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STUDIES OF VITAMIN B₁₂ METABOLISM IN SHEEP

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Studies Of Vitamin B₁₂ Metabolism In Sheep

by T.M. Gruner

Abstract

Vitamin B₁₂ deficiency has been difficult to diagnose, mainly due to the vitamin's lack of biological significance in serum in which it is usually assayed. This research has investigated the marker of vitamin B₁₂/cobalt (Co) deficiency in sheep, methylmalonic acid (MMA), in comparison with serum and liver vitamin B₁₂ concentrations in farm situations where vitamin B₁₂ deficiency is expected in order to establish more accurate reference ranges for serum and liver vitamin B₁₂, and MMA. In addition, an attempt was made to ascertain the vitamin B₁₂ requirements of pre-ruminant (PR) lambs, and to determine whether metabolic demand for vitamin B₁₂ influences tissue concentrations. Furthermore, since the vitamin is active in biological tissues in form of its coenzymes, 5'-deoxyadenosylcobalamin and methylcobalamin, a preliminary assessment of variation in the distribution of these coenzymes in liver in different situations has been sought.

The first trial was set up to find out if the addition of propionate to the PR lamb's diet stimulated the uptake and/or storage of vitamin B₁₂ in the liver as a reflection of the need to deal with the incoming propionate. Sixteen ten day old lambs (Dorset Down/Coopworth cross-bred) were housed indoors soon after birth and fed on milk replacer. For half of the lambs 7.5 % (w:w) of the milk powder was replaced by propionate. Within each group, four lambs were treated with 250 µg vitamin B₁₂ twice weekly. Supplementation with vitamin B₁₂ increased liver concentrations from ~ 250 to ~ 900 nmol/kg fresh tissue, but there was no effect of propionate. Propionate addition did, however, result in increased plasma vitamin B₁₂ concentrations in vitamin B₁₂ supplemented groups, values being 3323 and 2355

pmol/l in propionate supplemented and control groups, respectively. This suggested that diet could influence plasma vitamin B₁₂ concentrations.

An attempt was made to quantify the PR lamb's ability to absorb vitamin B₁₂ from the alimentary tract by comparing the ability of intra-muscular (IM) and oral vitamin B₁₂ to raise plasma and liver vitamin B₁₂ concentrations. Twenty-seven three to four day old lambs from a farm with marginal Co status were housed indoors and fed on milk replacer. They were divided into three groups: control (n=3), IM treatment (n=12) and oral treatment (n=12). The two treatment groups were further subdivided into five sub-groups. These received, respectively, 0.2 (n=3), 0.4 (n=2), 0.8 (n=2), 1.6 (n=2) and 3.2 µg OH-cbl/d (n=3). The oral groups received tenfold the amount of the comparable IM groups, on the assumption that if oral absorption of the vitamin is about 10 % both groups would show similar increases in plasma and liver vitamin B₁₂ concentration. None of the IM groups showed any significant change in plasma or liver vitamin B₁₂. In the oral groups only the group on the highest dose of vitamin B₁₂, viz 32 µg/d, showed increases in plasma and liver concentrations. It was concluded that either absorption of vitamin B₁₂ was greater than 10 % or that the vitamin was retained better when administered orally. The amount retained in the livers of the lambs in the highest oral group was calculated to represent ~ 7.5 % of the dose.

In a follow-up 24 h trial, 14 of the above lambs were divided into three groups: Control (n=3), oral (n=6) and IM (n=5) treatment. The IM group received 3.2 µg OH-cbl and the oral group tenfold the amount as single doses at 0800 h. Blood samples were taken at regular intervals throughout the 24 h period and assayed for vitamin B₁₂. Vitamin B₁₂ concentrations in the IM group rose steeply within the first hour after injection to a concentration that was calculated to reflect 100 % uptake of the vitamin. It rose more slowly over about 8 h in the oral group. From the area under the curve absorption of the oral dose was estimated to be ~ 7 %.

The next experiment involved a farm where Co deficiency had been reported previously. In the first year, 50 pregnant two-tooth Halfbred ewes were divided randomly into two groups of 25. One group received a Co bullet plus 1000 µg OH-cbl IM, the other group remained unsupplemented. In the following year the trial was repeated. Ewes from the previous year's trial (by then four-tooths) were augmented

by a new cohort of pregnant two-tooths to make up numbers to 75. After lambing the lambs were divided into four groups: first by their dams' vitamin B₁₂ treatment, then half of each group received injections of vitamin B₁₂ at approximately three weekly intervals while the other half remained untreated. The trials lasted about five months, from mid-pregnancy until weaning.

Pasture Co was at its lowest at lambing in both years, 0.09 and 0.10 µg/g DM, respectively. In the first year, vitamin B₁₂ concentrations in the untreated ewes rose from 340 to 950 pmol/l in plasma and decreased in liver from 330 to 170 nmol/kg fresh tissue. In the Co treated group, vitamin B₁₂ concentrations in plasma rose from 500 to 1550 pmol/l and in liver from 310 to 560 nmol/kg fresh tissue. In the second year, vitamin B₁₂ concentrations in serum in the unsupplemented groups fell from 500 to 260 pmol/l around lambing before rising again to starting values at weaning, and liver vitamin B₁₂ concentrations fell from 450 at the start to 230 nmol/kg fresh tissue at the end of the trial. Serum vitamin B₁₂ concentrations in the two-tooth supplemented group rose from < 500 to > 3000 pmol/l whereas in the four-tooth supplemented group serum vitamin B₁₂ levels started at ~ 2800 and rose to nearly 5000 pmol/l. The supplemented four-tooths maintained higher liver vitamin B₁₂ concentrations throughout compared to the supplemented two-tooths, viz 680 compared to below 400 at the start and 900 versus 650 nmol/kg fresh tissue at weaning, respectively. MMA in the untreated groups rose to 15 and to 8 µmol/l during early lactation in the first and second years, respectively, whereas MMA in the treated groups stayed below 3 µmol/l in the first season and below 1.5 µmol/l in the second season.

There was a liveweight response to treatment in the ewes as the unsupplemented groups showed a significantly lower weight gain during the trials than the supplemented groups, viz 10.0 versus 13.6 kg in the first year, and 10.6 versus 13.3 kg in the four-tooths and 9.9 versus 12.1 kg in the two-tooths in the second year. There was also a significant difference in faecal egg count (FEC) in the first year. FEC in the untreated group was higher during lactation than in the treated group, viz 590 versus 170 eggs per gram wet faeces (epg), respectively. In the second year, the two-tooths had a higher FEC than the four-tooths, viz 120 versus 40 epg

during the same time span, respectively. While there was a trend for treatment having an effect on FEC similar to that in the first year it was not significant.

Supplementation of ewes in the first year increased mean milk vitamin B₁₂ concentrations at lambing from 800 to 1400 pmol/l and at weaning from 1750 to 4000 pmol/l. In the second year, Co bullet treatment increased milk vitamin B₁₂ concentrations in the four-tooths and two-tooths from 1500 and 2300 to 4000 and 2900 pmol/l at lambing, and from 1800 and 1400 to 6200 and 4500 pmol/l at weaning, respectively.

Treatment of ewes increased vitamin B₁₂ concentrations in the lambs which were not themselves supplemented. Plasma values in the first year increased from 160 to 325 pmol/l soon after birth and from 650 to 900 pmol/l at weaning, and liver values from 75 to 140 nmol/kg fresh tissue soon after birth and from 150 to 240 nmol/kg fresh tissue at weaning. In the second year, plasma vitamin B₁₂ concentrations increased from 160 to 380 pmol/l soon after birth and from 500 to 700 pmol/l at weaning, and in liver from 130 to 260 nmol/kg fresh tissue soon after birth and from 220 to 340 nmol/kg fresh tissue at weaning. There was also a significant effect of ewe supplementation on lamb MMA in 1997/1998 when values decreased from 19 to 8 µmol/l around the time of rumen development. MMA in the second year stayed below 3 µmol/l throughout in all groups of lambs. There was no difference in LWG between any groups of lambs. FEC was lowest in the group where both ewes and lambs were supplemented and highest in the group where neither ewes nor lambs were treated.

Further investigations were conducted on farms in Southland with lambs post-weaning in order to compare changes in serum and liver vitamin B₁₂ with serum MMA and LWG to determine the critical time and level of deficiency. In the first year, three farms with 50 lambs each participated. Lambs from each farm were allocated to five groups of 10 animals each. The first group received a Co bullet at weaning, and each month another group was treated with a Co bullet. The lambs were weighed monthly, and blood and liver samples were taken prior to treatment and each subsequent month from five lambs of the first supplemented group. The trial lasted about four months.

Serum vitamin B₁₂ concentrations in lambs at weaning were between 500 and 1000 pmol/l. Although supplementation increased serum levels for the first month this was followed by a drop to near or below starting concentrations. An exception was Farm 3 where serum vitamin B₁₂ concentrations rose again at the end of the trial. Liver vitamin B₁₂ concentrations also showed an overall decline from starting levels (200 to 300 nmol/kg fresh tissue) to the end of the trial (100 to 200 nmol/kg fresh tissue). MMA started around 2 µmol/l and reached between 6 and 7 µmol/l in the untreated lambs on Farms 1 and 3 two months after weaning before decreasing to around 3 µmol/l at the end of the trial, whereas the treated lambs maintained MMA concentrations around 2 µmol/l. On Farm 2 MMA started just below 5 µmol/l, decreased to around 1 µmol/l for treated and untreated lambs one month later and rose again to between 2.5 and 4 µmol/l, respectively, at the end of the trial. LWG was below average for all lambs (between 0.20 and 0.04 kg/d except for Farm 1 in the first month after weaning) but no significant differences were noted between treated and untreated lambs on any of the farms.

Another trial was conducted on one of these farms in the following year. One hundred lambs were divided into two groups of 50 each at weaning and sampled monthly for about six months. One group was treated with two Co bullets, the other group remained untreated. Pasture Co was between 0.04 and 0.07 µg/g DM, yet serum levels for the untreated group stayed ~ 500 pmol/l throughout the trial. Serum vitamin B₁₂ concentrations for the treated group started at ~ 500 pmol/l, rose to ~ 2500 pmol/l before falling back to ~ 2000 pmol/l. Liver vitamin B₁₂ concentrations for the untreated and treated groups were 529 and 427 nmol/kg fresh tissue at weaning, respectively. This decreased for both groups to ~ 350 nmol/kg fresh tissue one month after weaning. In the untreated lambs liver values decreased further to ~ 290 nmol/kg fresh tissue whereas they increased to ~ 450 nmol/kg fresh tissue for the treated group at the end of the trial. MMA concentrations started between 2 and 3 µmol/l for both groups and increased to 4.5 µmol/l for the untreated group one month later before falling back to 3.2 µmol/l. In the treated group MMA decreased to ~ 1 µmol/l and stayed at that level throughout the trial. There was no difference in weight gain.

In order to obtain an understanding of the distribution of corrinoids in biological tissues a High Performance Liquid Chromatography method was developed. The sensitivity of the analytical method meant that it was only practical to assay mainly liver samples because of the higher vitamin B₁₂ concentrations than in other tissues. The general finding was that the coenzyme 5'-deoxyadenosylcobalamin (ado-cbl) constituted the highest proportion of corrinoids in liver (45 %), followed by analogues (28 %), OH-cbl (24 %) and lastly methylcobalamin (3 %). Ado-cbl did tend to be proportionately higher in supplemented than in unsupplemented animals (56 and 42 %, respectively), whereas biologically non-active analogues tended to be higher in untreated than in treated sheep (29 and 21 %, respectively).

It was concluded that in the farm trials Co deficiency was only mild or not present although deficiency would have been predicted from the low vitamin B₁₂ concentrations in serum and liver and from raised MMA values. Therefore, currently used thresholds in New Zealand appear to be too high for vitamin B₁₂, and overseas values for MMA do not seem to be appropriate. Revised marginal ranges of 100 to 200 pmol/l for serum, 100 to 200 nmol/kg fresh tissue for liver and 10 to 20 µmol/l for MMA are suggested. Further, this work shows that Co bullets were effective in elevating blood and liver vitamin B₁₂ concentrations for longer than one year.

In the trials with preruminant lambs it was found that maintenance requirements were met by the vitamin B₁₂ content of milk replacer. There is evidence from indoor and farm trials that vitamin B₁₂ from milk was much more readily absorbed than vitamin B₁₂ from supplements. It was estimated that suckling lambs probably require between 1200 and 4000 pmol vitamin B₁₂/d, depending on age.

Key words: sheep, lambs, cobalt, vitamin B₁₂, cobalamins, corrinoids, coenzymes, analogues, methylmalonic acid, faecal egg count, propionate, reference ranges, requirements, HPLC.

