^{b}	13.5	<0.001
35	$<50^{a}$	116^{b}	108^{b}	153^{c}	16.6	<0.001
56	59^{a}	157^{b}	150^{b}	167^{b}	24.2	<0.001
70	32^{a}	154^{b}	137^{b}	149^{b}	20.2	<0.001
84	50^{a}	158^{b}	163^{b}	181^{b}	22.6	<0.001
100	60^{a}	157^{b}	137^{b}	166^{b}	17.0	<0.001
112	44^{a}	115^{b}	110^{b}	162^{c}	21.7	<0.001
126	79^{a}	133^{b}	140^{b}	162^{b}	16.0	<0.001
140	63^{a}	134^{b}	143^{b}	212^{c}	21.2	<0.001
154	62^{a}	113^{b}	120^{b}	161^{c}	14.5	<0.001
168	$>2500^{a}$	168^{b}	153^{b}	208^{b}	34.3	<0.001
182	458^{a}	181^{b}	168^{b}	257^{b}	53.4	<0.001

Table 3.8. Serum MMA Concentrations (μmol/l)

Day No.	Cobalt Treatment Group Number				S.E.D	p
	1	2	3	4		
-63	1.8	1.6	<3	2	1.346	0.809
-47	10	3	8	8	4.0	0.330
-14	6	11	13	10	3.26	0.259
0	(28)	(18)	(31)	(12)	-	-
14	49^{a}	9^{b}	5^{b}	10^{b}	5.73	<0.001
35	48^{a}	6^{b}	6^{b}	6^{b}	3.08	<0.001
56	40^{a}	5^{b}	5^{b}	4^{b}	5.51	<0.001
70	58^{a}	5^{b}	2^{b}	12^{b}	15.16	0.004
84	60^{a}	4^{b}	2^{b}	7^{b}	5.47	<0.001
100	100^{a}	15^{b}	4^{b}	15^{b}	18.32	<0.001
112	94^{a}	8^{b}	8^{b}	5^{b}	9.85	<0.001
126	107^{a}	7^{b}	6^{b}	6^{b}	14.32	<0.001
140	94^{a}	11^{b}	20^{b}	12^{b}	17.36	<0.001
154	121^{a}	13^{b}	7^{b}	9^{b}	16.15	<0.001
168	$<1^{a}$	6^{b}	9^{b}	4^{ab}	2.615	0.033
182	$<1^{a}$	5^{b}	-	3^{ab}	1.579	<0.001

Day zero is date of first Cobalt supplementation and the values in brackets were used as covariates for the statistical analysis.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Fig.3.9 Serum GOT Activity (SF/ml)

Day No.	Cobalt Treatment Group Number				S.E.D	p
	1	2	3	4		
0	76^{ab}	83^{b}	67^{a}	69^{a}	4.59	0.008
14	65	95	70	70	14.25	0.189
100	68	67	68	47	10.45	0.750
154	130^{a}	77^{b}	66^{b}	63^{b}	15.07	<0.001
182	152^{a}	78^{b}	62^{b}	60^{b}	32.6	0.033

Day zero is date of first Cobalt supplementation.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Fig.3.10. Liveweight (kg)

Day No.	Cobalt Treatment Group Number				S.E.D	p
	1	2	3	4		
-63	17.5[a]	18.2[ab]	18.0[ab]	18.4[b]	0.429	0.19
-47	18.3	18.8	18.4	18.8	0.352	0.38
-27	18.0	18.5	18.0	18.1	0.384	0.51
-14	18.3[ab]	18.8[b]	18.0[a]	18.1[ab]	0.347	0.16
0	(18.7)	(18.2)	(19.0)	(19.0)	-	-
7	18.6	18.7	18.6	18.7	0.282	0.93
14	18.2	18.3	18.8	18.4	0.425	0.53
21	18.4	18.6	18.8	18.6	0.522	0.88
28	18.4	18.3	18.6	18.3	0.506	0.90
35	18.7	19.0	19.4	18.9	0.430	0.464
43	18.8[a]	18.6[a]	20.1[b]	19.3[ab]	0.418	0.013
50	18.7	18.7	19.7	19.5	0.567	0.180
56	18.4	18.4	19.4	19.2	0.544	0.146
63	18.5[a]	18.9[ab]	19.9[b]	19.5[ab]	0.638	0.146
70	18.5	19.0	20.0	19.8	0.725	0.168
77	18.6	19.4	20.3	20.2	0.855	0.181
84	18.6[a]	19.7[ab]	20.8[b]	20.4[ab]	0.884	0.111
91	18.7[a]	20.0[ab]	21.4[b]	21.2[b]	0.896	0.025
100	18.2[a]	20.2[ab]	21.6[b]	21.3[b]	1.217	0.051
105	18.2[a]	20.7[b]	22.0[b]	21.6[b]	1.094	0.012
112	18.0[a]	21.7[b]	22.6[b]	22.6[b]	1.268	0.006
119	18.4[a]	22.2[b]	23.5[b]	23.0[b]	1.226	0.002
126	17.5[a]	22.5[b]	23.9[b]	23.5[b]	1.349	<0.001
135	17.3[a]	24.6[b]	25.2[b]	25.1[b]	1.390	<0.001
140	16.8[a]	24.4[b]	25.2[b]	24.7[b]	1.499	<0.001
144	16.6[a]	25.1[b]	25.5[b]	25.2[b]	1.517	<0.001
154	16.0[a]	25.7[b]	26.9[b]	26.6[b]	1.447	<0.001
161	15.7[a]	26.6[b]	27.0[b]	27.3[b]	1.620	<0.001
168	17.4[a]	26.1[b]	27.4[b]	27.3[b]	1.920	<0.001
175	18.2[a]	27.2[b]	28.3[b]	28.2[b]	1.679	<0.001
182	18.7[a]	26.8[b]	29.0[b]	28.8[b]	1.564	<0.001

Day zero is date of first Cobalt supplementation and the values in brackets were used as covariates for the statistical analysis.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Fig.3.11. Plasma Vitamin E Concentrations (μmol/l)

Day No.	Vitamin E Treatment Groups		S.E.D	p
	-E	+E		
-47	3.22	3.36	0.446	0.747
0	1.73	1.86	0.238	0.569
14	1.85	1.78	0.227	0.741
35	1.62	1.65	0.269	0.905
56	1.61	1.56	0.247	0.821
64	1.32	1.29	0.229	0.909
70	1.40	1.50	0.213	0.670
84	1.18	1.12	0.246	0.820
100	1.16	1.22	0.180	0.771
112	1.12	1.29	0.188	0.378
126	1.17	1.16	0.156	0.976
140	1.32	1.36	0.219	0.848
154	1.60	1.67	0.266	0.811
161	1.73	1.71	0.259	0.938
163	1.80	1.81	0.289	0.972
168	1.70	1.78	0.298	0.785
175	1.68^{a}	3.77^{b}	0.512	<0.001
182	1.82^{a}	4.39^{b}	0.405	<0.001

Day 169 is the date of first vitamin E treatment.

Fig.3.12. Whole Blood GSH-Px Activity (μ/ml at 30^{0}C)

Day No.	Cobalt Treatment Group Number				S.E.D.	p
	1	2	3	4		
-63	40.1	60.8	49.7	44.3	12.86	0.429
-47	14.7	16.2	11.6	12.4	4.28	0.702
-27	42.3	57.8	44.1	48.5	10.36	0.465
-14	10.3^{a}	24.3^{b}	14.1^{ab}	11.3^{a}	6.06	0.115
0	28.5	38.0	31.2	31.2	5.48	0.374
14	37.3	44.5	45.8	37.9	7.64	0.579
35	38.5	43.5	33.0	32.7	6.30	0.293
56	17.6^{a}	28.4^{b}	20.3^{a}	19.2^{a}	3.79	0.043
70	23.8	30.4	24.4	25.3	3.46	0.236
84	23.4	29.8	23.8	24.0	3.14	0.166
100	25.0	32.4	27.2	28.2	3.71	0.281
112	29.8^{a}	38.2^{b}	34.2^{ab}	34.8^{ab}	3.10	0.093
126	48.7	51.2	47.1	52.0	3.63	0.528
140	42.6	40.5	38.6	40.1	2.88	0.579
154	47.1	45.8	41.9	42.4	4.54	0.611
168	43.2	40.1	35.9	39.1	3.52	0.253
182	43.9^{b}	40.2^{ab}	34.4^{a}	39.8^{ab}	4.13	0.186

Day zero is date of first Cobalt supplementation.
Values with different superscripts were significantly different from others in the same row ($p<0.05$)

Fig. 3.13. Plasma CK Activity (U/l at 30°C)

Day No.	Cobalt Treatment Group Number				S.E.D	p
	1	2	3	4		
14	107	67	81	71	39.2	0.736
84	70	69	93	94	14.9	0.310
161	180[a]	102[ab]	76[b]	73[b]	39.4	0.043
182	387	111	82	72	154.6	0.166

Fig.3.14. Serum Copper, Magnesium and Calcium Concentrations.

Mineral Day No.	Cobalt Treatment Group Number				S.E.D.	p
	1	2	3	4		
Cu μmol/l						
-47	12.6	13.5	13.0	11.6	1.368	0.527
-27	17.7	18.4	18.3	18.1	1.440	0.963
-14	12.4	14.0	13.2	12.8	1.022	0.460
56	12.9	14.0	13.2	12.4	1.192	0.591
Mg mmol/l						
-47	1.14	1.20	1.24	1.17	0.0522	0.244
-14	1.10[a]	1.05[a]	1.13[b]	1.10[a]	0.0324	0.122
56	0.95	1.00	1.02	0.90	0.0589	0.210
64	0.99[ab]	1.09[bc]	1.15[c]	0.98[a]	0.0501	0.007
70	1.01	1.00	1.05	1.00	0.0479	0.703
Ca mmol/l						
-47	2.91	2.90	2.96	2.75	0.1011	0.228
-14	2.74[ab]	2.77[b]	2.63[a]	2.74[ab]	0.0537	0.076
56	2.52	2.54	2.55	2.56	0.0272	0.455
64	2.48	2.57	2.50	2.55	0.0741	0.528
70	2.30[a]	2.38[ab]	2.38[ab]	2.48[b]	0.0568	0.034

Day zero is date of first Cobalt supplementation.
Values with different superscripts were significantly different from others in the same row ($p < 0.05$).