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Abbreviations

ABD-F	ammonium 7-fluorobenz-2-oxa-1,3-diazole-4-sulphonate
ado	5'-deoxyadenosyl
ANOVA	analysis of variance
ATP	adenosine triphosphate
BCFA	branched chain fatty acid
b.w.	body weight
C	carbon
cbi	cobinamide
cbl	cobalamin
CdAc	cadmium acetate
ci	Curie
CI	Confidence Interval
Cl	chlorine
CN	cyano
Co	cobalt
CoA	coenzyme A
CS	chick serum
Ctrl	control
d	day(s)
DM	dry matter
DMB	dimethylbenzimidazole
DTT	dithiothreitol
E	ewe(s)
EDTA	ethylenediaminetetra-acetic acid
EMA	ethylmalonic acid
epg	eggs per gram (faeces)
Fe	iron
FEC	faecal egg count
FID	flame ionisation detection
FIGLU	formiminoglutamic acid
g	gram
g	gyro
GC	gas chromatography
GI	gastro-intestinal
h	hour(s)
H	hydrogen
HC	haptocorrin
HCd	partially degraded HC
hcy	homocysteine
HPCE	high performance capillary electrophoresis
HPLC	high performance liquid chromatography
HS	heparin sepharose
IBC	intracellular cobalamin binding proteins
ICP-MS	inductively coupled plasma source mass spectrometer

i.d.	inner diameter
IF	Intrinsic Factor
IM	intramuscular(ly)
IV	intravenous(ly)
JML	Johnstone Memorial Laboratory
k	kilo
K	potassium
L	lamb(s)
l	litre
LSD	least significant difference
LWG	liveweight gain
m	milli
M	molar
me	methyl
MeTHF	methyl-tetrahydrofolate
min	minute(s)
MMA	methylmalonic acid
mol	moles
M _r	molecular weight
MS	mass spectrometry
n	nano / number (of samples or animals)
N	nitrogen
Na	sodium
NSE	2-tooth supplemented ewe(s)
NUE	2-tooth unsupplemented ewe(s)
O	oxygen
o.d.	outer diameter
OH	hydroxo
ONFA	odd numbered fatty acid
OSE	4-tooth supplemented ewe(s)
OUE	4-tooth unsupplemented ewe(s)
OWLD	ovine white liver disease
p	pico
PA	pernicious anaemia
PC	phosphatyl choline
PE	phosphatyl ethanolamine
Pr	propionate
PR	pre-ruminant
revs	revolutions
RIA	radioimmunoassay
RIDA	radioisotope dilution assay
RRF	relative response factor
S	sulphur / supplemented
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SE	standard error / supplemented ewe(s)
sec	second(s)
SED	standard error of the difference

SEM	standard error of the mean
SI	small intestines
SIM	select ion monitoring
SL	supplemented lamb(s)
TC	transcobalamin
TCA	trichloroacetic acid
TEA	triethylamine
THF	tetrahydrofolate
TLC	thin layer chromatography
Tris	Tris(hydroxymethyl)-methyllamine
Trt	treatment
UBBC	unsaturated B ₁₂ binding capacity
UE	unsupplemented ewe(s)
UL	unsupplemented lamb(s)
v	volume
VFA	volatile fatty acids
w	weight
μ	micro

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CHAPTER 1

Introduction

Vitamin B₁₂ deficiency in sheep was first reported in New Zealand as ‘bush sickness’ at the end of the 19th century. However, its relationship to cobalt (Co) was not established until the 1930s. The disease manifests itself in ill-thrift and loss of appetite which lead to decreases in liveweight, poor wool growth, anaemia, a watery eye discharge, increased susceptibility to infection, and increased perinatal mortality in lambs (Grace, 1994). A large part of New Zealand’s economy is based on (sheep) farming, therefore livestock production issues are paramount to the farmer. Andrews (1972) mapped the areas where Co deficiency occurred in New Zealand (Figure 1.1). Figure 1.2 (Andrews, 1956) shows the extent to which sheep could be affected.

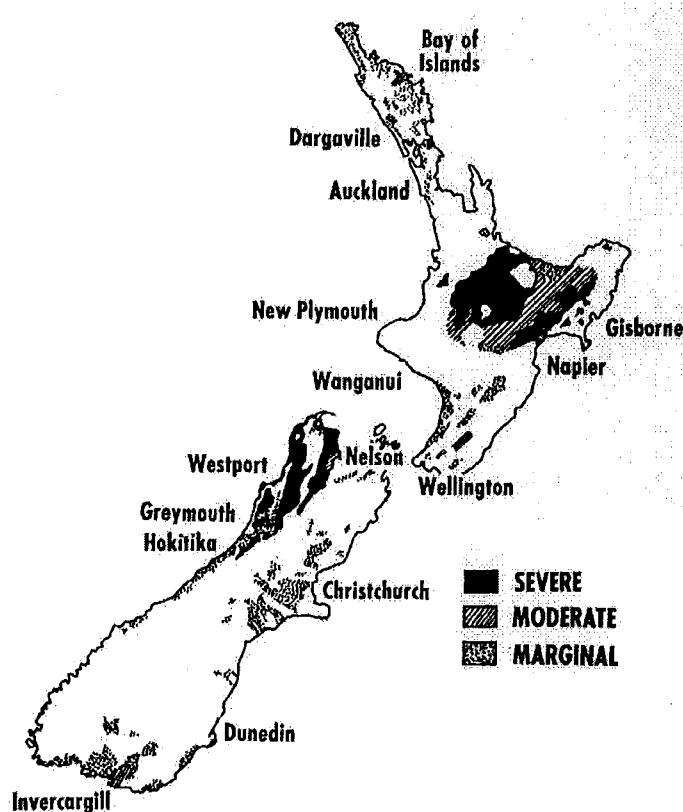


Figure 1.1: Distribution of Co deficient areas in New Zealand (from Andrews, 1972)



Figure 1.2: Co deficient sheep (top) and Co supplemented sheep (bottom) from the same flock (from Andrews, 1956)

Even today, definite diagnostic tools are lacking to ascertain when an animal or flock will become Co deficient. Animal health laboratories conduct mainly serum vitamin B₁₂ assays to determine adequacy or deficiency in livestock. However, the vitamin plays no active role in blood and while analysis of serum levels can give an indication of status it has proved an unreliable test for the amount of vitamin B₁₂ the animal has available for cellular metabolism and therefore responsiveness to supplementation. Further, vitamin B₁₂-like compounds without metabolic activity, called analogues, have returned falsely high results in serum tests. Reference ranges for vitamin B₁₂ deficiency in plasma and liver vary between countries and tend to be higher in New Zealand than in Australia and Northern Ireland.

Vitamin B₁₂ deficiency is complex because of the vitamin's occurrence in the tissues in the form of two coenzymes which are required for mainly two different metabolic reactions, the conversion of propionate to succinate and homocysteine to methionine. The metabolic by-products of these reactions, methylmalonic acid (MMA) and formiminoglutamic acid (FIGLU), measured in urine, were used in the past as another possible gauge of deficiency. However, workers in Northern Ireland sought better and more sensitive tools to diagnose the deficiency and developed a serum MMA test that is highly sensitive as a marker of deficiency. They also measured homocysteine (hcy) in serum. The reference values they established were mainly derived from barley-fed sheep, though, therefore attempts have been made in this thesis to find reference values for grass-fed sheep in New Zealand.

So far there is little understanding of the different forms vitamin B₁₂ is active in. Therefore a method has been developed in our laboratory to allow for the separation of corrinoids in biological samples. By gaining a greater understanding of the amounts of the coenzymes in each metabolic pathway a further step towards clarification of where and when deficiency may arise could be achieved.

For farm animals, cost of treatment needs to be balanced with productivity increases. Farmers have supplemented with vitamin B₁₂/Co bullets or used Co salts for top-dressing in areas where Co content of soil has been marginal or low and have not gained the liveweight response expected. More accurate diagnosis is needed to determine when treatment becomes necessary and profitable.

Another undefined area is the amount of vitamin B₁₂ that is needed by pre-ruminant (PR) lambs. They are monogastrics and their requirement for the vitamin may be quite different than that of mature sheep. Deficiency of vitamin B₁₂ is most commonly seen in lambs soon after weaning. Therefore, research described in this thesis was carried out with PR lambs, ruminant lambs, and ewes in order to

- > establish more accurate reference values for vitamin B₁₂ in lambs and mature sheep,
- > get a better understanding of the status of individual coenzyme forms of vitamin B₁₂ and
- > ascertain the vitamin B₁₂ requirements of PR lambs.

CHAPTER 2

Literature review

Part 1: Biochemistry and metabolism

2.1 History of vitamin B₁₂

Vitamin B₁₂ is one of the more recent vitamins to be discovered and structurally analysed. Yet as early as the beginning of the nineteenth century, a shepherd, James Hogg, realised that 'pining' of sheep was not due to an unknown infection, but could be cured by shifting the sheep to a different pasture on different soil (Mills, 1980). In the late 19th century this disease was recorded in New Zealand and later named 'bush sickness'. First, it was thought of as being caused by iron deficiency until early in the 20th century it was shown in Australia that iron-free limonite could cure the disease. It was discovered in 1935 that the potent element in limonite was cobalt (Co) (Andrews, 1972).

Already, early in the 19th century Combe, a Scottish physician, suspected that a certain type of anaemia in humans was probably due to a malfunctioning of the digestive and assimilative organs. It was not until 1926, however, that George Minot and William Murphy discovered that large amounts of raw liver, or its juice, taken daily could cure this anaemia that would, if left untreated, lead to death. They received a Nobel Prize for their work. In 1929, Castle suggested the existence of an 'intrinsic factor' in gastric juice which was necessary in combination with the 'extrinsic factor' found in raw liver to avoid this anaemia.

In the 1940s, Mary Shorb identified a species of bacterium which required this 'extrinsic factor' for its growth. The growth response of these bacteria was subsequently widely adopted as a method for the analysis of this 'extrinsic factor'. Finally, in 1948, the 'extrinsic factor', being vitamin B₁₂, was first isolated in crystalline form in both the United States, by Edward Rickes and Karl Folkers, and in Great Britain, by E. Lester Smith. Around that time it was also shown that *Actinomyces* spp. (soil bacteria) produced this vitamin. In 1957, a combination of

chemical and x-ray-diffraction methods were employed to elucidate the structure of vitamin B₁₂, which was finally determined by Dorothy Hodgkin in 1964, using x-ray crystallography, for which she was awarded a Nobel Prize.

2.2 Structure of vitamin B₁₂

Vitamin B₁₂ is a water-soluble vitamin and is unique among the vitamins in that it contains an essential mineral, viz Co. The structure of the vitamin (Figure 2.1) is similar to the porphyrin ring of haemoglobin or chlorophyll. The cobalt ion, in its 1+, 2+ or 3+ valency, is surrounded by a tetrapyrrole ring system, called a corrin ring. This contains seven carboxylic amide groups (-CH₂CONH₂ or -CH₂CH₂CONH₂), in which two of these rings are joined directly and not by a methene bridge. The four inner nitrogens are coordinated to the central atom, Co. 'True' vitamin B₁₂ (cobalamin or cbl) contains a ribonucleotide, 5,6-dimethylbenzimidazole (DMB) containing ribose 3-phosphate and aminoisopropanol, in an α -N-glycosyl linkage with D-ribose, as a lower ligand (α -face). The bond between the nucleotide and the corrin ring is achieved by joining the other nitrogen atom of the nucleotide to the cobalt atom, and by an ester linkage between the 3-phosphate group of the nucleotide and one of the carboxylic amide groups from the corrin ring.

The sixth coordination position, or upper ligand (β -face), is taken up by a functional group which determines its biological activity in mammals. In the isolated molecule, the upper ligand is a cyano (CN) group (vitamin B₁₂ is also known as cyanocobalamin: CN-cbl), with the chemical formula C₆₃H₈₈CoN₁₄O₁₄P and a molecular weight of 1355.42. The dark red crystals are hygroscopic and may absorb up to 12 % water. The hydrated crystals are stable in air. However, the cyanide is present as an artefact of isolation only; inside the cell, the CN group is replaced by a hydroxyl group or water, giving rise to hydroxocobalamin (OH-cbl) or aquo-cobalamin. Cobalt is in the +3 oxidation state in OH-cbl and is reduced by a flavoprotein reductase in two steps to the +1 state in which it can be active as a coenzyme. The two mammalian coenzymes are methylcobalamin (me-cbl) and 5'-deoxyadenosylcobalamin (ado-cbl). Traces of sulfitocobalamin have also been found in mammalian tissue.

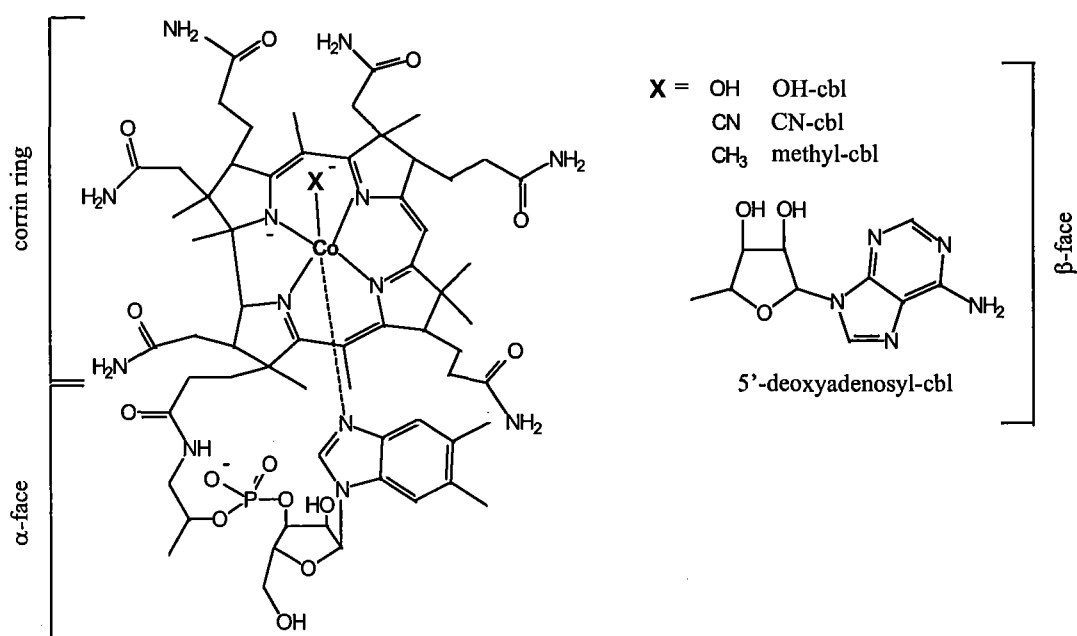


Figure 2.1: The structure of cobalamins (vitamin B₁₂)

2.3 Analogues

Vitamin B₁₂ belongs to a class of compounds called corrinoids. Various Co-containing complexes resembling vitamin B₁₂ have been discovered in biological tissues and rumen contents. They differ from the vitamin in the α -position. Either they do not have a nucleotide at all (termed cobinamides - cbi), or they have a modified nucleotide (termed cobamides). The ones that are metabolically active in the body are 'true' vitamin B₁₂ or cbl, whereas the inactive ones are termed 'analogues'. Although their metabolic significance is unclear, Porter discovered as early as 1953 that they seem to be needed by the micro-organisms in the rumen. This was later confirmed by Kondo *et al.* in 1980. According to Mills (1980), analogues are produced when bacteria in the rumen do not or cannot synthesise the complete nucleotide and substitute an alternative base instead.

Studies with rats (Mills, 1980) have shown that vitamin E can inhibit the nucleotide substitution and therefore may play a role in analogue production. Diets high in metabolisable energy are linked to higher amounts of analogues (Bigger *et al.*, 1976; Ørskov, 1994).

2.3.1 Origins of analogues

Kondo *et al.* (1980) tried to isolate analogues from rabbit tissues in order to compare them to synthetically produced corrinoids with alterations (in respect to cbl) in the corrin ring or the nucleotide. However, they were not successful in identifying the structures and origins of these analogues. They postulated that these analogues were not an artefact of isolation, but could be contained in plant material which the animal ingests. They could also be the result of bacterial degradation or produced from cbl degradation just prior to absorption. Analogues could account for as much as 25 % of total corrinoids.

Gimsing and Beck (1989) suggested another possibility for the existence of analogues. Diagnostic kits used to co-determine cobalamins and folates incorporated an alkaline extraction step. The manufacturers also added dithiothreitol (DTT) to stabilise the folates. However, cbIs are sensitive to alkaline conditions and reduced thiols, therefore they could be degraded during extraction, thus producing analogues. This degradation was demonstrated by Gimsing and Beck (1989), who used the alkaline methods (pH between 9.3 and 12.8, depending on the method used) suggested by the kit manufacturers. They found that up to 60 % of CN-cbl was lost in the procedure, and even more when DTT was added.

2.3.2 Isolation and classification of analogues in biological tissues

Hine and Dawbarn (1954) compared different microbiological (bioautographic) methods (see 2.10.1) for vitamin B₁₂ and attributed the differences in results to the presence of analogues. Gawthorne (1969) then tried to isolate and identify the different forms of analogues in rumen fluid. He used paper ionophoresis in dilute acetic acid in addition to the microbiological assays to separate the various analogues. This method only separates compounds that have different ionophoretic mobilities and does not pick up ionophoretically neutral molecules. However, Gawthorne (1969) managed to detect nine different corrinoids, of which he identified five by comparing their ionophoretic mobilities to crystalline standards or to values in the literature. They were: 5,6-dimethylbenzimidazolyl cobamide or 'true' vitamin B₁₂, adenyI-cobamide cyanide (pseudo-vitamin B₁₂), 2-methyladenyI cobamide

cyanide (Factor A), cobinamide (Factor B), and guanylcobamide cyanide. (He used the term 'cobamide' to denote any type of nucleotide, including the one for 'true' vitamin B₁₂ - DMB.) He found that around 80 % of total vitamin B₁₂ activity in rumen fluids was due to 'true' vitamin B₁₂ and Factor A (Gawthorne, 1970a), whereas the other compounds he could not identify only contributed around 3 %. Further, he compared the growth response of Factor A to 'true' vitamin B₁₂ in *Escherichia coli* and obtained similar responses for both substances. However, when he used sheep plasma instead of rumen fluid, he noted very little or no vitamin B₁₂ activity other than 'true' vitamin B₁₂, and attributed the difference to Intrinsic Factor (see 2.5.1.1) which selectively facilitates absorption of 'true' vitamin B₁₂ only (Gawthorne, 1970a).

Following this research, Gawthorne (1970b) found that addition of Co to rumen fluid increased vitamin B₁₂ activity only if the sheep was Co deficient, but had no effect on a Co-replete animal. Depending on the type of corrinoid (and hence nucleotide) he used, the organism would then include this nitrogen base to make the corresponding cobamide, at the expense of other cobamides.

2.3.3 Concentrations of analogues in biological tissues

Analogue concentrations are usually low in human and animal tissues, compared to rumen contents. Corrinoids in bile consist of ado-cbl, me-cbl and a large amount of analogues, all bound to haptocorrin (see 2.5.2.2), with the major cbl in bile being ado-cbl (Linnell, 1975). Human bile corrinoid concentrations are about four times higher than in plasma, the difference being considered to be entirely due to analogue content (Adjalla *et al.*, 1993; Nicolas and Guéant, 1994). Human blood contains between 9 and 40 % analogues (Djalali *et al.*, 1990; van Kapel *et al.*, 1983; Beck, 1982). Sheep blood appears to contain very few analogues (~ 1.5 % - Gawthorne, 1970a; 8 to 14 % - Schultz, 1987a), in contrast to cattle (Paterson and MacPherson, 1990a) where up to 50 % of total plasma vitamin B₁₂ can be due to analogues (Halpin *et al.*, 1984; Schultz, 1987a).

2.3.4 Disturbance in the balance between cobalamins and analogues

When vitamin B₁₂ concentrations in the body drop, analogue concentrations do not necessarily decrease (Kondo *et al.*, 1980). This disturbs the balance between cbl and analogues, which is considered to have the effect of interfering with growth and development by inhibiting the cbl dependent enzymes (Kondo *et al.*, 1980). The extent of damage in the system that is caused by this imbalance may also depend on the nature of the analogues and their tissue distribution, determining whether there are haematological or neurological symptoms, or both (Kondo *et al.*, 1980). In humans, relatively high levels of analogues have been associated with neurological abnormalities which could be due to demyelination of nerve fibres (Adjalla *et al.*, 1993 and 1994; Lambert *et al.*, 1992). No analogues have been found in mammalian milk (Adjalla *et al.*, 1994; Gregory, 1954).

2.4 Metabolic pathways

Although there are at least ten different biochemical reactions in biological systems which require vitamin B₁₂ (Table 2.1), most of them occur only in certain bacteria. There are only two forms of the vitamin that are active as coenzymes in mammalian metabolism: ado-cbl, which is formed when the Co⁺ displaces the triphosphate group from ATP in a nucleophilic reaction, and me-cbl. Another form of vitamin B₁₂ was discovered in 1958 which was devoid of the 5,6-dimethylbenzimidazole, but contained the 5'-deoxyadenosyl group as upper and as lower ligand. It has coenzyme activity only in certain mud bacteria (mainly in *Clostridium tetanomorphum*) which are unable to make the ribonucleotide. It was called pseudovitamin B₁₂ by H. A. Barker (Lehninger, 1975).

Reactions outlining the major requirements for vitamin B₁₂ are in bold print in Table 2.1 (from Lambert and Nicolas, 1990; Barker, 1972):

Table 2.1: Biochemical reactions requiring cobalamins (vitamin B₁₂)

Coenzyme	Broken substrate binding	Enzyme	Nomenclature	Reaction
ado-cbl	C-C	Glutamate mutase	5.4.99.1	Glutamic acid $\longleftrightarrow$ β -Methylaspartate
		Methylmalonyl-CoA mutase	5.4.99.2	L-Methylmalonyl-CoA $\longleftrightarrow$ Succinyl-CoA
		Methyleneglutarate mutase	5.4.99.4	α -Methyleneglutarate $\longleftrightarrow$ β -Methylitaconate
		1-2 Propanediol hydro-lyase	4.2.1.28	Ethylene glycol $\longrightarrow$ Acetaldehyde + H ₂ O
	C-O	Glycerol dehydratase	4.2.1.30	1-2 Propanediol $\longrightarrow$ Propionaldehyde + H ₂ O
				Glycerol $\longrightarrow$ 3-Hydroxypropionaldehyde + H ₂ O
		Ribonucleotide triphosphate reductase	1.17.4.2	Ribonucleotide $\longrightarrow$ 2-Deoxyribose + H ₂ O
	C-N	Ethanolamine ammonia-lyase	4.3.1.7	Ethanolamine $\longrightarrow$ Acetaldehyde + NH ₄ ⁺
				2-Aminopropanol $\longrightarrow$ Propionaldehyde + NH ₄ ⁺
		Aminomutases	5.4.3.-	NH ₂ displacement: α or β -Lysine (from C ₆ to C ₅) Ornithine (from C ₆ to C ₄) Leucine (from C ₂ to C ₃)
me-cbl		Methionine synthetase	2.1.1.13	Homocysteine $\longrightarrow$ Methionine
		Methane synthetase	2.1.1.-	N ⁵ -methyltetrahydrofolate $\longrightarrow$ Tetrahydrofolate
		Acetate synthetase	2.1.1.-	H ⁺ $\longrightarrow$ CH ₄ CO ₂ $\longrightarrow$ Acetate

2.4.1 5'-deoxyadenosyl-cobalamin

The coenzymes have various functions, e.g. proton transfer in ribonucleotide reduction, in hydrogen (H) and in methyl transfer. Common among the enzymatic reactions requiring ado-cbl is a 1,2 shift of a hydrogen atom from one carbon (C) of the substrate molecule to the next. There is usually a reverse or 2,1 shift of another group, like a hydroxyl, amino, alkyl or carboxyl group, for instance. The exact mechanism of these reactions is not known, but it has been postulated that ado-cbl reacts with a hydride ion of the substrate molecule which then replaces the methylene carbon atom of the ado group. Thus, the hydride ion is shifted to the adjacent carbon atom of the substrate, accompanied by the shift of the other group which is being transferred. One possible explanation is that the Co-C bond of the ado-group is split in a homolytic cleavage reaction, where Co is oxidised to the +2 state, forming an ado free radical. Therefore the role of vitamin B₁₂ is to provide free radicals for the abstraction of hydrogen atoms, which can be accomplished due to the weakness of the Co-C bond (Stryer, 1995).

In mammals, ado-cbl is needed for methylmalonyl mutase in the isomerization of methylmalonyl-CoA to succinyl-CoA. The whole thioester group (-O-S-CoA) from carbon 2 of methylmalonyl-CoA is shifted to the methyl carbon atom, in exchange for a hydrogen atom (Figure 2.2):

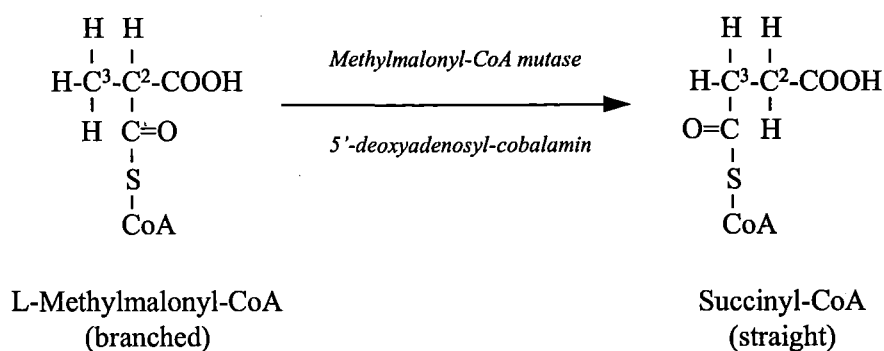


Figure 2.2: The role of 5'-deoxyadenosyl-cobalamin in proton transfer

This reaction is particularly important in ruminants because they are heavily dependent on gluconeogenesis for their glucose requirements. Precursors for this are the volatile fatty acids (VFA) acetate, propionate and butyrate which are the end-product of forage fermentation in the rumen. Of these, propionate is the main substrate for gluconeogenesis in roughage-fed animals, providing 50 to 80 % of this need (Brockman, 1993; van Houtert, 1993). Lactate accounts for approximately 15 % (Brockman, 1993), with the remainder being supplied by amino acids and glycerol. Direct absorption of glucose is not a major contribution to energy needs (Roe *et al.*, 1966).

There are two major ways in which propionate is formed from carbohydrate in the rumen: In roughage-fed animals, the succinate (dicarboxylic acid or randomised) pathway is favoured (which requires vitamin B₁₂ as a cofactor), whereas in animals fed on high levels of concentrates the acrylate (glyoxalate, direct reductive or non-randomised) pathway predominates. This pathway is independent of vitamin B₁₂ and seems to be triggered by high amounts of starch in the diet (van Houtert, 1993; Kennedy *et al.*, 1991a).

Of the propionate produced in the rumen, only about half is absorbed, mainly via passive diffusion, into the portal vein and carried to the liver, the rest is metabolised further, mainly to lactate, in the rumen or during absorption. More than 90 % of the absorbed propionate is taken up by the liver, the major site of gluconeogenesis, and most of it is converted to glucose. It has been estimated (Gardiner, 1977) that as much as 7.5 µg/d of vitamin B₁₂ is required for this

metabolic reaction. Therefore, very little propionate is found in the blood (other than the portal vein) or other tissues (Brockman, 1993).

Propionate is acylated in the liver to propionyl-CoA. Propionyl-CoA is carboxylated to the (D)-stereoisomer of methylmalonyl-CoA and then epimerized to its (L)-stereoisomer. (L)-methylmalonyl-CoA is rearranged by methylmalonyl-CoA mutase (E.C. 5.4.99.2), which requires *ado-cbl* as a coenzyme, to succinyl-CoA. (This reaction is also necessary for the removal of odd-numbered and branched-chain fatty acids.) In vitamin B₁₂ deficiency the conversion cannot proceed. Methylmalonic acid (MMA) is formed from (L)-methylmalonyl-CoA by the action of (L)-methylmalonyl-CoA hydrolase (Kovachy *et al.*, 1983) and accumulates in the tissues, blood and urine (Figure 2.3). This takes place in the mitochondria along the pathway outlined in Figure 2.4.

Succinate enters the tricarboxylic acid (Krebs) cycle (Figure 2.5) to yield malate which is exported to the cytoplasm. There it is further metabolised via triose phosphate to glucose (van Houtert, 1993; Newsholme and Start, 1976; Matthews and van Holde, 1990).