Fig.3.15. Neutrophil Function (% kill)

Day No.	Cobalt Treatment Group Number				S.E.D.	p
	1	2	3	4		
14	23	27	23	29	4.34	0.459
35	24	23	28	37	7.12	0.231
56	36	26	32	32	4.61	0.238
70	21^{ab}	16^{a}	27^{b}	24^{ab}	4.77	0.196
84	25	23	26	21	4.67	0.741
100	15	14	20	19	3.96	0.473
112	22^{b}	19^{ab}	15^{a}	21^{b}	2.19	0.070
126	17	14	14	18	2.47	0.321
140	21	22	17	18	2.85	0.259

Day zero is date of first Cobalt supplementation.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table.3.16. Serum Vitamin B_{12} Concentrations (ng/l)

Day No.	Cobalt Treatment Group Number						S.E.D	p
	1	2	3	4	5	6		
-91	430^{ab}	429^{ab}	424^{ab}	484^{ab}	370^{a}	652^{b}	118.0	0.356
-77	271	198	193	242	213	266	69.8	0.776
-63	117	156	128	129	110	149	39.9	0.841
-49	74^{a}	123^{b}	91^{ab}	90^{ab}	83^{ab}	104^{ab}	22.1	0.330
-35	57	69	66	59	70	62	13.3	0.883
-22	61	51	110	63	39	56	40.5	0.597
0	(45)	(44)	(49)	(56)	(35)	(61)	-	-
14	40^{a}	50^{ab}	42^{ab}	53^{ab}	57^{ab}	63^{b}	10.25	0.231
28	34^{a}	56^{ab}	57^{ab}	62^{ab}	62^{ab}	80^{b}	14.3	0.111
42	36^{a}	37^{a}	55^{ab}	58^{ab}	37^{a}	77^{b}	13.1	0.058
56	30^{a}	39^{ab}	65^{b}	59^{ab}	48^{ab}	100^{c}	15.7	0.009
69	34^{a}	85^{b}	99^{bc}	68^{ab}	82^{b}	140^{c}	21.8	0.005
84	40^{a}	88^{b}	84^{b}	89^{b}	100^{bc}	135^{c}	18.2	0.003
97	32^{a}	57^{ab}	69^{b}	71^{b}	88^{b}	148^{c}	17.3	<0.001
111	52^{a}	58^{a}	71^{a}	57^{a}	86^{ab}	112^{b}	18.7	0.039
125	37^{a}	52^{ab}	57^{ab}	59^{bc}	79^{cd}	96^{d}	10.0	<0.001
139	36^{a}	50^{ab}	52^{ab}	64^{abc}	75^{bc}	95^{c}	15.6	0.022
153	359	51	55	71	90	103	387	0.560

Day zero is date of first Cobalt supplementation and the values in brackets were used as covariates for the statistical analysis.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table 3.17. Serum MMA Concentrations (μmol/l)

Day No.	Cobalt Treatment Group Number						S.E.D	p
	1	2	3	4	5	6		
-91	1.6	1.6	1.7	1.5	2.7	2.3	0.754	0.563
-77	6.3	5.5	6.2	4.0	5.2	6.3	1.543	0.687
-63	8.1^{ab}	6.4^{ab}	3.9^{a}	7.0^{ab}	11^{b}	8.6^{ab}	2.725	0.197
-49	14	9.5	11	8.8	16	12	3.92	0.431
-35	19	16	16	16	22	18	4.58	0.799
-22	16	16	18	18	22	20	4.67	0.711
0	(16)	(23)	(21)	(26)	(18)	(14)	-	-
14	21^{b}	11^{a}	13^{a}	10^{a}	8.3^{a}	9.6^{a}	2.640	0.001
28	23^{b}	12^{a}	13^{a}	7.3^{a}	7.9^{a}	8.5^{a}	3.044	<0.001
42	25^{b}	12^{a}	10^{a}	11^{a}	8.5^{a}	7.7^{a}	3.59	0.001
56	33^{b}	12^{a}	8.7^{a}	10^{a}	9.7^{a}	7.8^{a}	3.95	<0.001
69	35^{b}	14^{a}	10^{a}	11^{a}	11^{a}	8.7^{a}	2.590	<0.001
84	38^{b}	15^{a}	11^{a}	10^{a}	8.1^{a}	6.6^{a}	6.83	0.003
97	40^{b}	12^{a}	8.4^{a}	6.9^{a}	5.5^{a}	3.5^{a}	6.26	<0.001
111	63^{b}	19^{a}	12^{a}	11^{a}	8.2^{a}	6.4^{a}	12.23	0.002
125	52^{b}	22^{a}	13^{a}	11^{a}	7.0^{a}	7.5^{a}	10.00	0.002
139	58^{b}	19^{a}	14^{a}	12^{a}	7.1^{a}	7.9^{a}	15.32	0.026
153	38^{b}	14^{ab}	18^{ab}	11^{a}	9.4^{a}	10^{a}	12.24	0.172

Day zero is date of first Cobalt supplementation and the values in brackets were used as covariates for the statistical analysis.
Values with different superscripts were significantly different from others in the same row ($p<0.05$)

Table 3.18. Serum Succinate Concentrations (μmo'/l)

Day No.	Cobalt Treatment Group Number						S.E.D	p
	1	2	3	4	5	6		
-91	9.6	9.3	11	12	9.6	10	1.710	0.645
-77	9.3^{bc}	6.2^{a}	8.1^{ab}	11^{c}	8.1^{ab}	7.6^{ab}	1.097	0.045
-63	7.1^{a}	9.1^{b}	7.3^{ab}	7.4^{ab}	7.1^{a}	8.9^{ab}	0.881	0.205
-49	9.3^{a}	11^{b}	10^{ab}	9.3^{a}	10^{ab}	9.7^{a}	0.600	0.106
-35	9.8	12	11	11	11	12	1.348	0.568
-22	9.2	10	10	9.8	8.8	11	0.822	0.175
0	(11)	(12)	(9.9)	(8.7)	(9.4)	(11)	-	-
14	7.6	8.6	9.5	8.6	8.1	8.4	0.955	0.479
28	7.4	8.8	8.6	8.3	8.2	8.9	0.815	0.458
42	6.7^{a}	8.2^{b}	8.7^{b}	7.5^{ab}	7.9^{ab}	8.4^{b}	0.681	0.069
56	9.6^{a}	11^{ab}	21^{c}	17^{bc}	16^{bc}	13^{ab}	2.663	0.010
69	20	22	17	13	17	16	4.09	0.492
84	7.1^{a}	11^{ab}	8.4^{a}	8.2^{a}	12^{b}	11^{ab}	1.930	0.068
97	5.8^{a}	8.8^{b}	8.6^{b}	8.2^{b}	7.3^{ab}	8.3^{b}	1.039	0.042
111	6.9^{a}	8.8^{bc}	8.3^{b}	8.1^{b}	8.6^{b}	9.9^{c}	0.555	0.001
125	7.4^{a}	10^{b}	8.3^{ab}	7.1^{a}	8.4^{ab}	7.8^{a}	1.090	0.141
139	6.8^{a}	8.8^{b}	8.7^{ab}	7.7^{ab}	7.9^{ab}	9.0^{b}	0.922	0.188
153	5.9^{a}	8.7^{c}	8.2^{bc}	6.5^{ab}	6.9^{abc}	7.6^{abc}	0.978	0.084

Table.3.19. Serum GOT Activity (SF/ml)

Day No.	Cobalt Treatment Group Number						S.E.D	p
	1	2	3	4	5	6		
0	124^{b}	88^{ab}	69^{a}	68^{a}	78^{ab}	82^{ab}	23.7	0.235
14	105	107	81	75	96	105	21.9	0.572
28	101	98	72	76	83	119	26.6	0.515
42	75^{ab}	78^{ab}	66^{ab}	60^{a}	70^{ab}	84^{b}	9.0	0.167
56	96^{ab}	88^{ab}	74^{a}	64^{a}	73^{a}	130^{b}	22.2	0.090
69	111^{b}	103^{ab}	84^{ab}	78^{a}	91^{ab}	98^{ab}	15.4	0.326
84	75	85	70	68	77	76	9.5	0.547
97	75^{a}	112^{ab}	93^{a}	90^{a}	147^{b}	109^{ab}	20.2	0.035
111	74	91	76	74	91	82	9.6	0.262
125	112	90	77	76	77	80	21.4	0.507
139	110	115	86	83	98	99	16.5	0.379
160	104	85	72	66	76	86	23.6	0.671

Day zero is date of first Cobalt supplementation and the values in brackets were used as covariates for the statistical analysis.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table 3.20. Liveweight (kg)

Day No.	Cobalt Treatment Group Number						S.E.D	p
	1	2	3	4	5	6		
-91	20.1^{c}	19.4^{bc}	17.8^{a}	18.2^{ab}	18.8^{abc}	19.3^{abc}	0.717	0.06
-77	20.4	20.2	19.0	19.2	19.6	19.5	0.698	0.305
-63	20.8^{b}	19.8^{ab}	19.0^{a}	19.4^{a}	20.2^{ab}	19.7^{ab}	0.607	0.104
-49	21.0^{b}	19.5^{a}	19.2^{a}	19.7^{a}	19.5^{a}	20.1^{ab}	0.575	0.08
-35	20.2	19.9	19.4	19.6	20.0	20.0	0.403	0.341
-22	21.0	20.6	19.8	19.9	20.8	20.2	0.631	0.408
0	(19.6)	(19.8)	(20.5)	(20.0)	(19.1)	(19.8)	-	-
7	20.9	20.8	20.0	19.7	20.1	20.3	0.528	0.21
14	20.8	20.8	20.2	19.5	20.4	20.5	0.706	0.499
21	21.0^{ab}	21.2^{b}	20.5^{ab}	19.8^{a}	20.5^{ab}	20.7^{ab}	0.639	0.341
28	21.0	21.4	20.6	19.7	20.4	20.8	0.849	0.515
35	21.3	21.8	21.2	20.2	21.1	21.2	0.766	0.474
42	21.4	21.9	21.3	20.2	21.2	21.4	0.856	0.517
49	21.8	22.2	21.9	20.6	21.4	21.8	0.869	0.577
56	20.7	21.7	21.6	20.0	20.6	20.9	1.003	0.567
63	21.7	23.0	22.4	21.4	22.2	22.5	1.098	0.737
69	21.9	23.0	22.6	21.5	22.5	22.7	1.066	0.728
77	22.1	24.0	23.3	22.2	22.8	23.1	1.182	0.582
84	22.7	25.1	24.2	23.0	23.8	24.1	1.211	0.430
91	22.6^{a}	25.2^{b}	24.5^{ab}	23.8^{ab}	24.1^{ab}	24.2^{ab}	1.070	0.304
97	23.0	25.1	24.2	23.5	23.7	23.5	1.228	0.621
104	23.1^{a}	26.8^{b}	24.4^{ab}	23.2^{a}	24.4^{ab}	24.5^{ab}	1.485	0.222
111	23.6^{a}	27.6^{b}	25.7^{ab}	24.3^{ab}	25.0^{ab}	26.1^{ab}	1.647	0.258
118	24.1^{a}	27.9^{b}	27.1^{ab}	25.4^{ab}	25.6^{ab}	26.6^{ab}	1.688	0.318
125	22.9^{a}	27.4^{b}	26.4^{ab}	26.0^{ab}	25.4^{ab}	26.0^{ab}	1.703	0.237
132	23.7^{a}	29.2^{b}	27.8^{b}	25.9^{ab}	27.0^{ab}	27.9^{b}	1.876	0.114
139	24.1^{a}	30.0^{b}	29.0^{b}	27.4^{ab}	28.0^{ab}	28.9^{b}	2.064	0.130
146	24.6^{a}	30.6^{b}	29.0^{b}	27.4^{ab}	28.6^{ab}	29.0^{b}	1.974	0.108
153	25.2^{a}	31.0^{b}	29.2^{ab}	27.3^{ab}	28.3^{ab}	28.7^{ab}	1.912	0.129
160	25.9^{a}	32.2^{b}	30.1^{b}	28.3^{ab}	30.0^{ab}	30.5^{b}	1.971	0.077

Day zero is date of first Cobalt supplementation and the values in brackets were used as covariates for the statistical analysis.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table 3.21. Plasma Vitamin E Concentrations (μmol/l)

Day No.	Vitamin E Treatment Groups		S.E.D	p
	+E	-E		
-77	2.71	3.09	0.269	0.272
-63	1.99	2.02	0.238	0.867
-49	1.39	1.60	0.210	0.516
-35	1.26	1.46	0.1594	0.334
-22	1.27	1.27	0.1156	0.956
0	1.23	1.18	0.1411	0.654
14	1.30	1.31	0.1531	0.948
28	1.31	1.21	0.1525	0.503
42	1.60	1.39	0.1722	0.263
56	1.53	1.48	0.1854	0.790
69	1.54	1.60	0.200	0.778
84	1.60	2.11	0.269	0.449
97	2.40	2.33	0.288	0.819
105	51.1^{b}	2.20^{a}	3.57	<0.001
108	10.0	-	-	-
111	7.00^{b}	2.46^{a}	0.505	<0.001
125	5.70^{b}	2.88^{a}	0.424	<0.001
139	4.33^{b}	2.69^{a}	0.371	<0.001
153	4.68	2.99	0.456	0.001

Day 104 is the date of first Vitamin E treatment.

Table.3.22. Whole blood GSH-Px activity (μ/ml at 30^{0}C)

Day No.	Cobalt Treatment Group Number						S.E.D	p
	1	2	3	4	5	6		
-77	24^{ab}	26^{ab}	34^{b}	19^{a}	22^{ab}	30^{ab}	6.2	
-49	27^{ab}	24^{ab}	36^{b}	22^{a}	21^{a}	26^{ab}	5.9	0.210
0	33^{ab}	27^{ab}	39^{b}	22^{a}	25^{a}	30^{ab}	6.8	0.208
28	30	23	30	19	20	24	5.8	0.266
42	28	27	31	20	23	25	5.4	0.414
56	40	39	44	33	35	36	5.7	0.440
97	44^{b}	39^{ab}	44^{b}	31^{a}	33^{ab}	37^{ab}	5.8	0.180
111	31	28	33	26	25	28	5.5	0.697
139	41^{c}	27^{ab}	36^{bc}	23^{a}	28^{ab}	29^{ab}	5.6	0.058

Day zero is date of first Cobalt supplementation.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table 3.23. Plasma CK Activity (U/l at 30 C.)

Day No.	Cobalt Treatment Group Number						S.E.D	p
	1	2	3	4	5	6		
0	104	67	57	61	74	85	34.4	0.762
28	136	77	54	74	121	227	82.6	0.367
56	72^{ab}	104^{b}	63^{a}	56^{a}	65^{a}	71^{a}	15.4	0.080
97	51^{a}	70^{ab}	52^{a}	61^{a}	128^{b}	54^{a}	28.5	0.110
125	85^{b}	64^{ab}	52^{ab}	40^{a}	38^{a}	52^{ab}	17.2	0.111
153	186^{b}	80^{ab}	58^{a}	51^{a}	56^{a}	62^{ab}	60.2	0.249

Table.3.24. Serum Copper, Magnesium and Calcium Concentrations

Mineral Day No.	Cobalt Treatment Group Number						S.E.D	p
	1	2	3	4	5	6		
Cu μmol/l								
-91	14	15	15	16	14	17	1.497	0.410
0	12^{a}	13^{ab}	14^{ab}	14^{ab}	12^{a}	15^{b}	1.167	0.191
153	16^{ab}	16^{ab}	15^{ab}	13^{a}	15^{ab}	17^{b}	1.571	0.204
Mg mmol/l								
-91	0.96^{a}	0.97^{ab}	1.00^{ab}	1.07^{b}	1.00^{ab}	0.94^{a}	0.0515	0.227
0	1.03	1.00	0.98	1.01	0.97	0.96	0.0534	0.808
153	1.04^{bc}	1.06^{c}	0.96^{ab}	0.92^{a}	0.95^{ab}	0.88^{a}	0.0406	0.003
Ca mmol/l								
-91	2.3^{abc}	2.3^{abc}	2.4^{bc}	2.2^{ab}	2.2^{a}	2.5^{c}	0.0796	0.039
0	2.4^{c}	2.2^{a}	2.4^{bc}	2.3^{abc}	2.2^{abc}	2.2^{ab}	0.0939	0.136
153	2.0^{a}	2.2^{ab}	2.3^{b}	2.2^{b}	2.2^{b}	2.2^{b}	0.0610	0.028

Day zero is date of first vitamin E supplementation.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table 3.25. Differential Cell Counts (%)

Day No.	Cobalt Treatment Group Numbers						S.E.D	p
	1	2	3	4	5	6		
Neutrophills								
28	30	25	26	33	35	26	4.9	0.247
56	37	32	34	38	33	34	6.74	0.964
69	37	30	29	35	40	30	5.27	0.254
97	33	40	32	40	36	34	6.82	0.797
111	28	36	29	35	35	26	4.90	0.245
125	34	25	34	38	28	36	7.07	0.448
153	38^{c}	31^{abc}	27^{ab}	36^{bc}	28^{ab}	26^{a}	4.41	0.090
Lymphocytes								
28	67	71	70	64	62	72	4.70	0.283
56	62	64	62	60	65	64	6.56	0.980
69	62	66	70	63	58	66	5.56	0.403
97	64	58	64	58	62	64	7.16	0.907
111	68	63	69	62	63	71	5.21	0.431
125	62^{a}	77^{b}	64^{ab}	59^{a}	70^{ab}	61^{a}	7.10	0.154
153	60^{a}	66^{abc}	70^{bc}	61^{ab}	67^{abc}	72^{c}	4.42	0.098
Monocytes								
28	0.25^{a}	1.5^{ab}	3^{c}	1.75^{bc}	2^{bc}	1^{ab}	0.68	0.016
56	1.25^{a}	3^{b}	1^{a}	1^{a}	1.25^{a}	1^{a}	0.72	0.081
69	0.75	1.33	1.25	1	1.25	1.25	0.87	0.983
97	0.75	0.33	1.5	0.75	1	0.75	0.77	0.767
111	0.25	0	0.75	0	0.75	1	0.43	0.128
125	1	0.67	0.75	0.5	1	0.75	0.52	0.917
153	0.25	0.67	1.25	1.25	1	0.25	0.68	0.489
Eosinophills								
28	2	1.75	0.75	0.5	0.25	0.5	1.00	0.413
56	0	0.33	1	0.5	0.5	0.5	0.53	0.607
69	0.75	1.33	0.25	1	0.75	2	0.86	0.457
97	2	1	1.25	0.5	0.5	0.75	0.76	0.381
111	2.5	0.67	0.5	1.75	1	1	1.41	0.720
125	3.5	0.33	0.75	0.75	1.25	2.25	2.20	0.710
153	1.25	1.33	1.5	1.25	2.25	1	0.94	0.824
Basophills								
28	0.25	0.75	0.25	0.75	0.25	0.5	0.68	0.929
56	0.25	0	1	0.5	0.5	0.75	0.53	0.507
69	0	0.67	0.25	0.25	0.5	1	0.46	0.349
97	0.5	1	0.75	0.75	0.75	1.25	0.64	0.892
111	0.5	0	0.75	1	0.5	0.75	0.53	0.543
125	0.5^{a}	0.33^{a}	0.5^{a}	1.75^{b}	0.5^{a}	0.5^{a}	0.47	0.067
153	0.5	1.67	0.25	1	1.25	0.5	0.69	0.341

Day zero is date of first vitamin E supplementation.
Values with different superscripts were significantly different from others in the same row ($p < 0.05$).