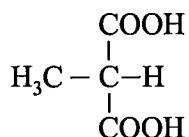


Figure 2.3: Methylmalonic acid

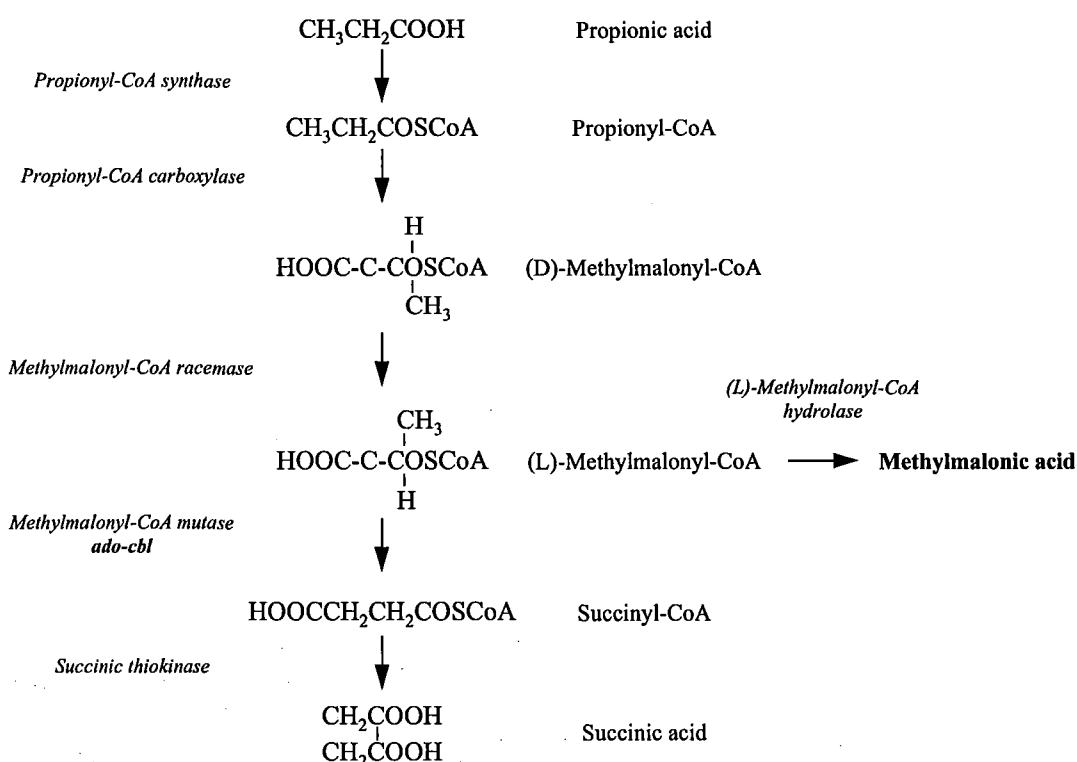


Figure 2.4: The role of 5'-deoxyadenosyl-cobalamin in propionic acid metabolism

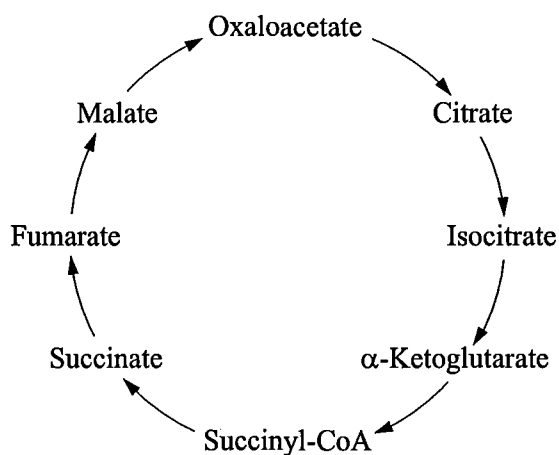


Figure 2.5: Tricarboxylic acid cycle (Krebs cycle)

2.4.2 Methylcobalamin

Me-cbl, the other coenzyme form of vitamin B₁₂, is required for the synthesis of methionine from homocysteine (Figure 2.6).

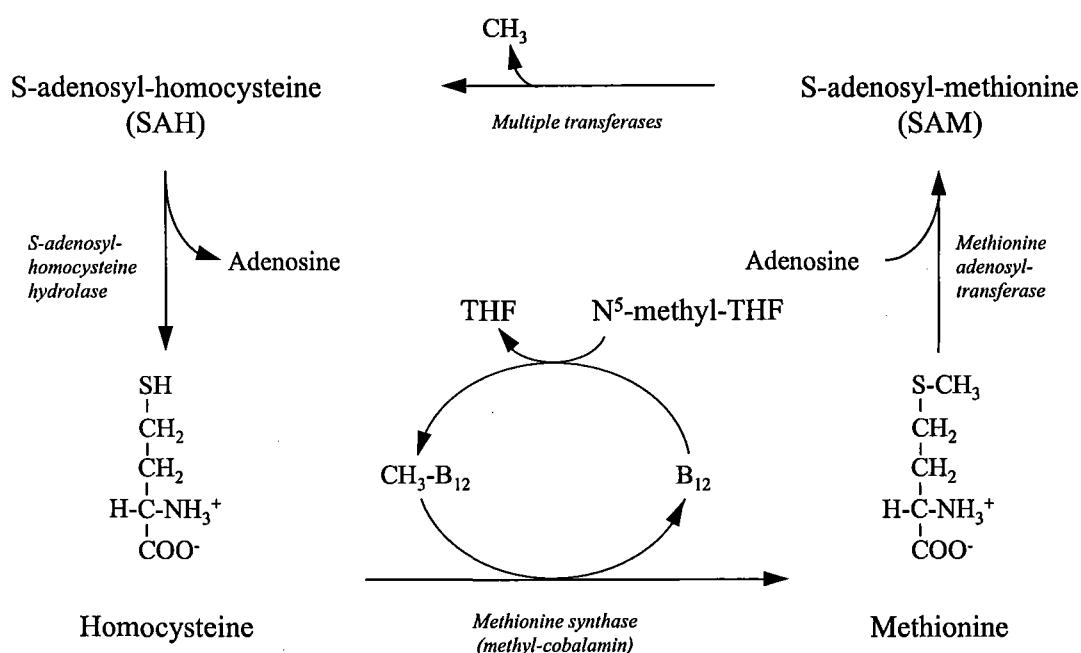


Figure 2.6: The role of methyl-cobalamin in methyl transfer

Methyl groups are generated *de novo* through the reduction of N⁵,N¹⁰-methylenetetrahydrofolate to N⁵-methyltetrahydrofolate. With the aid of methionine synthase (E.C. 2.1.1.13) me-cbl transfers its methyl group to homocysteine to yield methionine. Methionine is converted to S-adenosyl methionine (SAM) which is a methyl group donor for other metabolic reactions. S-adenosyl-homocysteine (SAH) is formed which hydrolyzes to form homocysteine, which can either be degraded to form cysteine or remethylated to methionine. With low vitamin B₁₂ concentrations, SAM concentrations are expected to be low (Gawthorne and Smith, 1974). Vitamin B₁₂ deficiency also leads to elevated levels of plasma hcy due to the failure of me-cbl to convert hcy to methionine.

One metabolic pathway that depends on methylation is phosphatyl choline (PC) synthesis, which can be accomplished in two ways: the direct pathway using free choline, or indirectly through the step-by-step methylation of phosphatidyl ethanolamine (PE) by SAM. For mammals, sources of methyl groups include choline, betaine and methionine, but although these compounds are present in the ruminants' diet, they are degraded by the rumen bacteria. Consequently, sheep and cattle are particularly susceptible to methyl group deficiencies, and PC synthesis depends solely on the indirect pathway.

A link between folate metabolism and vitamin B₁₂ exists through the methyltetrahydrofolate-homocysteine transmethylation reaction which regulates tetrahydrofolic acid (Gawthorne, 1968). The methyl group transferred during the conversion of hcy to methionine originates from methyltetrahydrofolate (MeTHF), which is converted to tetrahydrofolate (THF). THF in turn gains a formimino group from FIGLU and is converted to 5-formimino-THF, leaving a glutamate residue. If there is no vitamin B₁₂ to receive the methyl group from MeTHF, then THF, and also 5-formimino-THF, cannot be formed and so FIGLU and homocysteine accumulate (Figure 2.7).

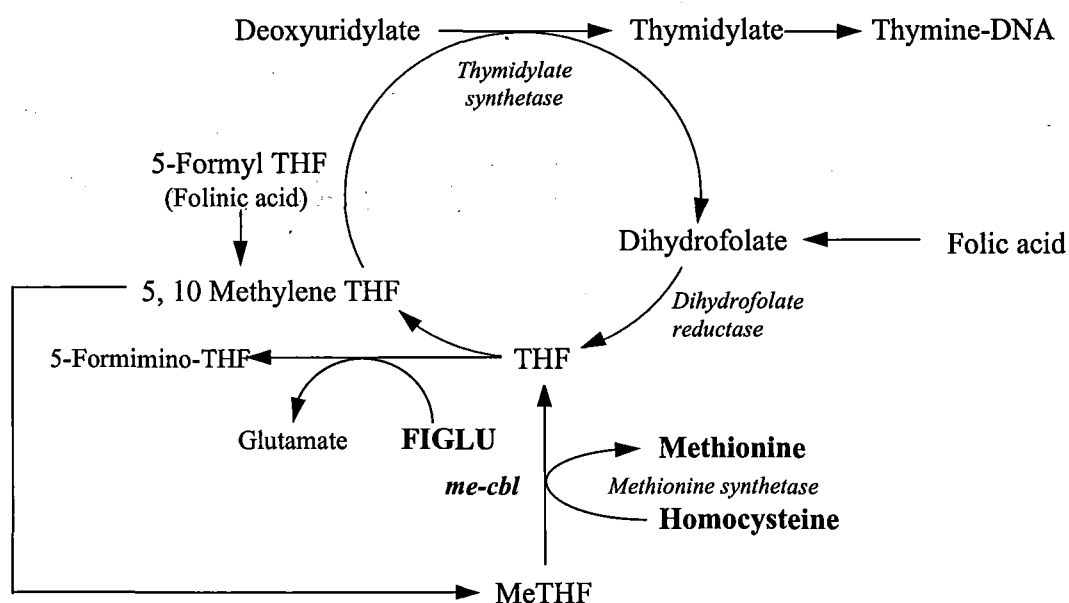


Figure 2.7: Metabolic pathways involving methylcobalamin

Methionine can be degraded by oxidation, via homocysteine, cystathione and α -ketobutyrate, to propionyl-CoA and further to succinyl-CoA, requiring methylmalonyl-CoA as an intermediate. Succinyl-CoA then follows the pathway described above. In that way, methionine can be glycogenic.

2.5 Absorption and assimilation of vitamin B₁₂ and analogues

Absorption, retention and elimination of cbl and analogues is influenced by the interplay and availability of dietary intake, digestive secretions, and saturation of blood and tissue stores. Most of the studies have been done on humans (Gräsbeck, 1984; Guéant and Gräsbeck, 1990), and very few on ruminants. *In vivo*, the vitamin is normally found attached to one of a number of binding proteins. Free (unbound) vitamin B₁₂ is found only in negligible amounts. The binding proteins are quite distinctive in their function and site of action.

In Western countries, the daily absorption of vitamin B₁₂ in humans has been estimated at 4 to 20 µg (Heyssel *et al.*, 1966; Clementz and Schade, 1990; Scott, 1977), with physiological requirements considered to be 0.5 to 5 µg (Belaïche and Cattani, 1989; Clementz and Schade, 1990; Festen, 1991; Seetharam and Alpers, 1994). Human food sources high in vitamin B₁₂ include products from those animals that ingest large numbers of micro-organisms or store accumulated cbl (shellfish, egg yolk and organ meat). Up to 100 µg of vitamin B₁₂ per 100 g of wet weight can be found in food. The maximum amount of vitamin B₁₂ absorbed at one meal is about 1.5 to 2 µg, as studied on various animal models. A small amount of cbl can be absorbed by passive diffusion (Scott, 1997; Herbert, 1968a). The rest is eliminated. The Recommended Daily Allowance for most countries and endorsed by the WHO is 1 to 2 µg per day (Gräsbeck, 1984; Scott, 1997). Cooking and the action of digestive enzymes help to free the vitamin from food and make it available for absorption (Nicolas and Guéant, 1995; Carmel, 1994).

The human body contains about 3 to 5 mg half of which is stored in the liver (Clementz and Schade, 1990; Schneider and Stroinski, 1987). This amount would last several years before stores became depleted if vitamin B₁₂ intake or uptake stopped (Belaïche and Cattani, 1989). Total body half-life has been estimated to be one to four years (Reizenstein, 1959a; Schneider and Stroinski, 1987).

Ruminants usually have a far greater need for vitamin B₁₂ than humans (Gardiner, 1977). It has been estimated that sheep need approximately 11 µg/day (Marston, 1970). Their rumen micro-organisms, notably *Selenomonas ruminantium*

and *Peptostreptococcus elsdenii* (Gardiner, 1977), produce from 400 to 1000 μg vitamin B_{12} a day of which only 3 to 5 % appears to be absorbed (Marston, 1970; Gardiner, 1977). Approximately 1 mg is stored in the liver (Gardiner, 1977), with a maximum capacity for storage of 1.4 $\mu\text{g}/\text{kg}$ ($\sim 1000 \text{ nmol}/\text{kg}$) fresh tissue (Marston, 1970).

2.5.1 Digestive Tract

2.5.1.1 Gastric phase

Several authors (Ellenbogen, 1975; Gräsbeck, 1979; Jacob *et al.*, 1980; Beck, 1982; Nicolas and Guéant, 1994) have given an overview of the path of vitamin B_{12} from the uptake in the stomach through to the final metabolic sites: the cells (Figure 2.8).