Table.3.26. Serum Vitamin B_{12} Concentrations (ng/l)

Day No.	Cobalt Treatment Group Number				S.E.D.	p
	1	2	3	4		
-56	586	556	577	452	78.8	0.356
-42	430^{bc}	396^{b}	558^{c}	260^{a}	63.9	0.003
-28	228^{ab}	253^{ab}	265^{b}	134^{a}	59.4	0.181
-14	121	127	143	93	29.6	0.457
0	(84)	(78)	(63)	(110)	-	-
21	53^{a}	63^{a}	70^{a}	97^{b}	11.8	0.010
35	37^{a}	34^{a}	47^{a}	85^{b}	12.1	0.008
49	52^{ab}	46^{a}	44^{a}	71^{b}	11.8	0.147
63	30^{a}	31^{a}	47^{b}	61^{b}	6.5	<0.001
78	66^{ab}	49^{a}	48^{a}	69^{b}	8.8	0.053
91	79	59	89	65	26.6	0.668
105	48	62	74	66	15.1	0.384
118	52^{a}	56^{a}	82^{b}	78^{b}	6.9	<0.001
133	65^{ab}	59^{a}	76^{ab}	82^{b}	9.2	0.078
147	48^{ab}	46^{a}	74^{c}	68^{bc}	9.9	0.017
161	67	46	69	84	20.2	0.325

Table.3.27. Serum MMA Concentrations (μmol/l)

Day No.	Cobalt Treatment Group Number				S.E.D.	p
	1	2	3	4		
-56	2.2	2.0	2.2	2.3	0.47	0.896
-42	2.0	1.7	2.4	1.9	0.49	0.570
-28	5.1	3.7	8.2	3.9	2.40	0.346
-14	8.4	5.9	8.0	6.5	2.86	0.769
0	(8.1)	(7.2)	(28)	(5.1)	-	-
21	17^{b}	13^{b}	18^{b}	3.7^{a}	3.46	0.002
35	32^{c}	20^{b}	15^{ab}	7.0^{a}	4.94	<0.001
49	38^{b}	19^{ab}	30^{ab}	7.5^{a}	13.01	0.110
63	42^{c}	28^{bc}	21^{ab}	11^{a}	7.52	0.002
78	52^{b}	32^{ab}	44^{b}	9.6^{a}	16.23	0.064
91	58^{b}	26^{ab}	55^{b}	14^{a}	18.19	0.066
105	44^{c}	16^{ab}	34^{bc}	10^{a}	9.51	0.006
118	75^{b}	57^{ab}	51^{ab}	20^{a}	24.3	0.1444
133	60	42	65	14	27.3	0.267
147	104^{b}	57^{ab}	79^{ab}	18^{a}	33.0	0.074
161	136^{b}	54^{ab}	21^{a}	16^{a}	45.4	0.039

Day zero is date of first Cobalt supplementation and the values in brackets were used as covariates for the statistical analysis.

Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table.3.28. Serum Succinate Concentrations (μmol/l)

Day No.	Cobalt Treatment Group Number				S.E.D.	p
	1	2	3	4		
-56	11	9.0	9.2	9.9	1.12	0.418
-42	16^{b}	11^{ab}	12^{ab}	7.9^{a}	3.22	0.127
-28	7.5^{b}	6.6^{ab}	7.2^{ab}	6.0^{a}	0.61	0.131
-14	11	12	10	11	1.81	0.789
0	(6.9)	(7.5)	(7.4)	(8.7)	-	-
21	8.1	7.2	6.8	7.6	0.72	0.289
35	7.2	6.8	6.7	7.6	0.61	0.491
49	6.3	6.8	5.9	6.4	0.60	0.550
63	13	10	9.6	9.9	1.77	0.242
78	8.1	7.6	8.9	10	1.75	0.467
91	14	14	13	16	1.71	0.415
105	13	11	11.4	11	1.22	0.86
118	8.9^{b}	9.1^{b}	6.8^{a}	8.6^{b}	0.85	0.04
133	11	8.0	10	9.5	1.67	0.317
147	11	9.3	9.9	11	1.49	0.681
161	10	7.6	6.8	8.8	1.97	0.395

Table.3.29. Serum GOT Activity (SF/ml)

Day No.	Cobalt Treatment Group Number				S.E.D.	p
	1	2	3	4		
21	125	98	80	87	24.9	0.319
49	141	128	99	102	24.4	0.264
78	73	78	66	75	10.2	0.683
105	98	106	70	76	25.6	0.450
133	156^{b}	76^{ab}	61^{a}	69^{ab}	44.8	0.159
161	337	56	46	48	166	0.248

Day zero is date of first Cobalt supplementation and the values in brackets were used as covariates for the statistical analysis.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table.3.30. Liveweight (kg)

Day No.	Cobalt Treatment Group Number				S.E.D.	p
	1	2	3	4		
-56	20.1	20.5	19.9	20.3	0.48	0.587
-42	20.1	20.4	19.4	20.5	0.59	0.303
-28	20.8	20.5	20.0	21.1	0.55	0.254
-14	21.3^{b}	21.2^{ab}	20.6^{a}	21.7^{b}	0.29	0.011
0	(20.5)	(20.4)	(20.9)	(19.8)	-	-
7	20.6	20.9	20.7	20.4	0.28	0.325
21	20.2	20.5	20.2	20.0	0.32	0.432
28	20.6	20.9	20.5	20.6	0.41	0.822
35	20.5	21.0	20.0	20.6	0.64	0.494
42	20.0	20.7	20.1	20.6	0.74	0.756
49	20.4	21.2	20.4	21.0	0.84	0.707
56	20.3	21.0	20.4	20.8	0.95	0.871
63	20.3	21.6	20.1	21.8	0.97	0.225
70	20.7	21.3	20.3	21.7	0.93	0.461
78	20.7	21.6	20.8	22.5	1.10	0.360
84	20.6^{a}	21.8^{ab}	21.1^{ab}	23.0^{b}	1.13	0.198
91	20.4^{a}	21.6^{ab}	21.0^{a}	23.8^{b}	1.06	0.028
98	20.4^{a}	21.6^{a}	21.7^{a}	24.2^{b}	1.10	0.019
105	20.2^{a}	22.1^{ab}	22.1^{ab}	24.4^{b}	1.33	0.040
112	19.9^{a}	22.3^{ab}	22.1^{ab}	25.0^{b}	1.41	0.018
118	19.5^{a}	22.6^{a}	22.2^{a}	25.7^{b}	1.44	0.004
126	20.0^{a}	23.2^{b}	23.0^{ab}	26.3^{c}	1.44	0.004
133	20.2^{a}	23.6^{bc}	23.4^{ab}	26.8^{c}	1.57	0.006
140	20.4^{a}	23.8^{b}	24.5^{bc}	27.9^{c}	1.63	0.002
147	20.2^{a}	24.1^{b}	24.4^{bc}	28.0^{c}	1.76	0.003
154	20.3^{a}	24.7^{b}	24.6^{b}	29.1^{c}	1.77	0.001
161	20.4^{a}	25.1^{b}	25.0^{b}	28.8^{b}	1.92	0.004

Day zero is date of first Cobalt supplementation and the values in brackets were used as covariates for the statistical analysis.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table.3.31. Plasma CK Activity (U/l at 30°C)

Day No.	Cobalt Treatment Group Number				S.E.D.	p
	1	2	3	4		
21	84	55	63	56	23.2	0.572
49	91	76	59	50	20.8	0.230
78	72	75	48	47	19.9	0.343
105	92	89	56	52	29.6	0.399
133	547^{b}	60^{a}	47^{a}	46^{a}	222	0.089
161	570	59	45	53	263	0.155

Table.3.32. Whole Blood GSH-Px Activity (μ/ml at 30°C)

Day No.	Cobalt Treatment Group Number				S.E.D.	p
	1	2	3	4		
-56	77	71	73	77	7.6	0.801
-28	90	78	80	85	6.4	0.216
0	85	82	80	91	6.8	0.396
35	73	76	71	83	6.1	0.223
63	53	69	62	66	8.6	0.323
91	54^{a}	58^{ab}	63^{ab}	72^{b}	8.7	0.206
118	46^{a}	57^{a}	69^{ab}	81^{b}	10.6	0.023
147	48^{a}	69^{b}	74^{b}	95^{c}	10.0	0.002
161	67^{a}	93^{b}	87^{ab}	111^{b}	11.8	0.013

Day zero is date of first Cobalt supplementation.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table.3.33. Serum Copper, Magnesium and Calcium Concentrations

Mineral Day No.	Cobalt Treatment Group Number 1	2	3	4	S.E.D.	p
Cu μmol/l						
-56	24.5	23.2	23.7	23.8	1.26	0.777
0	12.9	13.5	12.3	13.7	1.06	0.551
161	12.4	11.1	10.7	12.9	1.68	0.529
Mg mmol/l						
-56	0.9	0.9	1.0	0.9	0.059	0.474
0	1.0^{ab}	1.0^{bc}	1.1^{c}	1.0^{a}	0.034	0.025
161	1.0	1.0	1.0	1.0	0.045	0.517
Ca mmol/l						
-56	2.1^{a}	2.1^{ab}	2.1^{a}	2.3^{b}	0.093	0.132
0	2.7	2.7	2.7	2.7	0.070	0.932
161	2.5	2.6	2.4	2.6	0.088	0.208

Table.3.34. Serum IgG Titre (mg/100ml)

Day No.	Cobalt Treatment Group Number 1	2	3	4	S.E.D.	p
161	4100	4800	4083	3050	1692	0.78

Day zero is date of first Cobalt supplementation.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

APPENDIX II

Table 4.1. Composition of Hay

	Contents						
	1	2	3	4	5	Mean	(S.E.)
DM g/kg	846	813	854	877	838	846	10.4
Co mg/kg	0.04	0.05	0.09	0.08	0.06	0.06	$9.3*10^{-3}$
Vitamin E	5.4	8.3	9.6	4.7	11.1	7.8	1.22
Se mg/kg	0.03	0.04	0.66	-	-	0.04	$8.8*10^{-3}$
CP g/kg	66	68	88	130	88	88	11.5
OM g/kg	952	945	939	958	940	947	3.6
IVOMD%	61.2	59.4	55.5	50.5	51.8	55.7	2.08
In vitro D	58.3	56.2	52.2	48.4	48.7	52.8	1.98
M/D	9.1	8.7	8.0	7.3	7.4	8.1	0.35
Ca g/kg	2.8	3.0	3.5	3.3	3.8	3.3	0.18
P g/kg	1.4	1.5	1.6	1.4	2.0	1.6	0.11
Mg g/kg	0.8	0.8	1.0	0.8	1.0	0.9	0.05
K g/kg	15.1	15.5	15.2	13.2	17.0	15.2	0.61
Na g/kg	0.18	1.01	1.36	0.83	2.12	1.1	0.319
Cu mg/kg	3.18	3.37	4.62	3.94	3.94	3.81	0.253
Mo mg/kg	0.49	0.37	0.37	0.11	0.19	0.31	0.068
S g/kg	1.06	1.17	1.04	0.9	1.14	1.06	0.047
Zn mg/kg	18.5	20.9	24.4	27.8	22.7	22.9	1.58
Fe mg/kg	85.3	62.4	100.00	67.4	73.7	77.8	6.75
Mn mg/kg	30.3	101.0	125.00	103.00	244	121	34.7
Available Cu mg/kg	0.29	0.31	0.44	0.42	0.38	0.37	0.03
PUFA							
16:1	3.3	3.3	1.8	2.6	2.1	2.6	0.31
18:1	5.1	5.6	7.2	8.2	10.0	7.2	0.89
18:2	20.7	19.5	19.0	21.7	18.6	19.9	0.57
18:3	38.9	38.4	30.8	31.0	24.8	32.8	2.64
20:3	2.0	2.0	1.9	1.8	3.4	2.2	0.30
20:4	-	-	0.1	0.1	-	0.1	0
20:5	3.0	1.3	3.2	2.0	3.5	2.6	0.41
22:5	1.0	0.8	1.7	2.6	1.8	1.6	0.32

Table 4.2. Composition of Concentrates.

	SOYA	BARLEY	NaOH treated BARLEY			
			1	2	Mean	(S.E.)
DM g/kg	859	838	795	797	796	1
Co mg/kg	0.25	0.04	0.01	0.02	0.02	$5*10^{-3}$
Vitamin E	1.52	6.9	0.5	0.7	0.6	0.1
Se	0.18	-	-	-	-	-
CP g/kg	527	96	91	90	90.5	0.5
OM g/kg	926	979	937	933	935	2
IVOMD%	93.5	84.0	90.1	90.6	90.4	0.25
In vitro D	86.5	82.2	84.3	84.4	84.4	0.05
M/D	13.8	13.2	13.5	13.5	13.5	-
Ca g/kg	3.5	0.5	0.4	0.5	0.45	0.05
P g/kg	7.0	3.5	3.3	3.5	3.4	0.1
Mg g/kg	3.5	1.3	1.2	1.1	1.2	0.05
K g/kg	26	4.48	3.89	3.92	3.90	0.02
Na g/kg	0.07	<0.1	22.1	22.1	22.1	-
Cu mg/kg	17.7	4.41	5.06	4.68	4.87	0.19
Mo mg/kg	4.29	0.13	0.10	0.11	0.10	$5*10^{-3}$
S g/kg	4.75	0.9	0.95	0.87	0.91	0.04
Zn mg/kg	55.9	21.6	24.6	23.6	24.1	0.5
Fe mg/kg	162.00	72.3	93	61.8	77.4	15.6
Mn mg/kg	45.5	18	18	17.0	17.5	0.5
Available Cu mg/kg	0.29	0.44	0.51	0.47	0.49	0.02
PUFA						
16:1	0.52	0.1	-	-	-	-
18:1	16.0	15.9	14.4	15.5	15.0	0.55
18:2	53.5	46.6	53.8	55.6	54.7	0.9
18:3	6.7	4.2	6.7	5.9	6.3	0.4
20:3	-	-	-	0.1	-	-
20:4	-	0.1	-	0.3	-	-
20:5	-	1.0	0.9	0.5	0.7	0.2
22:6	-	3.4	-	0.05	-	-

Table 4.3. Composition of Propcorn Barley.

	Contents					
	1	2	3	4	Mean	(S.E.)
DM g/kg	851	810	768	778	802	18.7
Co mg/kg	0.05	0.08	0.05	0.03	0.05	0.010
Vitamin E	4.08	1.52	9.38	3.1	4.52	1.70
Se	0.05	0.01	0.01	-	0.02	0.01
CP g/kg	122	103	113	113	113	3.88
OM g/kg	972	971	971	976	972	1.19
IVOMD%	84	82.3	82.3	80.1	82.2	0.80
In vitro D	81.7	79.9	79.9	78.2	79.9	0.71
M/D	13.1	12.8	12.8	12.5	12.8	0.12
Ca g/kg	0.6	0.8	0.6	0.5	0.6	0.06
P g/kg	3.5	3.8	4.1	4.0	3.85	0.13
Mg g/kg	1.3	1.3	1.4	1.3	1.3	0.025
K g/kg	5.13	4.9	4.89	4.23	4.81	0.17
Na g/kg	0.10	0.19	0.11	<0.1	0.12	0.02
Cu mg/kg	4.78	5.26	4.64	5.63	5.08	0.23
Mo mg/kg	0.46	0.21	0.32	0.26	0.31	0.05
S g/kg	1.55	1.27	1.32	1.14	1.32	0.09
Zn mg/kg	25.3	24.2	33.2	36.8	29.9	3.06
Fe mg/kg	126.00	138.00	191	152	152	14.1
Mn mg/kg	16.2	21.4	24.1	23.1	21.2	1.76
Available Cu mg/kg	0.48	0.53	0.46	0.56	0.51	0.02
PUFA						
16:1	-	1.3	0.5	-	0.9	0.4
18:1	12.8	20.9	15.5	14.8	16	1.73
18:2	56.1	38.3	51.0	56.5	50.5	4.25
18:3	7.1	4.8	6.5	6.4	6.2	0.49
20:4	-	0.7	0.3	-	0.5	0.2
20:5	-	1.0	0.9	-	0.95	0.05
22:6	-	0.4	-	-	-	-

N.B. Urea contains <0.01 mg/kgDM Cobalt.

Table 4.4. Plasma Vitamin E Concentrations (μmol/l)

DAY No.	VITAMIN E TREATMENT GROUPS			S.E.D	p
	CONTROL	ORAL	INJECTION		
-70	1.87	1.87	2.18	0.90	0.92
-63	0.78	0.72	1.18	0.37	0.44
-56	0.92	0.80	0.95	0.24	0.82
-49	1.06	0.83	0.96	0.17	0.48
-42	1.14	1.04	1.16	0.14	0.73
-35	1.27	1.24	0.94	0.37	0.62
-28	1.49^{b}	1.24^{ab}	0.95^{a}	0.20	0.10
-21	1.50	1.27	1.44	0.16	0.41
-14	1.60	1.44	1.61	0.18	0.60
-7	1.60^{b}	1.30^{ab}	1.20^{a}	0.13	0.06
0	(1.70)	(1.48)	(1.65)	-	-
1	1.67^{a}	4.28^{a}	35.07^{b}	2.03	<0.001
7	1.75^{a}	6.99^{b}	6.53^{b}	0.49	<0.001
14	1.89^{a}	8.71^{c}	4.68^{b}	0.79	0.001
21	1.85^{a}	8.86^{c}	4.06^{b}	0.71	<0.001
22	-	-	20.3	-	-
28	1.70^{a}	9.02^{c}	6.88^{b}	0.36	<0.001
35	1.78^{a}	9.04^{c}	5.07^{b}	0.48	<0.001
42	1.57^{a}	9.57^{c}	3.66^{b}	0.58	<0.001
49	1.62^{a}	9.96^{c}	3.56^{b}	0.52	<0.001
56	1.59^{a}	10.1^{b}	2.62^{b}	0.51	<0.001
63	1.41^{a}	7.44^{c}	2.71^{b}	0.40	<0.001
70	1.82^{a}	6.95^{c}	3.05^{b}	0.34	<0.001
71	-	-	22.7	-	-
77	1.70^{a}	7.99^{b}	7.95^{b}	0.36	<0.001
85	2.09^{a}	6.55^{c}	4.89^{b}	0.25	<0.001
91	1.78^{a}	8.18^{c}	5.16^{b}	0.62	<0.001