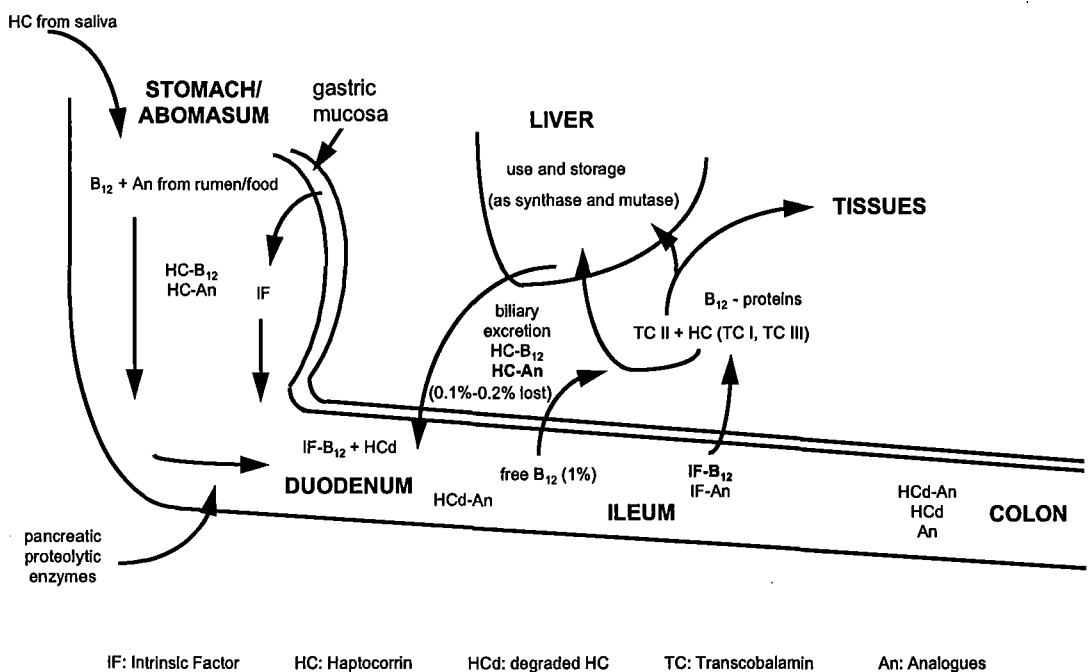


Figure 2.8: Pathway of vitamin B_{12} transport in the body

Vitamin B_{12} , once liberated from food, binds to a protein called haptocorrin (HC) (see 2.5.2.2) (Nexø *et al.*, 1994). HC is secreted by salivary and gastric glands and therefore is present in gastric juice, with an unsaturated vitamin B_{12} binding

capacity (UBBC) of 15 to 45 nmol/l (Guéant and Gräsbeck, 1990), being 4 to 10 % of the total gastric UBBC. At the acid pH of the stomach vitamin B₁₂ preferentially binds to HC as it is liberated from food (Carmel, 1994 and 1995). However, other, as yet unidentified, factors seem to play a role in the release of cbl from food (Carmel, 1994).

Jacob *et al.* (1980) found that in humans, monkeys, cats, rabbits, guinea pigs and cattle the parietal cells of the gastric mucosa produce a spherical glycoprotein. This protein, which contains 9 to 15 % carbohydrate, of which 13 to 18 % is sialic acid (Guéant and Gräsbeck, 1990), has a molecular weight of around 50,000 to 60,000. Sometimes this figure has been estimated at 100,000 to 120,000, presumably due to the formation of dimers in the presence of vitamin B₁₂ (Jacob *et al.*, 1980). This glycoprotein was Castle's 'intrinsic factor' (IF) (with vitamin B₁₂ being the 'extrinsic factor'). The production of IF is dependent on the presence of hydrochloric acid and pepsin in the stomach. Gastric atrophy, hypochlorhydia or achlorhydia, gastric resection or removal, all result in loss of IF (Jacob *et al.*, 1980; Seetharam and Alpers, 1994; Guéant and Gräsbeck, 1990; Stabler, 1995). If antibodies are produced against IF pernicious anaemia (PA) results which represents an auto-immune disease.

In sheep, it is the pyloric abomasal parietal cells that secrete IF, with only small amounts of it being found in fundic secretions (McKay and McLeay, 1979). In rodents, the main cells of the gastric mucosa synthesise IF (Guéant and Gräsbeck, 1990). IF combines with cbl in the upper small intestines and delivers the vitamin to the distal ileum for absorption (Booth, 1967).

The secretion of IF is stimulated by the presence of food, primarily protein, in the stomach. IF secretion increases in the presence of insulin, gastrin and histamine, and histamine H₂-receptor antagonists have been known to reduce IF secretion in humans (Jacob *et al.*, 1980; Guéant and Gräsbeck, 1990). Under basal conditions, concentrations of IF in gastric juice typically range from 2 to 40 nmol/l, increasing to 50 to 100 nmol/l in response to stimulation. These amounts are in excess of what is considered necessary to bind cbl (Hall, 1989; Guéant and Gräsbeck, 1990; Nicolas and Guéant, 1995).

IF binds vitamin B₁₂ on a one-to-one basis via the benzimidazole ring as well as the corrin ring (Jacob *et al.*, 1980). For 'true' vitamin B₁₂, the affinity of IF

appears to be strongest for OH-cbl and weakest for ado-cbl. Yet this bond is up to 60 times weaker for analogues (Guéant and Gräsbeck, 1990; Nicolas and Guéant, 1995). The IF-cbl complex is stable in the presence of proteases. Only IF that is not bound to vitamin B₁₂ becomes partially degraded by pepsin and chymotrypsin (Jacob *et al.*, 1980). The IF that escapes degradation is potentially available in the ileum to combine with vitamin B₁₂ that has been produced there by some bacteria or was present in bile and thus can become available for reabsorption (Kapadia *et al.*, 1976; Guéant and Gräsbeck, 1990; Nicolas and Guéant, 1995).

2.5.1.2 Duodenal-jejunal phase

HC has up to 50 times greater affinity for vitamin B₁₂ than IF in the acid conditions of the stomach (Allen *et al.*, 1978; Belaïche and Cattán, 1989; Guéant and Gräsbeck, 1990; Seetharam and Alpers, 1994). Therefore, the HC-cbl complex, which is stable at low pH, and free IF advance to the duodenum, where HC is degraded by pancreatic enzymes (chymotrypsin, trypsin and elastase working together) and bile in an alkaline milieu (Belaïche and Cattán, 1989; Guéant and Gräsbeck, 1990; Seetharam and Alpers, 1994; Nexø *et al.*, 1994; Nicolas and Guéant, 1995). The liberated vitamin B₁₂ then binds to IF which will carry it to the distal ileum for absorption.

This mechanism is still poorly understood since absence of pancreatic enzymes does not necessarily result in vitamin B₁₂ malabsorption; vitamin B₁₂ is found to be complexed with IF in those cases too (Jacob *et al.*, 1980). However, malabsorption of cbl has been found in 40 % of people with chronic pancreatitis and in 100 % of people with cystic fibrosis, which is corrected by administration of pancreatic extracts (Guéant *et al.*, 1984a; Belaïche and Cattán, 1989). It seems that there may be another, non-proteolytic, component in pancreatic juice that could be involved in the breakdown of HC. A low gastric pH is needed to stimulate pancreatic secretions, including bicarbonate (Marcoullis *et al.*, 1980). Both bile and pancreatic enzymes are necessary for cbl uptake (von der Lippe, 1976).

As digestive and biliary HC are degraded by pancreatic enzymes, the cbl they carry would be available for absorption again by binding to unsaturated IF (Guéant

and Gräsbeck, 1990; el Kholty *et al.*, 1991; Nicolas and Guéant, 1995). Some controversy exists about the degradation of HC in the duodenum and its binding capacity thereafter. Hall (1989) claimed that all HC was degraded by pancreatic enzymes, thus being unavailable for further binding in the intestines. No explanation was given, though, for the fact that HC and partially degraded HC (HCd), with or without attached cbl or analogues, has been found in stools. Figure 2.9 shows the different mechanism of cbl binding to IF and HC (the shaded area denotes the carbohydrate mantle), illustrating that HC is more vulnerable to proteolytic digestion.

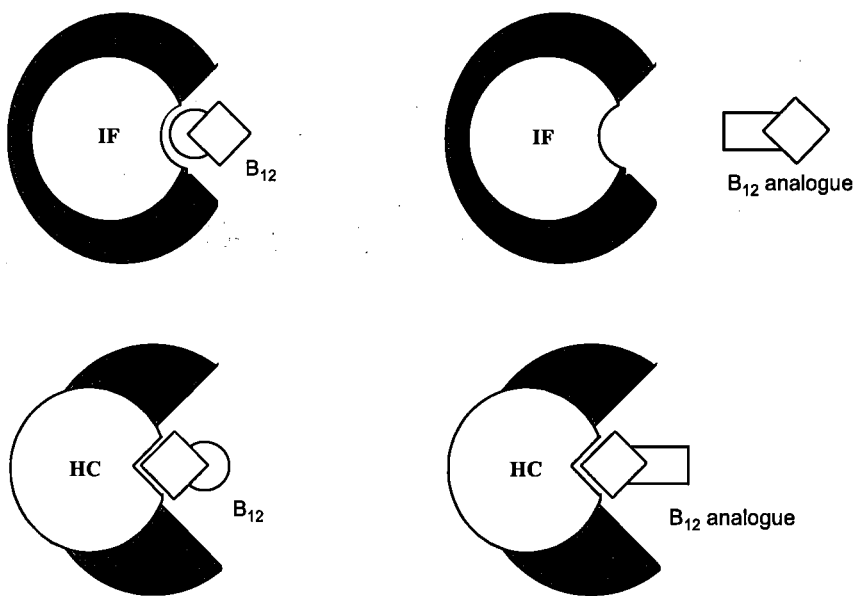


Figure 2.9: Binding of cobalamins to Intrinsic Factor and to haptocorrin (adapted from Guéant and Nicolas, 1990)

2.5.1.3 Ileal phase

The IF-cbl complex is absorbed only in the terminal ileum (Watson, 1968; Carmel *et al.*, 1969; Schjónsby and Anderson, 1974; Nexø *et al.*, 1994) by very few specialised membrane lipoproteins (Jacob *et al.*, 1980). The reaction between receptor and IF-cbl complex depends on the presence of calcium ions, and to a lesser extent magnesium ions (Beesley and Bacheller, 1980), and is optimal between pH 6.4 and 8.4 (Jacob *et al.*, 1980; Nicolas and Guéant, 1994). Bile acids seem to be

necessary for the IF-cbl complex to bind to the surface receptors in the terminal ileum (Kanazawa *et al.*, 1985; Seetharam *et al.*, 1992).

Transit through the ileal enterocyte occurs against a concentration gradient (Lindenbaum, 1979) and thus requires energy. In humans, the time from ingestion to appearance in the blood has been estimated at 5 to 8 h by Linnell and Matthews (1984) and Allen (1976) and 1.5 to 3 h by el Kholty *et al.* (1991). The exact mechanism by which the IF-cbl complex is absorbed is not yet clear. One possibility is that the whole complex binds to a receptor and enters the mucosal cells at the base of the microvilli by endocytosis with the subsequent release of cbl from IF (Belaïche and Cattani, 1989). Another possibility is that IF stays attached to the cell wall receptor while cbl enters the cell (Belaïche and Cattani, 1989). This process is a limiting factor in cbl absorption (Scott, 1997).

The ileal receptor has a high degree of specificity for cbl (Scott, 1997); however, it does not selectively bind IF-cbl complexes only. To a minor extent IF can also carry vitamin B₁₂ analogues which may be absorbed (Brandt *et al.*, 1977; Kolhouse and Allen, 1977a). Since varying amounts of analogues are present in plasma of different mammals, another route of absorption, independent of IF, has been suggested (Shaw *et al.*, 1989; Seetharam and Alpers, 1994) which needs further investigation.

Certain bacteria in the small intestines are capable of producing cbl and analogues which they require for their growth (Allen, 1976) and which in turn could facilitate bacterial proliferation (Shaw *et al.*, 1989; Nicolas and Guéant, 1995). Alternately, bacteria could use the host's cbl for their own requirements (Donaldson, 1975), producing inactive analogues in the process. Some bacteria may bind the IF-cbl complex, thus making it unavailable for absorption (Festen, 1991). This not only reduces the cbl available to the host, leading to deficiency, but also may allow for the absorption of some of these analogues into the system (Brandt *et al.*, 1977).

These analogues could then be taken up preferentially by HCD and thus excreted. By eliminating analogues in this way, HCD confers antimicrobial activity (el Kholty, *et al.*, 1991), freeing IF to selectively absorb the 'true' vitamin B₁₂ (Ford, 1974; Kolhouse and Allen, 1977a) and depriving the bacteria of an essential nutrient. Administration of antibiotics in the case of small bowel bacterial overgrowth will

restore cbl absorption to normal. The same is true for parasitic infestation, anthelmintics will re-establish normality (Belaïche and Cattán, 1989). Hence HC may play a vital role in the immune system of the body (Osifo *et al.*, 1982).

In sheep the few calculated absorption efficiencies of cbl range from 3 to 38 %. The lower the cbl production, the higher the estimate of absorption and retention. Diets capable of producing larger quantities of cbl but with slower intestinal passage appear to be associated with the greatest cbl absorption, mainly from the small intestine, with some free cbl being considered to be absorbed passively from the rumen (Marston, 1970; Gardiner, 1977; Rickard and Elliot, 1978; Smith and Marston, 1970).

2.5.2 Blood

2.5.2.1 Transcobalamin II

Once vitamin B₁₂ has been absorbed into the bloodstream, it is then carried by serum proteins termed transcobalamins (Katz and O'Brian, 1979; Hall, 1979). The majority move electrophoretically with the β -globulins and these appear to bind most of the absorbed vitamin B₁₂. They are called transcobalamin II (TC II) (Jacob *et al.*, 1980; Kumar and Meyer, 1980) and take up vitamin B₁₂ from IF in the ileal enterocyte. The TC II-cbl complex, as well as free TC II, is then released into the portal blood (Rothenberg and Quadros, 1995; Fedosov *et al.*, 1996). Approximately 90 % of TC II (Hall, 1989) is present in plasma as apo-TC II (TC II without cbl attached to it) which accounts for about 80 % of the total UBBC (Allen, 1976; Fräter-Schröder *et al.*, 1979; Haus *et al.*, 1979; van Kapel *et al.*, 1988; Rothenberg and Quadros, 1995). In humans, blood contains around 0.2 μ g/l of vitamin B₁₂.

TC II, unlike IF, is not a glycoprotein. It has a molecular weight of 36,000 - 38,000 (Hom *et al.*, 1966). Like IF, one molecule of TC II binds one molecule of vitamin B₁₂, and it has a similar site of interaction with cbl, namely the benzimidazole and the corrin rings. However, since TC II also binds pseudovitamin B₁₂ and to some extent dimethylbenzimidazole, the type of attachment of vitamin B₁₂ to the two proteins is different (Jacob *et al.*, 1980).