Day zero is date of first vitamin E treatment, and the values in brackets were used as covariates for the statistical analysis.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table 4.5. Plasma Vitamin E Concentrations (μmol/l)

DAY No. Vit B_{12} Treat	VITAMIN E TREATMENT GROUPS			S.E.D	p
	CONTROL	ORAL	INJECTION		
1 -	1.51^{a}	3.70^{a}	36.73^{b}	2.92	0.510
+	1.84^{a}	4.85^{a}	33.17^{b}		
7 -	1.69^{a}	5.84^{b}	7.21^{bc}	0.70	0.030
+	1.81^{a}	8.13^{c}	5.60^{b}		
14 -	1.75^{a}	7.87^{c}	5.04^{bc}	1.14	0.304
+	2.04^{a}	9.56^{c}	3.93^{ab}		
21 -	1.86^{a}	7.50^{b}	4.37^{a}	1.02	0.122
+	1.84^{a}	10.2^{c}	3.52^{a}		
28 -	1.53^{a}	7.82^{c}	7.89^{c}	0.52	0.005
+	1.88^{a}	10.2^{d}	5.87^{b}		
35 -	1.56^{a}	7.60^{c}	5.60^{b}	0.70	0.028
+	1.99^{a}	10.5^{d}	4.54^{b}		
42 -	1.30^{a}	8.17^{c}	4.01^{b}	0.83	0.077
+	1.84^{a}	11.0^{d}	3.32^{ab}		
49 -	1.56^{a}	9.70^{c}	3.60^{b}	0.75	0.852
+	1.69^{ab}	10.2^{c}	3.52^{b}		
56 -	1.55^{a}	9.23^{b}	3.00^{a}	0.74	0.140
+	1.64^{a}	11.0^{b}	2.24^{a}		
63 -	1.29^{a}	6.42^{b}	2.83^{a}	0.57	0.079
+	1.52^{a}	8.47^{b}	2.59^{a}		
70 -	1.74^{a}	6.07^{c}	3.21^{b}	0.50	0.069
+	1.91^{a}	7.83^{c}	2.88^{ab}		
77 -	1.20^{a}	6.63^{b}	9.04^{c}	0.52	0.003
+	2.19^{a}	9.35^{c}	6.85^{b}		
85 -	1.95^{a}	5.82^{b}	5.28^{b}	0.36	0.019
+	2.23^{a}	7.29^{c}	4.50^{b}		
91 -	1.83^{a}	6.63^{b}	5.68^{b}	0.89	0.050
+	1.72^{a}	9.72^{c}	4.63^{b}		

Day zero is date of first vitamin E treatment, and the values in brackets were used as covariates for the statistical analysis.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).
-= no B12 injection, +=received B12 injection

Table 4.6. EST, 1% Tween (% haemolysis)

DAY No.	VITAMIN E TREATMENT GROUPS			S.E.D	p
	CONTROL	ORAL	INJECTION		
-28	32	18	22	14.5	0.64
-7	45	28	36	15.2	0.55
0	27	14	21	13.4	0.66
1	46	30	44	19.1	0.67
7	38	20	25	13.3	0.42
14	46	36	50	19.0	0.77
21	28	15	25	13.6	0.64
28	46	29	38	21.6	0.73
35	38	20	29	20.0	0.68
42	46	20	38	24.0	0.57
49	44	20	37	24.4	0.65
56	53	37	45	26.9	0.84
63	46	21	38	22.3	0.55

Table 4.7. EST, 3% Tween (% haemolysis)

DAY No.	VITAMIN E TREATMENT GROUPS			S.E.D	p
	CONTROL	ORAL	INJECTION		
-28	62	52	51	15.1	0.74
-7	68	69	68	12.3	1.0
0	62	68	54	15.2	0.69
1	64	44	47	15.7	0.42
7	56	24	34	15.1	0.18
14	62	49	60	20.2	0.80
21	59	52	55	26.6	0.97
28	61	27	44	20.4	0.32
35	70	44	57	18.7	0.44
42	68	36	58	22.7	0.40
49	76	54	76	17.8	0.43
56	75	55	70	22.2	0.67
63	76	36	68	18.2	0.14

Day zero is date of first vitamin E supplementation.
Values with different superscripts were significantly different from others in the same row ($p < 0.05$).

Table 4.8. EST, 5% Tween (% haemolysis)

DAY No.	VITAMIN E TREATMENT GROUPS			S.E.D	p
	CONTROL	ORAL	INJECTION		
-28	77	80	76	11.0	0.90
-7	76	79	76	8.7	0.92
0	78	78	76	12.2	0.99
1	75	41	47	16.6	0.15
7	74	54	57	13.1	0.32
14	89	84	86	5.9	0.68
21	85	78	79	14.7	0.88
28	72	45	59	17.8	0.38
35	79	61	69	15.3	0.53
42	80	56	77	18.0	0.40
49	74	62	83	15.0	0.31
56	85	76	83	15.3	0.83
63	84	52	78	15.7	0.18

Table 4.9. Plasma CK Activity (U/l at 30°C)

DAY No.	VITAMIN E TREATMENT GROUPS			S.E.D	p
	CONTROL	ORAL	INJECTION		
-70	45	45	60	10.6	0.34
-56	28	33	32	6.7	0.75
-42	32	32	48	15.1	0.48
-28	34	33	36	7.6	0.89
-14	77	66	88	36.9	0.84
0	48	55	64	26.4	0.84
1	45^{a}	51^{a}	124^{b}	24.9	0.03
7	37	50	31	8.1	0.12
14	36	34	30	3.7	0.38
28	38	35	34	2.0	0.13
42	44	44	45	7.7	1.00
56	34	32	29	4.6	0.58
70	35	35	33	4.5	0.87
85	59	48	55	15.1	0.79

Day zero is date of first vitamin E treatment.
Values with different superscripts were significantly different from others in the same row ($p < 0.05$).

Table 4.10. Serum GOT Activity (SF/ml)

DAY No.	VITAMIN E TREATMENT GROUPS			S.E.D	p
	CONTROL	ORAL	INJECTION		
-70	75	56	64	15.2	0.50
-63	50	59	55	7.7	0.52
-56	62	79	69	12.6	0.45
-49	52	52	60	7.5	0.52
-42	61	65	70	5.4	0.36
-35	67	62	62	4.9	0.50
-28	63	60	64	2.9	0.35
-14	62	59	63	5.5	0.81
-7	50	54	58	3.6	0.13
0	59	59	59	2.9	1.0
7	55	58	53	4.0	0.39
14	69	65	69	4.4	0.52
21	62	64	67	3.9	0.57
28	66	67	65	3.8	0.87
35	66	66	64	4.4	0.86
42	60	59	66	3.4	0.13
49	57	60	56	3.5	0.54
56	58	60	58	4.8	0.88
63	59	58	60	4.4	0.88
70	70	62	68	2.6	0.07
77	66	53	64	6.5	0.17
85	62	52	70	8.1	0.16
91	88	61	70	12.1	0.16

Day zero is date of first vitamin E supplementation.
Values with different superscripts were significantly different from others in the same row ($p < 0.05$).

Table 4.11. Serum Vitamin B_{12} Concentrations (ng/l)

DAY No.	VITAMIN B_{12} TREATMENT GROUPS		S.E.D	p
	-	+		
-70	215	151	54.6	0.284
-28	71	46	10.6	0.057
7	63[b]	37[a]	6.0	0.004
14	75	47	12.0	0.056
14 p.m.	-	1250	-	-
15	-	1250	-	-
16	67[a]	673[b]	47.6	<0.001
21	108[a]	225[b]	18.4	<0.001
28	87[a]	155[b]	20.0	0.014
42	56[a]	78[b]	8.5	0.039
49	57[a]	193[b]	24.3	<0.001
56	48[a]	111[b]	16.4	0.009
63	36[a]	72[b]	11.5	0.020
70	30[a]	65[b]	6.4	0.002
77	25[a]	203[b]	25.5	<0.001
85	32[a]	131[b]	16.5	<0.001
91	18[a]	142[b]	7.3	<0.001

Day zero is date of first vitamin E supplementation.
Values with different superscripts were significantly different from others in the same row ($p < 0.05$).

Table 4.12. Serum MMA Concentrations (μmol/l)

DAY No.	VITAMIN B_{12} TREATMENT GROUPS		S.E.D	p
	-	+		
-70	0.4	0.4	0.04	0.49
-63	0.6	0.6	0.24	0.95
-56	0.7	0.7	0.22	0.72
-49	0.9	1.0	0.28	0.57
-35	0.9	1.2	0.38	0.49
-21	0.9	1.3	0.48	0.47
-14	1.3	1.7	0.75	0.66
0	1.1	1.2	0.23	0.68
7	1.7	1.7	0.60	0.98
14	1.3	1.6	0.35	0.48
14 p.m.	-	1.9	-	-
15	-	0.8	-	-
16	1.6^b	0.7^a	0.17	0.002
21	1.4^b	0.5^a	0.13	<0.001
28	1.7^b	0.6^a	0.18	<0.001
42	2.0^b	0.7^a	0.19	<0.001
49	2.5^b	0.4^a	0.54	0.008
56	3.0^b	0.5^a	0.71	0.012
63	3.2^b	0.6^a	0.84	0.020
70	3.8^b	0.5^a	1.19	0.032
77	4.1^b	0.4^a	0.78	0.003
85	4.2^b	0.4^a	1.21	0.014
91	5.0^b	1.0^a	1.25	0.018

Day zero is date of first vitamin E treatment.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table 4.13. Serum Succinate Concentrations (μmol/l)

DAY No.	VITAMIN B_{12} TREATMENT GROUPS		S.E.D	p
	-	+		
-70	13.5	15.0	1.25	0.30
-63	8.3	9.1	1.33	0.58
-56	9.2	9.7	0.61	0.44
-49	8.4	9.1	0.82	0.42
-35	6.2	6.6	0.75	0.57
-21	4.8	4.7	0.23	0.73
-14	4.0	3.5	0.56	0.45
0	10.7	9.4	0.89	0.21
7	12.7	12.4	1.92	0.87
14	8.4	9.0	0.45	0.24
14 p.m.	-	9.3	-	-
15	-	9.1	-	-
16	8.4	7.4	0.76	0.20
21	5.9	6.8	0.78	0.30
28	10.2	10.2	1.092	0.94
42	7.3	8.2	0.72	0.25
49	5.9	6.2	0.78	0.74
56	5.9	6.9	0.81	0.25
63	6.8	8.1	1.07	0.25
70	5.7^a	7.3^b	0.57	0.03
77	8.1	8.2	0.94	0.95
85	5.2^a	7.6^b	0.83	0.03
91	6.4	7.7	0.79	0.15

Day zero is date of first vitamin E supplementation.
Values with different superscripts were significantly different from others in the same row ($p < 0.05$).