The main site of TC II synthesis is considered to be the liver, by hepatocytes, although Rothenberg and Quadros (1995) suggest venous endothelial cells as an additional biosynthetic site. Cells of other tissues seem to be capable of its production too, including fibroblasts, macrophages and possibly enterocytes (Chanarin *et al.*, 1978). Injection of large amounts of vitamin B₁₂ saturates TC II (converting it to holo TC II) and initiates cell absorption, with the degree of saturation rapidly dropping to half its post-injection concentration (Hall, 1989). The half-life of cbl in blood has been estimated at about six days (Reizenstein, 1959a).

In humans, TC II seems to be specific for 'true' vitamin B₁₂. It also has been found to bind 6 to 20 % of the endogenous (~ 300 ng/l) vitamin B₁₂ (Das *et al.*, 1991; Allen, 1976). Only TC II is considered to facilitate the uptake of vitamin B₁₂ into the cells. Absence of TC II manifests itself in the first 3 to 6 weeks of life in the form of megaloblastic anaemia, failure to thrive, repeated infections and neurologic changes.

The TC II-cbl complex (holo-TC II) is estimated to have a half-life of 5 (Chanarin *et al.*, 1978) to 90 min (Tröscher and Menke, 1985) and is cleared by the liver (Chanarin *et al.*, 1978). TC II has been isolated by Allen and Majerus (1972) through affinity chromatography together with ion exchange chromatography and gel filtration. The TC II-cbl complex can be separated again by dialysis (Allen and Majerus, 1972). It is most stable at a pH range from 4 to 11 (Allen, 1976).

2.5.2.2 *Haptocorrin*

Body fluids such as saliva, tears, milk, amniotic fluid, cerebro-spinal fluid, blood and bile all contain proteins capable of binding corrinoids (Hall, 1989; el Kholty *et al.*, 1991; Seetharam and Alpers, 1994; Fedosov *et al.*, 1996). These proteins have all been termed HC, cobalophilins, R-proteins or R-binders (because they move rapidly with the α -globulins on electrophoresis). HC has a molecular weight of approximately 120,000 (Hom *et al.*, 1966). The HC-cbl complex has a half-life of about nine days, considerably longer than that of TC II (Hardwicke and Jones, 1966).

The majority of HC is found in the upper digestive tract. In plasma, it is sometimes called TC I or TC III, to differentiate it from TC II (Kumar and Meyer,

1980). TC III in blood is largely unsaturated and is believed to be an artifact of the isolation process (Jacob *et al.*, 1980). TC I, TC III as well as HC from other body fluids are similar in protein content and differ only by their sialic acid and/or fucose content. They behave in the same way immunologically (Allen, 1976; Fräter-Schröder *et al.*, 1979; Yang *et al.*, 1982; Nicolas and Guéant, 1994).

Each HC molecule, like IF and TC II, binds only one molecule of vitamin B₁₂. However, it also seems to bind analogues. Although sites of vitamin B₁₂ attachment to HC are most likely the corrin and dimethylbenzimidazole rings, the mechanism of binding is different from the other vitamin B₁₂-binding proteins (Figure 2.3). Of the three binders, HC is considered to have the greatest affinity for analogues and IF the least (Muir and Chanarin, 1983).

Most of the analogues are either inactive in or potentially toxic to the host organism (Kolhouse and Allen, 1977a; Adjalla *et al.*, 1994). Therefore, they appear to be barred from gaining entry to the tissues by being taken up by HC which delivers them exclusively to asialoglycoprotein receptors in the hepatocytes for excretion (Kolhouse and Allen, 1977a; Jacob *et al.*, 1980, Seetharam and Alpers, 1994). Bile is the preferred means of excretion, in faeces. It has been estimated that 70 to 90 % of corrinoids in faeces are analogues (Adjalla *et al.*, 1993) and that UBBC in bile is 1 µg/l (Linnell, 1975).

HC does not always exclude analogues from entering the bloodstream (Thorndike and Beck, 1984). Since each binder can carry analogues to some extent, analogues are capable of entering tissues (Kolhouse and Allen, 1977a), so it is possible that they occur in biological tissues in higher amounts than can be measured (Kondo *et al.*, 1980). If the nucleotide moiety is sufficiently similar to that in cbl, even IF will bind the analogue and carry it to the tissues (Lambert *et al.*, 1992). The few analogues that are bound to IF or TCII are taken to the tissues in the same way as is 'true' vitamin B₁₂ (Adjalla *et al.*, 1993).

These analogues present in human and animal blood can block the uptake of cbl by tissues by competing for binding and absorption sites on cells. They are taken up preferentially by HC. Since a higher concentration of analogues has been found in portal compared to peripheral circulation (el Kholty *et al.*, 1991), analogues may originate from intestinal absorption rather than from endogenous vitamin B₁₂

metabolism (Shaw *et al.*, 1989). The finding of a negative correlation between the concentration of analogues in bile and in blood supports the hypothesis that one function of HC is to remove unwanted analogues from the system (Shaw *et al.*, 1989).

However, the complete range of functions of HC has probably not yet been fully elucidated (Nexø *et al.*, 1994). Its role in metabolism is still rather unclear as humans with congenital defects in HC production do not necessarily seem to have any abnormality in cbl metabolism or storage (Allen, 1976). It has been shown that some HC-cbl can enter the cell with subsequent conversion of the cbl to its coenzyme forms, but this process is slow and inefficient. Compared to TC II-cbl, this is considered to be negligible (Hall, 1989).

2.5.2.3. *Endogenous cobalamins*

Whereas the role of TC II is to carry exogenous vitamin B₁₂ to the cells for metabolism or storage, HC evidently carries about 90 % of the endogenously derived vitamin B₁₂ in the plasma but contributes very little to the overall movement of vitamin B₁₂ in humans (Jacob *et al.*, 1980; Seetharam and Alpers, 1994). HC is the most abundant binding protein in plasma (Seetharam and Alpers, 1994). 80 % is found as holo HC (Hall, 1989); apo HC contributes only up to 10 % to the total UBBC (Allen, 1976). It binds cbl more tightly than TC II. However, since TC II is present in blood mainly in the apo form, this will bind cbl first. HC does not seem to have any transport function; it may act more like a blood storage of cbl, releasing cbl sparingly as and when the necessity arises (Herbert, 1968b). There may be exceptions to this in situations where IF activity is low, as in infancy. There is some evidence that suckling animals may have HC-cbl receptors which allow uptake of vitamin B₁₂ bound to HC in milk across the gut wall (Seetharam and Alpers, 1994).

Little is as yet known about the release and subsequent distribution of intracellular cbIs. HC could be involved in carrying cbl out of the cell according to Hall and Finkler (1971). Plasma does contain the coenzyme forms of cbl. In humans, at least 50 % is in the form of me-cbl, the remainder being ado- and OH-cbl, with up to 8 % of CN-cbl in some individuals (Linnell and Matthews, 1984; Linnell *et al.*,

1969). Alternatively, cbl could have been converted to coenzymes in the lengthy passage through the ileal enterocytes as they have been found attached to TC II in hepatic portal blood (Belaïche and Cattani, 1989; Rothenberg and Quadros, 1995). Nexø *et al.* (1979) found that me-cbl was mostly attached to HC and ado-cbl to TC II.

However, Gimsing *et al.* (1982) found that the predominant form of cbl in plasma was the one injected or given orally and that cbl was converted to its coenzyme forms in the tissues. This may not apply when considering the comparatively small amount of cbl taken up and released from food. That some release of cbl from cells is happening, though, is obvious from the fact that in cbl deficiency a steady decline of stored cbl is occurring. One possibility is that free cbl leaves the cell (Allen, 1976) and is taken up by either apo TC II or apo HC in the blood (Cooksley *et al.*, 1974; Hall, 1989).

Linnell and Matthews (1984) showed that plasma me-cbl is disproportionately lowered in pernicious anaemia, veganism and towards the end of pregnancy while total plasma vitamin B₁₂ can still be within the normal range. Therefore me-cbl could be an early indicator of impending deficiency in these cases. In foetal blood, me-cbl is present at much higher concentrations than that in maternal blood (Matthews, 1979; Linnell, 1975).

Seventy percent of injected ⁵⁷Co-cbl is apparently cleared from human plasma within the first ten minutes, with the rest clearing slowly over several days (Allen, 1976). This supports the view that cbl is taken up by cells very rapidly and then recirculated (Hall, 1975; Allen, 1976). All the absorbed cbl must pass through the liver first before it appears in systemic blood which contains no more than 2 to 10 % of the *de novo* absorbed vitamin B₁₂ (Allen, 1976).

2.5.2.4 *Animal Studies*

Schultz and Judson (1985) have studied plasma cbl binders in sheep and calves. They found that in sheep the major plasma binder of injected cbl was TC II, whereas in calves it was HC and TC 0 (a cbl binder eluting in the void volume). The concentration of apo TC II in human and sheep plasma was higher than apo HC. The

reverse was observed for mature cattle (Schultz, 1987b). Although calves have a higher total UBBC, they do not have as high blood levels of cbl as adult animals. The difference appears to lie in their higher amounts of apo HC (Schultz, 1987b).

Polak *et al.* (1979) found that in cows ~ 50 % of UBBC was due to TC II and 50 % to HC. This may reflect a higher rate of vitamin B₁₂ absorption in the bovine. When comparing free and bound vitamin B₁₂ in serum, Kato (1960) found that 30 to 50 % of vitamin B₁₂ was in the unbound form in calves, ~ 10 % in mature cattle and less than 5 % in humans. While endogenous vitamin B₁₂ in humans is carried mainly attached to HC, TC II is the main plasma cbl binder in most mammals, including sheep (Linnell *et al.*, 1979).

2.5.3 Tissues

The role of TC II is to carry vitamin B₁₂ to the various tissues and to promote its uptake. There are specific receptors for the TC II-cbl complex on the cell surface which allow it to cross the cell membrane intact by pinocytosis. Once inside the cell, TC II is broken down by lysosomes after they fuse with the pinocytotic vesicles, freeing the vitamin B₁₂ and thus making it available for cell metabolism (Allen, 1976; Kolhouse and Allen, 1977a; Jacob *et al.*, 1980; Hall, 1989; Idriss and Jonas, 1991). This step is magnesium dependent (Idriss and Jonas, 1991). The liberated cbl can then be either returned to the plasma for transport to other metabolic sites or for elimination from the system, or it is converted to the active coenzyme forms and stored (Kolhouse and Allen, 1977b; Jacob *et al.*, 1980).

Significant amounts of vitamin B₁₂ are converted to ado-cbl (in mitochondria) or to me-cbl (in cytosol) via a two-step reduction of the Co moiety (Cooksley *et al.*, 1979; Kolhouse and Allen, 1977b; Gimsing *et al.*, 1982; Idriss and Jonas, 1991; Seetharam and Alpers, 1994; Nexø *et al.*, 1994). This occurs primarily in the liver where the vitamin accumulates in significant concentrations. Ado-cbl, the major portion of biologically active vitamin B₁₂ in the cells (Linnell and Matthews, 1984; Linnell, 1975), becomes associated with the mitochondria where it is bound to another protein: S-methylmalonyl-CoA mutase (EC 5.4.99.2). OH-cbl and me-cbl are mainly found in the cytoplasm, the latter being attached to methionine synthase (EC

2.1.1.13). Therefore, the mutase and the synthase have been called intracellular cobalamin-binding proteins (IBC) ($M_r > 100,000$) and play a major role in the intracellular retention and compartmentalisation of cbl (Kolhouse and Allen, 1977b).

The distribution of cbl among its coenzyme forms in human cells is reversed from those observed in plasma: me-cbl accounts for only about 10 % of total cbl, with CN-cbl being virtually absent (Linnell and Matthews, 1984). Liver me-cbl declines steadily throughout life in humans, from 35 % of total cbl in the foetus to 2 % in old age. Ado-cbl in adult liver is about 1000 μg , being ~ 70 % of cbl (Linnell, 1975; Tröschner and Menke, 1985). Of total body cbl, ado-cbl constitutes one fifth to one half (Linnell, 1975).

An overview of the rather complex transport system is given in Figure 2.10, adapted to ruminants. For monogastrics, the process would start one step later, at the abomasum/stomach site.