Table 4.14. Liveweight (kg)

DAY No.	VITAMIN E TREATMENT GROUP MEANS			S.E.D	p
	CONTROL	ORAL	INJECTION		
-70	141	144	150	4.28	0.18
-63	144	143	148	3.77	0.41
-56	150	146	154	3.17	0.12
-49	158	155	162	3.11	0.19
-42	163	161	169	3.27	0.12
-35	169^{ab}	166^{a}	173^{b}	2.35	0.08
-28	179	177	182	1.87	0.12
-21	183	185	186	1.50	0.18
-14	189	188	190	1.83	0.59
-7	195	193	196	1.30	0.23
0	(195)	(202)	(198)	-	-
7	203	205	204	1.50	0.39
14	213	215	215	1.88	0.57
21	219	223	221	2.53	0.32
28	227	227	226	2.44	0.94
35	228	230	228	2.60	0.60
42	231	234	234	2.82	0.45
49	238^{a}	245^{b}	241^{ab}	1.69	0.03
56	251	258	248	4.31	0.17
63	250	261	250	4.31	0.09
70	256	268	258	5.36	0.14
77	263^{a}	274^{b}	262^{a}	4.16	0.06
85	268	278	266	7.65	0.33
91	271	284	271	10.3	0.44

Day zero is date of first vitamin E treatment, and the values in brackets were used as covariates for the statistical analysis.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table 4.15. Neutrophil Function Test (% kill)

DAY No.	VITAMIN E TREATMENT GROUPS			S.E.D	p
	CONTROL	ORAL	INJECTION		
E.coli					
70	64[b]	68[b]	54[a]	2.8	0.009
85	49	49	48	5.7	0.96
S.aureus					
70	61	62	62	12.8	1.0
85	61	64	56	4.7	0.26
C.albicans					
77	16	16	16	1.6	1.00
91	17	14	16	1.9	0.46

Day No.	VITAMIN B_{12} TREATMENT GROUPS		S.E.D.	p
	-	+		
E.coli				
70	59[a]	66[b]	2.3	0.03
85	47	49	4.6	0.73
S.aureus				
70	59	64	10.5	0.68
85	62	59	3.8	0.46
C.albicans				
77	14[a]	18[b]	1.3	0.05
91	16	16	1.5	0.92

Day zero is date of first vitamin E supplementation.
Values with different superscripts were significantly different from others in the same row ($p < 0.05$).

Table 4.16. Whole Blood GSH-Px Activity (μ/ml at 30°C)

DAY No.	VITAMIN E TREATMENT GROUPS			S.E.D	p
	CONTROL	ORAL	INJECTION		
-70	54	70	70	11.0	0.31
-63	39	55	49	9.0	0.26
-56	43	59	52	9.0	0.28
-49	40	55	47	7.8	0.26
-42	42	54	51	7.9	0.34
-35	38	49	44	6.6	0.32
-28	41	52	48	8.8	0.50
-21	41	53	48	6.8	0.29
-14	38	54	47	7.5	0.19
-7	29	43	35	7.9	0.30
0	29	43	35	6.0	0.13
7	29	41	32	5.3	0.12
14	25	37	30	5.4	0.18
21	26	34	27	5.3	0.33
28	25	34	28	3.8	0.15
35	24	31	24	4.5	0.28
42	23	29	23	2.8	0.12
49	18	24	19	2.5	0.13
56	18	24	19	3.4	0.27
63	18	22	18	2.4	0.27
70	17	22	19	2.2	0.18
77	15	19	16	1.8	0.16
85	16	17	16	2.2	0.86

Day zero is date of first vitamin E supplementation.
Values with different superscripts were significantly different from others in the same row ($p < 0.05$).

Table 4.17. Serum Copper, Magnesium and Calcium Concentrations.

DAY No.	VITAMIN E TREATMENT GROUPS			S.E.D	p
	CONTROL	ORAL	INJECTION		
Cu umol/l					
-70	12.5	9.8	10.8	1.94	0.41
21	12.5	11.5	11	1.41	0.59
91	13^{b}	9^{a}	11.5^{ab}	1.19	0.04
Mg mmol/l					
-70	0.93	0.92	0.90	0.07	0.91
21	0.91	0.96	0.94	0.04	0.46
91	0.90	0.90	0.93	0.04	0.72
Ca mmol/l					
-70	2.8	2.9	3.0	0.08	0.27
21	2.6	2.6	2.5	0.05	0.11
91	2.2^{a}	2.4^{ab}	2.5^{b}	0.09	0.11

Day zero is date of first vitamin E supplementation.
Values with different superscripts were significantly different from others in the same row ($p < 0.05$).

APPENDIX III

Table.5.1 Diet Compositions

	Straw Pellet	Complete Pellet
DM g/kg	841	885
Co mg/kg	0.06	0.13
Se mg/kg	0.05	0.10
CP g/kg	35	93
OM g/kg	888	926
IVOMD %	59.5	74.3
In vitro D	52.8	68.8
M/D	8.2	11.0
Ca g/kg	5.0	5.7
P g/kg	0.6	3.1
Mg g/kg	0.8	2.0
K g/kg	10.1	7.32
Na g/kg	27.9	14.9
Cu mg/kg	2.65	4.26
Mo mg/kg	0.64	0.55
S g/kg	1.65	-
Zn mg/kg	6.96	18.4
Fe mg/kg	195	289
Mn mg/kg	40.1	33.9
Available Cu mg/kg	0.21	-

Table.5.2. Concentrate Refusals (%)

Month (Pre)	Treatment Group			S.E.D.	p
	C	B	M		
Dec	2.1	1.9	5.1	2.10	0.290
Jan	2.3	2.4	2.8	1.34	0.935
Feb	1.2	1.4	1.4	0.70	0.971
April	32	8.0	29	22.8	0.545
May	59	31	52	22.1	0.444
June	39	70	64	18.6	0.302
Week No. (Post)					
1	56^{ab}	33^{a}	85^{b}	21.0	0.096
2	12^{a}	6.8^{a}	87^{b}	10.3	<0.001
3	14^{a}	5.6^{a}	78^{b}	12.1	<0.001
4	12^{a}	1.9^{a}	79^{b}	9.5	<0.001

Table.5.3. Forage Refusals (%)

Month (Pre)	Treatment Group			S.E.D.	p
	C	B	M		
Dec	63	61	48	16.0	0.623
Jan	50	55	48	13.5	0.77
Feb	60^{b}	38^{a}	60^{b}	5.4	0.038
April	72	61	62	16.5	0.777
May	65	65	53	18.4	0.767
June	77	59	44	17.6	0.277
Week No. (Post)					
1	72	60	50	19.7	0.557
2	79	70	44	19.2	0.224
3	70	63	55	17.9	0.693
4	69	59	45	18.4	0.444

Values with different superscripts were significantly different from others in the same row ($p<0.05$).

For pre treatment means groups C and M n=4, group B n=5, except in June n=2 for all groups
For post treatment means n=4.

Table.5.4. Serum Vitamin B_{12} Concentrations Post Treatment (ng/l)

Day No.	Treatment Group			S.E.D.	p
	C	B	M		
0	106	64	61	50	0.616
1	339^{b}	2500^{c}	56^{a}	90	<0.001
2	678^{b}	2255^{c}	70^{a}	266	<0.001
3	1010^{ab}	1805^{b}	76^{a}	492	0.020
6	1038^{b}	655^{ab}	70^{a}	410	0.112
7	918^{b}	495^{ab}	70^{a}	310	0.065
8	953^{b}	2500^{c}	73^{a}	222	<0.001
9	1195^{b}	2500^{c}	65^{a}	365	<0.001
10	1173^{b}	2095^{b}	65^{a}	417	0.003
13	1303^{b}	959^{ab}	74^{a}	431	0.048
14	1268^{b}	818^{ab}	69^{a}	406	0.045
21	1353^{b}	880^{ab}	78 $(140)^{a}$	384	0.029
28	1438^{b}	1128^{b}	70 $(170)^{a}$	427	0.032

Table.5.5. Serum MMA Concentrations Post Treatment (μmol/l)

Day No.	Treatment Group			S.E.D.	p
	C	B	M		
0	92	74	140	56.9	0.519
1	82	51	130	44.6	0.259
2	94	99	82	49.4	0.943
3	129	33	100	47.7	0.175
6	16	96	147	69.7	0.218
7	39	26	50	24.5	0.637
8	22	96	149	75.8	0.292
9	12^{a}	20^{a}	159^{b}	48.7	0.025
10	31^{a}	15^{a}	171^{b}	42.8	0.010
13	30^{a}	39^{a}	211^{b}	57.7	0.020
14	29^{a}	31^{a}	206^{b}	59.9	0.025
21	7^{a}	5^{a}	215 $(151)^{b}$	45.8	0.005
28	8^{a}	4^{a}	235 $(87)^{b}$	52.1	0.023

Day zero is date of first treatment.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).
Figures in brackets refer to sheep recieving 5,6-dimetylbenzimidazole treatment.