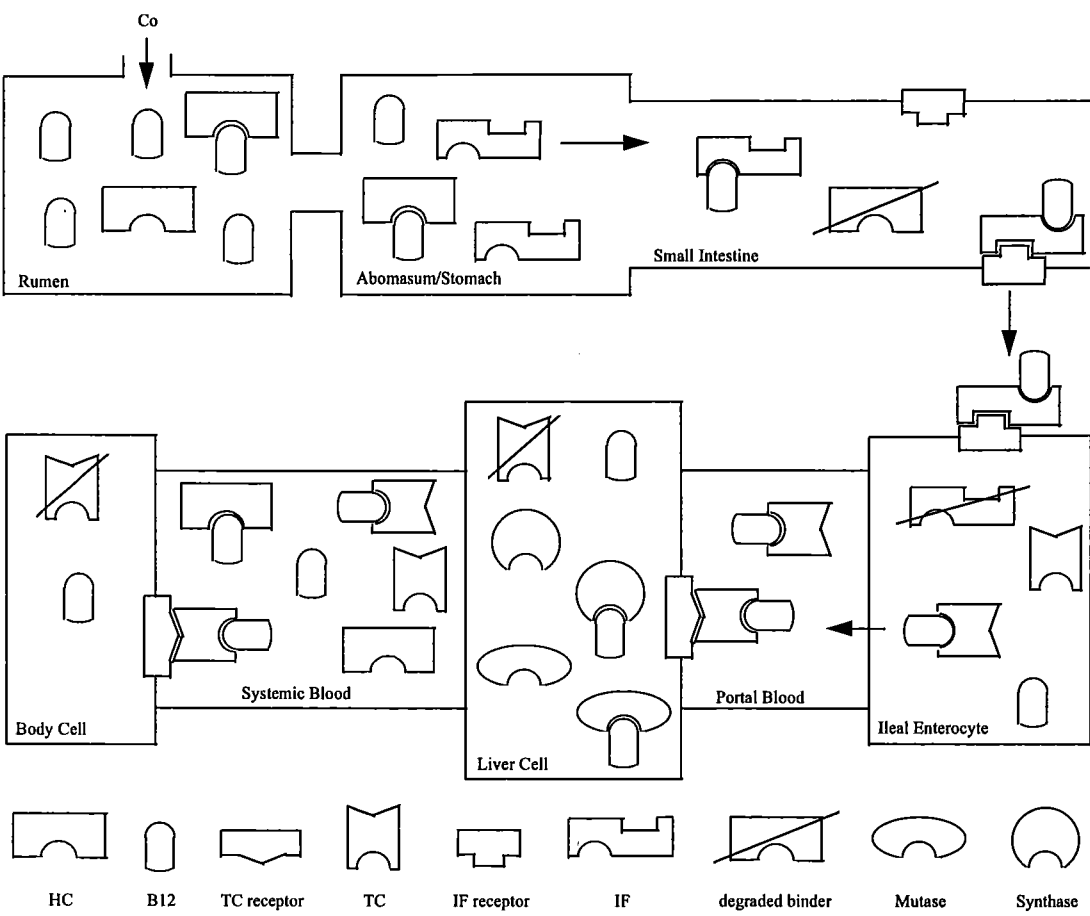


Figure 2.10: Overview of cbl transport and absorption (adapted from Hall, 1989)

2.6 Excretion

2.6.1 Urine

The majority of surplus cbl, bound and unbound, appears to be eliminated through the kidneys and only occurs when the total binding capacity of TC II is exceeded (Cooksley *et al.*, 1974). This is the basis of the Schilling test, in which patients are given radiolabeled vitamin B₁₂ (containing ⁵⁷Co) orally, followed by unlabeled vitamin B₁₂ as an intramuscular (IM) injection to flush the ⁵⁷Co-vitamin B₁₂ out. If radioactivity in a 24 h urine collection is less than 8 % of the oral dose it has not been absorbed and malabsorption of vitamin B₁₂ is suspected (Clementz and Schade, 1990).

The immediate excess after administration of supra-physiological amounts of vitamin B₁₂ is eliminated in urine. Cbl is probably detached from its binder during passage through the kidney (Reizenstein, 1959b). The kidney is also capable of reabsorbing TC II-cbl so that little is lost to the system (Nicolas and Guéant, 1994). Beck (1982) found that under normal metabolic conditions, less than 250 ng/day of cbl was eliminated in the urine.

2.6.2 Faeces

Okuda *et al.* (1958) postulated another route of excretion for unwanted cbl - via bile and faeces - as there was a discrepancy between the amounts of absorbed vitamin B₁₂ and that excreted in urine. Dawbarn and Hine (1955) found that urinary excretion was around 1 % of faecal excretion. Therefore bile was considered to be the main path of excretion (Smith and Loosli, 1957). Smith and Marston (1970) found that 5 to 44 µg of cbl was excreted daily in the faeces of cbl deficient sheep and 86 to 300 µg in sheep supplemented with 1 mg Co/d. Gräsbeck *et al.* (1958) injected a small amount of ⁵⁶Co-cbl into human volunteers. They concluded that a) the main route of cbl elimination in humans is via bile and faeces, and b) there must be an entero-hepatic circulation, reabsorbing some of the cbl initially excreted by bile.

Reizenstein (1959c) measured corrinoids in faeces and found 1.3 µg/day cbl and 7.4 µg/day total corrinoids. The difference would be suggestive of analogues.

Other absorption trials with labeled cbl have shown excretion of excess cbl and analogues (Guéant *et al.*, 1984a and 1984b; Shaw *et al.*, 1989) in stool samples at a concentration of about 1 µg/day in humans. They were exclusively attached to HC or Hcd and were found in bile 24 to 72 h after ingestion. The total amount of cbl excreted in bile has been estimated at up to 9 µg/day or 0.1 to 0.2 % of the body's total vitamin B₁₂ (Heyssel *et al.* 1966; Gräsbeck, 1984; Festen, 1991; Nicolas and Guéant, 1995), depending on how much is stored in the tissues (Allen, 1976; Belaïche and Cattani, 1989; Seetharam and Alpers, 1994).

HC-cbl originating from the body and present in bile can thus be freed when HC is degraded in the intestinal lumen and made available for IF binding and absorption (Nicolas and Guéant, 1995). The entero-hepatic circulation therefore serves to regulate cbl balance in three ways: 1) the elimination of unwanted analogues, 2) maintaining normal cbl balance (Green *et al.*, 1981), and 3) retention of cbl in times of shortage.

Part 2: Nutritional and other factors affecting vitamin B₁₂ status in humans and animals

2.7 Sources of cobalt / vitamin B₁₂ and dietary requirements

Apart from vitamins A and D, vitamin B₁₂ is the only other vitamin not found in plants. The only exception is fermented foods, like seaweeds and tempeh, where it has been detected in trace amounts. Vitamin B₁₂ synthesis from Co is accomplished by only a few species of micro-organisms. Anaerobic bacteria found in sewage sludge, manure, soil and dried estuarine mud are rich in this vitamin. In animals, the vitamin is synthesised by bacteria in the intestinal tract (rumen or gut).

Only traces of the vitamin are required by animals. Vitamin B₁₂ is found mainly in the liver, kidneys and bones, but is generally widely distributed throughout the body. There is little variation between different animal species and humans in respect to the amount of vitamin B₁₂ in the various organs and tissues (Underwood, 1971).

In the following sections the traits assayed are expressed in number of moles (or sub-units thereof) per volume when comparisons are made between authors. Therefore values expressed as weight per volume have been converted to moles per volume and rounded.

2.7.1 Ruminants

The ruminant is entirely dependent on rumen microbial synthesis of vitamin B₁₂ from dietary Co. Yet minimum requirements for bacteria do not satisfy the need of the host animal for vitamin B₁₂. The total requirements of sheep for vitamin B₁₂ are not clearly established. However, Marston (1970), using six month old lambs, calculated that these animals needed about 11 µg/day of vitamin B₁₂ for maintenance. This figure has been used since as a guideline for other researchers.

The requirements of PR lambs for vitamin B₁₂ are even less defined (Ramos *et al.*, 1994; Fisher and MacPherson, 1991; Grace *et al.*, 1986). Therefore, no standards have been set for pregnant and lactating ewes or PR lambs regarding their daily vitamin B₁₂ requirements. Quirk and Norton (1987) found that milk production depended on the Co status of the ewe. They therefore suggested an increase of the minimum dietary Co intake from 0.08 mg/d to 0.15 mg/d for optimal milk production in lactating ewes.

The less Co there is in a sheep's diet, the more efficient the conversion of Co into vitamin B₁₂ (Smith and Marston, 1970). The same authors found that dietary Co tends to be more readily utilised by the animal than Co from supplementation. It has been estimated that on average 13 % of dietary Co is converted into vitamin B₁₂ in the rumen (in sheep, this amounts to about 700 µg of vitamin B₁₂ a day), of which about 5 % only are absorbed (Marston, 1970; Grace, 1994).

2.7.1.1 Cobalt content of soil

There are various factors influencing the amount of Co in the diet of ruminants. Available Co concentrations in the soil fluctuate according to the season: they decrease during spring and summer, when growth rate is high, and increase during late autumn and winter. Available Co concentration also depends on the type

of soil: if it is sandy or derived from igneous rocks, such as granite, Co is low, whereas soils derived from basalt are high in Co (Grace, 1994). Well-drained soils tend to lose up to seven times more Co compared to waterlogged pastures (Minson, 1990). If the soil becomes too alkaline, plant uptake of Co is reduced. High soil levels of manganese interfere with Co uptake by plants (Metherell, 1989).

Although there is a correlation between Co in the soil and the Co status of a ruminant, there is no clear indication of when an animal may become deficient in Co and subsequently in vitamin B₁₂. Generally it has been found (Grace, 1994) that if the total soil Co content is less than 2 mg/kg, or the acetate-extractable (available) soil Co concentration is lower than 0.25 mg/kg air-dried soil, deficiency in the animal is likely.

2.7.1.2 *Cobalt content of plants*

Plants contain much less Co than soils, and the amount varies among different species (0.08 to 0.31 mg Co/kg DM - Gardiner, 1977). Analysis of different pasture plants shows higher Co content in clovers and legumes than in grasses. It is therefore difficult to establish clear estimates as to how much Co an animal will ingest. A minimum dietary concentration of 0.07 - 0.08 mg Co/kg dry matter (DM) has been suggested to meet the Co requirements of sheep of 0.1 mg/day, with lambs requiring slightly higher amounts of 0.11 mg Co/kg DM (Andrews *et al.*, 1958). Cattle seem to need less Co and still thrive on 0.06 mg Co/kg DM (Andrews *et al.*, 1958). However, if the pasture is kept short, the animal will ingest a certain amount of soil which can increase its Co intake markedly (Judson *et al.*, 1989).

2.7.1.3 *Other dietary factors*

Little is known about other factors, apart from the Co content of the diet, that influence the vitamin B₁₂ status of ruminants. Since an inverse relationship has been postulated between the number of micro-organisms in the rumen and the rate of forage degradation it is perhaps not surprising that the latter could have an influence on how much Co can be converted to vitamin B₁₂. Digestibility depends on the type of forage consumed by the animal, and the resulting rumen pH. A slightly acidic pH

seems to favour propionic acid production. Yet high amounts of propionic acid in the rumen, produced from rolled grain or starch, are sometimes linked to larger quantities of analogues. This may indirectly contribute to vitamin B₁₂ deficiency (Ørskov, 1994).

2.7.1.4 *Pregnancy and lactation*

Until lambs are able to produce their own vitamin B₁₂ as a result of rumen microbial synthesis, at approximately four to six weeks of age (Ramos *et al.*, 1994), they rely on their daily intake from milk and their body stores. PR lambs are able to absorb the lactose in milk and utilise it directly for their energy needs. They therefore may need less vitamin B₁₂ channelled into the conversion of propionate to succinate (Fisher and MacPherson, 1991; Rice *et al.*, 1989). However, they presumably do require vitamin B₁₂ for the methylation of homocysteine (Fisher and MacPherson, 1991) as part of the maintenance of methionine for wool and body growth. They also need to build up immunity after birth to the world outside the womb. Lowered immunity in vitamin B₁₂ deficient lambs, as indicated by depressed levels of neutrophils and serum IgG, has been found by Fisher and MacPherson (1989). In fact, the main coenzyme form of cbl found in (human) milk was me-cbl (Sandberg *et al.*, 1981). Since the digestive system of PR lambs functions like that of a monogastric it is possible that me-cbl could also be the main coenzyme requirement in lambs.

The vitamin is transferred to the lamb via the placenta (Quirk and Norton, 1987) and stored in the foetal liver during gestation (Grace, 1999a). Colostrum provides the newborn lamb with an initial excess which could also increase liver concentrations (Grace *et al.*, 1986). Grace (1999a) found that liver reserves in PR lambs from Co deficient dams were depleted within 58 days post partum.

Grace (1999a) also demonstrated a direct relationship between milk vitamin B₁₂ and plasma concentrations of the ewe. However, as milk vitamin B₁₂ concentrations are much higher than those in plasma the vitamin needs to be secreted into the milk against a concentration gradient. In turn, plasma vitamin B₁₂ concentrations in lambs have increased dramatically after colostrum intake (Halpin

and Caple, 1982). These researchers reported increases from around 1350 pmol/l to above 3000 pmol/l. The vitamin B₁₂ in milk is minimal in comparison to colostrum and may not necessarily always supply the lambs' daily needs (Halpin and Caple, 1982; O'Halloran and Skerman, 1961; Hart and Andrews, 1959). PR lambs, therefore, may need some reserves to draw on in order to maintain normal metabolic functions.

Ramos *et al.* (1994) measured serum vitamin B₁₂ levels in lambs during the first two weeks of life. Consumption of colostrum elevated serum vitamin B₁₂ threefold in the first 24 h compared to a control group fed on milk substitute. However, within four days the serum vitamin B₁₂ levels in the colostrum-fed group had fallen to below birth values and continued to decrease steadily throughout the trial. The same trend was reflected in milk vitamin B₁₂ levels which started at around 151,000 pmol/l and fell to below 5,000 pmol/l during the trial. Other workers (O'Halloran and Skerman, 1961; Halpin and Caple, 1982; Marca *et al.*, 1996; Grace, 1999a) reported similar results. Table 2.2 gives an overview of vitamin B₁₂ values in colostrum and milk.

Table 2.2: Vitamin B₁₂ content of colostrum and milk

Source	Days post partum		Colostrum (mean) (pmol/l)	Days post partum	Milk B ₁₂ (mean) (pmol/l)
Gregory, 1954				mid lact.	6200
O'Halloran & Skerman, 1961	S	1 - 4	5843	7 - 28	1107
	U		28192	7 - 28	13432
Ramos <i>et al.</i> , 1994		1 - 4	29496	8 + 16	6126
Marca <i>et al.</i> , 1996		1 - 7	14000	30	4910
Grace, 1999a	S			30 - 108	4400
Grace, 1999a	U			30 - 108	3357

S: Supplemented ewes

U: Unsupplemented ewes

In times of deficiency, the vitamin B₁₂ available to the lamb could be reduced by two mechanisms (Quirk and Norton, 1987). Co deficiency (and the accompanying increase in methylmalonic acid) is likely to decrease the ewe's appetite and hence food intake (Duncan *et al.*, 1981), and milk production could be compromised as a result. Also, there is less of the vitamin available to the offspring in the milk, which

may become insufficient for the lamb's metabolic needs (O'Halloran and Skerman, 1961). The lamb is therefore likely to have little of the vitamin stored in its liver and minimal or no reserves to draw on. This may explain why the young lamb is reported to be most prone to developing deficiency symptoms, such as failure to thrive (Andrews, 1972).