Table.5.6. Serum Succinate Concentrations Post Treatment (μmol/l)

Day No.	Treatment Group			S.E.D.	p
	C	B	M		
0	11.2	8.1	10.2	1.82	0.262
1	11.7	8.4	11.0	2.37	0.395
2	11.1	9.0	8.6	1.14	0.112
3	10.2	8.8	9.2	1.39	0.557
6	11.2	9.3	9.4	2.07	0.616
7	12.0	8.9	8.0	2.04	0.189
8	10.3	8.7	8.6	1.39	0.454
9	9.6	8.8	8.7	1.82	0.865
10	13.4	9.1	9.1	2.58	0.212
13	15.1	12.4	11.0	3.31	0.488
14	15.2^{b}	10.7^{ab}	9.4^{a}	2.40	0.088
21	10.8	7.0	8.7	1.82	0.169
28	14.8	8.3	11.4	3.57	0.239

Table.5.7. Rumen Succinate Concentrations (μmol/l)

Day No.	Treatment Group			S.E.D.	p
	C	B	M		
0	57	126	42	66	0.429
1	32	392	35	316	0.489
2	37	191	36	112	0.327
3	31	306	300	296	0.589
6	42	1338	37	644	0.119
7	50	1099	37	626	0.206
8	131	600	24	293	0.208
9	2631	315	57	1434	0.193
10	3222	585	69	2101	0.332
13	32	689	39	510	0.375
14	37	752	143	576	0.441
21	39	1100	48	559	0.148
28	47	1127	83	782	0.337

Day zero is date of first treatment.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table.5.8. Serum GOT Activity (SF/ml)

Date	Treatment Group			S.E.D.	p
	C	B	M		
7/1/92	66	67	61	8.5	0.722
24/3/92	64	51	62	9.0	0.307
31/3/92	62	66	74	9.8	0.497
28/4/92	59	56	78	12.3	0.209
26/5/92	45	87	88	54.6	0.664
23/6/92	83	59	107	34	0.373

Table.5.9. Liveweights (kg)

Date	Treatment Group			S.E.D.	p
	C	B	M		
18/7/91	84	82	82	12.0	0.984
23/8/91	75	75	75	10.7	0.998
11/9/91	76	73	72	12.8	0.959
2/10/91	75	72	70	12.6	0.925
12/11/91	71	72	70	9.5	0.969
17/12/91	74	74	72	9.7	0.960
7/1/92	73	73	73	9.5	0.999
18/2/92	74	73	73	9.3	0.986
8/4/92	75	75	76	7.1	0.993
12/5/92	73	73	74	6.9	0.972
26/5/92	72	70	74	7.5	0.866
9/6/92	72	72	73	6.8	0.990
23/6/92	71	73	72	7.6	0.962
30/6/92	68	69	67	7.6	0.975
14/7/92	70	70	62	7.1	0.525
28/7/92	72	73	64	7.0	0.500

Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table.5.10. Whole Blood GSH-Px Activity (μl at 30^{0}C)

Date	Treatment Group			S.E.D.	p
	C	B	M		
22/10/91	49	49	54	13.5	0.886
3/12/91	40	40	48	11.2	0.703
7/1/92	53	50	55	10.7	0.892
4/2/92	55	58	61	7.3	0.716
4/3/92	54	60	60	8.5	0.708
31/3/92	52	56	53	8.8	0.840
28/4/92	56	58	58	12.9	0.974
26/5/92	77	73	75	11.1	0.950
23/6/92	82	88	89	10.9	0.818
28/7/92	84	76	80	8.0	0.656

Table.5.11. Serum Copper, Magnesium and Calcium Concentrations.

Date	Treatment Group			S.E.D.	p
	C	B	M		
Cu μmol/l					
18/7/91	15.8^{b}	13.2^{a}	14.1^{ab}	1.2	0.108
26/5/92	11.6	10.4	11.2	2.0	0.815
23/6/92	10.5^{ab}	8.6^{a}	11.9^{b}	1.3	0.077
Mg mmol/l					
18/7/91	0.9	1.0	1.0	0.1	0.645
26/5/92	1.1	1.0	1.1	0.1	0.637
23/6/92	1.1	1.1	1.1	0.05	0.624
Ca mmol/l					
18/7/91	2.2	2.2	2.3	0.2	0.859
26/5/92	2.4	2.7	2.6	0.2	0.481
23/6/92	2.6	2.6	2.7	0.2	0.978

Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table.5.12. Rumen Acetate Percentage

Day No.	Treatment Group			S.E.D.	p
	C	B	M		
0	66.2	66.7	68.0	2.39	0.750
1	64.7	66.2	64.6	2.04	0.637
2	65.7	64.8	66.4	2.10	0.772
3	67.3	65.3	66.8	2.56	0.730
6	66.4	63.4	65.8	4.30	0.761
7	66.0	64.0	65.5	3.59	0.845
8	63.4	65.2	66.6	2.99	0.576
9	62.0	64.3	63.3	2.09	0.575
10	61.3	64.6	63.3	2.12	0.341
13	59.6^{b}	61.8^{ab}	65.5^{b}	1.90	0.036
14	60.8^{a}	64.0^{ab}	65.5^{b}	1.96	0.098
21	60.8^{a}	63.6^{b}	65.8^{b}	0.99	0.002
28	60.8^{a}	63.3^{a}	67.8^{b}	1.74	0.009

Table.5.13. Rumen Propionate Percentage

Day No.	Treatment Group			S.E.D.	p
	C	B	M		
0	20.6	20.2	18.7	2.32	0.703
1	21.0	21.5	22.3	2.08	0.829
2	20.9	22.8	20.1	2.16	0.484
3	19.6	21.8	19.9	2.18	0.591
6	21.9	24.0	19.2	3.19	0.369
7	22.8	23.2	19.3	3.15	0.425
8	24.0^{b}	23.0^{ab}	18.4^{a}	2.23	0.073
9	24.5	21.7	20.2	2.55	0.277
10	25.3	21.4	23.6	3.02	0.468
13	28.8^{b}	25.5^{b}	20.8^{a}	1.81	0.006
14	26.9^{b}	22.2^{ab}	21.3^{a}	2.22	0.069
21	26.3^{c}	22.8^{b}	19.5^{a}	1.00	<0.001
28	25.3^{b}	22.4^{ab}	19.5^{a}	1.98	0.048

Day zero is date of first treatment.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table.5.14. Rumen Butyrate Precentage

Day No.	Treatment Group			S.E.D.	p
	C	B	M		
0	8.51	8.77	8.87	1.14	0.950
1	9.77	8.07	9.02	1.11	0.332
2	8.80	7.95	9.07	0.95	0.494
3	8.68	8.34	8.80	1.42	0.947
6	7.76	8.24	10.1	1.68	0.373
7	7.23^{a}	8.31^{ab}	10.8^{b}	1.25	0.047
8	8.57	7.68	10.3	1.43	0.226
9	9.07^{a}	9.46^{ab}	11.6^{b}	1.06	0.087
10	9.10	9.47	8.62	0.94	0.672
13	7.65	8.18	9.01	0.64	0.155
14	8.28	9.13	8.65	0.50	0.293
21	8.34	8.89	9.45	0.699	0.381
28	9.41	9.43	8.18	0.94	0.359

Table.5.15. Rumen Acetate : Propionate Ratio

Day No.	Treatment Group			S.E.D.	p
	C	B	M		
0	3.28	3.40	3.76	0.55	0.676
1	3.12	3.11	2.89	0.395	0.810
2	3.23	2.85	3.43	0.48	0.499
3	3.48	3.03	3.54	0.55	0.626
6	3.54	2.67	3.45	0.90	0.590
7	3.19	2.80	3.48	0.70	0.637
8	2.69^{a}	2.90^{ab}	3.68^{b}	0.41	0.086
9	2.56	3.13	3.16	0.42	0.322
10	2.46	3.21	2.76	0.51	0.379
13	2.08^{a}	2.08^{a}	3.19^{b}	0.30	0.013
14	2.28	2.99	3.10	0.38	0.118
21	2.31^{a}	2.82^{b}	3.38^{c}	0.17	<0.001
28	2.43^{a}	2.87^{ab}	3.52^{b}	0.31	0.020

Day zero is date of first treatment.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).

Table.5.16. Rumen Acetate + Butyrate : Propionate Ratio.

Day No.	Treatment Group			S.E.D.	p
	C	B	M		
0	3.70	3.85	4.25	0.61	0.661
1	3.59	3.49	3.30	0.45	0.815
2	3.66	3.20	3.90	0.54	0.455
3	3.92	3.42	4.00	0.61	0.601
6	3.89	3.02	3.98	0.91	0.526
7	3.51	3.17	4.06	0.73	0.504
8	3.05^{a}	3.24^{a}	4.25^{b}	0.44	0.050
9	2.94	3.59	3.74	0.49	0.273
10	2.83	3.68	3.14	0.61	0.405
13	2.35^{a}	2.80^{a}	3.63^{b}	0.32	0.009
14	2.59	3.42	3.51	0.43	0.117
21	2.63^{a}	3.21^{b}	3.86^{c}	0.20	<0.001
28	2.81^{a}	3.29^{ab}	3.94^{b}	0.35	0.032

Day zero is date of first treatment.
Values with different superscripts were significantly different from others in the same row ($p<0.05$).