Quirk and Norton (1987) found evidence of vitamin B₁₂ deficiency in lambs born to and suckling from Co deficient ewes. Lambs had elevated FIGLU in their urine and a lower liveweight gain (LWG) than lambs from Co supplemented ewes. Duncan *et al.* (1981) reported low birth weight, failure to suckle or thrive and serum vitamin B₁₂ concentrations < 200 pmol/l.

The vitamin B₁₂ status of PR lambs is therefore determined by three factors: their liver stores (acquired while still in the womb), their colostrum intake and, later, their milk intake (Fisher and MacPherson, 1991). Ultimately, this hinges on the vitamin B₁₂ status of their dams: if the ewes have plenty of the vitamin their offspring are also likely to be replete.

2.7.2 Non-ruminants

In most non-ruminants, vitamin B₁₂ needs to be obtained from the diet. Only animal products (meat or dairy) contain sufficient vitamin B₁₂ to meet metabolic demands. Unless there are absorption problems (see 2.8), people and animals consuming meat in their diet do not suffer from vitamin B₁₂ deficiency. Vegetarians, even if they consume some dairy products in their diet, could be at risk of deficiency since these foods are not as high in the vitamin as is meat.

Although vitamin B₁₂ is also produced in the gut by micro-organisms, it is past the absorption site for the vitamin. Therefore it is lost in the faeces. Vegans and non-ruminant herbivores need to meet their vitamin B₁₂ requirements in different ways. One possibility is through coprophagy (as in the rabbit). Vegans need to supplement their diet with vitamin B₁₂.

2.8 Pathology of vitamin B₁₂ deficiency

Common causes of vitamin B₁₂ deficiency are inadequate dietary intake of the vitamin (as in vegans) or, in the case of ruminants, of cobalt. In humans, lack of transport proteins, notably IF, also leads to deficiency states.

2.8.1 Deficiency signs and symptoms

2.8.1.1 *Ruminants*

Since the requirements of sheep for vitamin B₁₂ are higher than those for cattle, and deficiency is more likely to occur in spring, lambs are the most susceptible ones to develop deficiency symptoms, followed by adult sheep, calves, and lastly, mature cattle (Gardiner, 1977; Andrews, 1972). Consequently, analysing the vitamin B₁₂ status of lambs gives the best indication of likely problems in other animals. If their vitamin B₁₂ status is adequate then other ruminants grazing the same pasture should also have a sufficient supply (Andrews, 1956).

The most obvious feature of deficiency in ruminants is the generalised phenomenon of ill-thrift. This encompasses inappetence, wastage of musculature (marasmus), listlessness, lethargy, weight loss, increased perinatal mortality, anaemia, enhanced susceptibility to infection due to a compromised immune system, a watery eye discharge, pale and fragile mucous membranes and skin, poor coat or wool growth. However, the nervous system of sheep tends to be resistant to vitamin B₁₂ deficiency (Kennedy *et al.*, 1992a), probably due to the fact that the depletion of vitamin B₁₂ from the gluconeogenic pathway will incapacitate the animal before neurological damage can set in (Fell *et al.*, 1985).

Later a fatty degeneration of the liver can occur in sheep, known as Ovine White Liver Disease (OWLD) (Ulvund, 1990a-f). The most characteristic symptoms of affected livers are their pale and friable appearance, the accumulation of lipid droplets in hepatocytes, necrosis and biliary hyperplasia (Kennedy *et al.*, 1994a). Post-mortem examination reveals extreme emaciation with total lack of body fat, whereas the liver is fatty and the spleen haemosiderised. The fatty infiltration of the

liver is typical of protein-calorie malnutrition and may well reflect failure of production of methionine for complete fatty acid oxidation (Smith *et al.*, 1974).

Other health problems linked to vitamin B₁₂ deficiency are phalaris staggers in sheep, and infertility and low milk production in cattle. The haemoglobin levels and red cell numbers are well below normal. The anaemia was found to be normocytic and hypochromic or normochromic in lambs, and microcytic and hypochromic in calves (Underwood, 1971; Smith, 1987). Although it has been shown that low vitamin B₁₂ status alone can lead to OWLD (Young, 1996), other factors may play a role in the development of this disease, like mycotoxicosis, low pasture molybdenum content or high pasture fructan content (Kennedy *et al.*, 1994a; Ulvund and Pestalozzi, 1990). If the deficiency is not corrected, it will be fatal.

2.8.1.2 *Humans*

Deficiency sets in gradually as the vitamin gets depleted from the tissues. Symptoms can be either haematological or neurological. Haematological changes lead to megaloblastic anaemia (which is virtually the same as in folic acid deficiency) with macrocytosis, hypersegmentation of neutrophil nuclei, bone marrow changes, and often pancytopenia (Carmel, 1990).

Nervous system symptoms include soreness and weakness in arms and legs, diminished reflex response and sensory perception, difficulty in walking (gait ataxia) and speaking, and jerking of limbs. This leads to irreversible damage of the central nervous system in humans if left untreated. As the depletion progresses, brain damage occurs, manifesting in sore mouth, numbness, shooting pains or pins-and-needles in limbs, memory defects, to psychotic episodes and dementia (Kirschmann, 1979). If the condition is treated within the first year of occurrence these symptoms, including dementia (Swain, 1995), can be reversed.

2.8.2 **Metabolic changes**

Plasma vitamin B₁₂ concentrations reflect the metabolic balance of vitamin B₁₂ from the production by rumen micro-organisms, the release from the liver, and excretion in faeces and urine. Concentrations usually range from < 80 to > 900

pmol/l (for sheep and cattle) and tend to decrease first as the animal becomes deficient (Figure 2.11, Phase 2a) as they reflect more directly the animal's rate of absorption of the vitamin. Liver stores (ranging from < 48 to > 1000 nmol/kg fresh tissue) can buffer low absorption of vitamin B₁₂ for some time (Phase 2b), and only when these stores are depleted does functional deficiency occur (Phase 3) (Sutherland, 1980; Clark and Ellison, 1993). However, Grace (1999b) postulated a linear relationship between serum and liver vitamin B₁₂ concentrations in sheep, implying that changes in vitamin B₁₂ status occur simultaneously in both tissues.

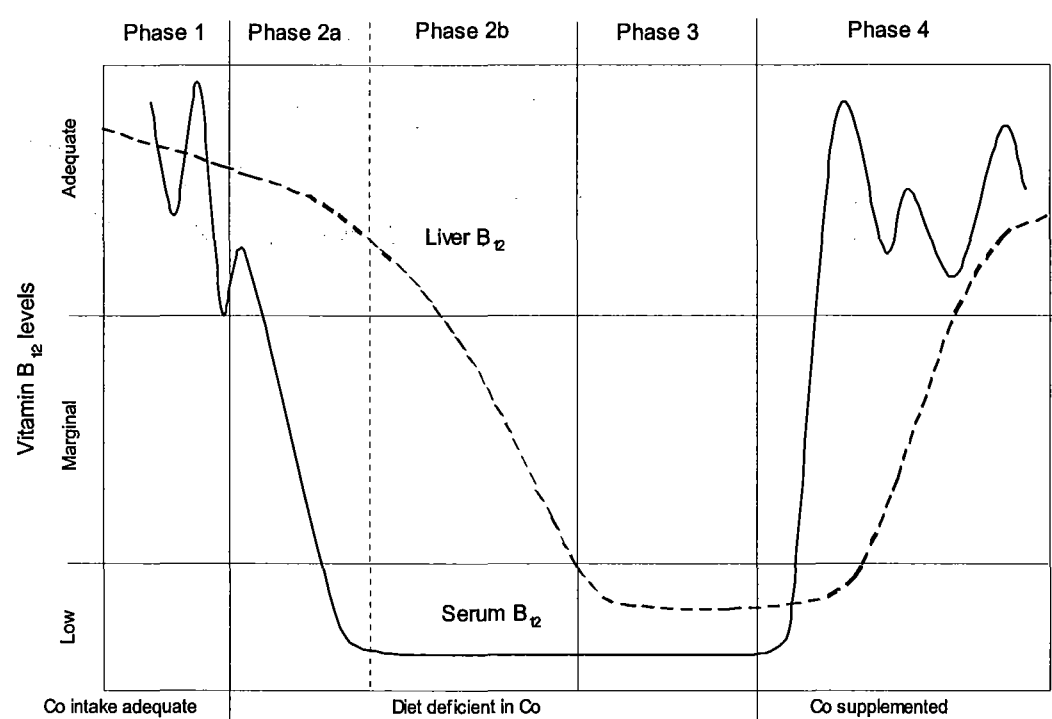


Figure 2.11: A model for the interrelationship between liver and serum levels of vitamin B₁₂ and dietary cobalt (from Sutherland, 1980)

Since vitamin B₁₂ is closely linked to gluconeogenesis from volatile fatty acids, one might expect hypoglycaemia to feature as a symptom. Yet blood glucose levels have not varied between vitamin B₁₂ deplete and replete sheep (Kennedy *et al.*, 1991a), except in very severe deficiency. The tight homeostatic regulation of blood glucose and capability for gluconeogenesis from amino acids and glycerol to supply carbon skeletons as substrates for the Krebs cycle may be responsible.

The failure of the liver to metabolise propionate has been considered to be the primary metabolic defect in vitamin B₁₂ deficiency (Marston *et al.*, 1961 and 1972; Smith and Marston, 1971). These researchers and others (Marston *et al.*, 1961; Farningham and Whyte, 1993) have found suppressed appetite and food intake as a result and attributed this to the high concentrations of unprocessed propionate. However, according to Kennedy *et al.* (1991b) the inhibition of propionate to succinate may not be the primary metabolic defect since they found it to be a late manifestation of the deficiency.

The possibility that alternative pathways for propionate metabolism in liver may occur in vitamin B₁₂ deficient sheep has been raised by Peters *et al.* (1983). The more recent work of Kennedy *et al.* (1991a; 1996) has suggested that severe Co deficiency may change the balance between propionate- and succinate-producing bacteria in the rumen, favouring the latter. Dramatic increases in succinate production have been observed on diets containing less than 20 µg Co/kg DM (Kennedy *et al.*, 1996). Since these researchers also demonstrated succinate absorption; this would short-circuit the methylmalonic acid - vitamin B₁₂ dependent pathways of gluconeogenesis from propionate and provide direct entry of succinate to the Krebs cycle. However, lowered MMA in these cases, as could be expected, has not been observed. It could therefore be possible that the inhibition of the me-cbl dependent pathway determines the severity of vitamin B₁₂ deficiency (Kennedy *et al.*, 1996).

2.8.3 Accumulation of odd-numbered, branched-chain fatty acids

Odd-numbered, branched-chain fatty acids accumulate in the tissues of vitamin B₁₂ deficient sheep (Kennedy *et al.*, 1994b). Normally, fatty acids up to C₁₈ are produced by condensation of malonyl-CoA units to acetyl-CoA (both containing two carbons). Accumulation of the 3-carbon substrates propionyl-CoA and MM-CoA - as occurs in vitamin B₁₂ deficiency - leads to their substitution for acetyl-CoA and malonyl-CoA, respectively, resulting in the assembly of odd-numbered, straight-chain and branched-chain fatty acids (ONFA and BCFA). This gives the fat of ruminants a soft, oily consistency.

MMA has often been observed to be elevated in moderate vitamin B₁₂ deficiency, but to fall back to near-normal values when the deficiency has progressed to a more advanced state. This second phase may well reflect use of MMA for fatty acid synthesis, or it may be due to reduced food intake. Their excessive accumulation could be responsible for the neurological damage that is seen in severe human vitamin B₁₂ deficiency (Kennedy *et al.*, 1994b).

However, accumulation of ONFA and BCFA is unlikely to be unique to vitamin B₁₂ deficiency. It is regularly observed in animals on diets rich in readily fermentable carbohydrate. Duncan *et al.* (1981) suggested that this could be due to an overload of the propionate pathway with the same metabolic consequences as a vitamin B₁₂ deficiency.

2.9 Supplementation

There are three possible ways to correct a Co/vitamin B₁₂ deficiency in ruminants: top-dressing the pastures with a Co-salt, oral administration of Co to the animal, or injections of vitamin B₁₂.

2.9.1 Topdressing pastures

Topdressing pastures with Co has become the most economical and widespread means of alleviating any vitamin B₁₂ deficiencies in the grazing animal in New Zealand. Depending on the degree of deficiency and the soil type, 100 - 150 g of Co sulphate per acre is generally enough for one to two years, and 500 g of Co per acre has lasted up to six years. Co salts or ores can be mixed with fertilizers and applied together.

2.9.2 Oral supplementation

2.9.2.1 Salt licks

If, however, the soil is extremely low in Co, or if it contains high levels of calcium carbonate or manganese (which fixes Co, thus making it unavailable to the

plant), topdressing may not be the most efficient and economical way of supplying Co. In that case, feeding a Co salt as part of a mineral supplement or giving it as a drench to the animal is more effective. A guideline for sheep would be 1 mg Co per head per day to ensure adequate supply (Lee and Marston, 1969). Weekly doses of 7 mg Co (as sulphate) were effective in increasing and maintaining serum and liver vitamin B₁₂ concentrations and growth rates (Andrews *et al.*, 1966). However, in order to maintain adequate vitamin B₁₂ concentrations on pastures deficient in Co this form of supplementation needs to be administered frequently, i.e. on a weekly basis (Lee and Marston, 1969; Andrews *et al.*, 1966; Grace, 1998). The reason for this is that Co is not readily stored in the tissues, and what is stored is not released again into the digestive tract for rumen micro-organisms to produce vitamin B₁₂.

2.9.2.2 Cobalt pellets

In order to ensure an adequate supply of vitamin B₁₂ to the animal on a daily basis, Co has to be continuously available to the rumen micro-organisms. Consequently, a slow-release form of Co (Co₃O₄) was introduced in form of an intraruminal Co pellet or bullet. While the claim is that it ensures adequacy of tissue vitamin B₁₂ for up to one year (Judson *et al.*, 1992 and 1995), this effectiveness has been questioned. Co pellets are unsuitable for PR lambs (the main target group for supplementation) because of their size. In addition, two problems were encountered: some animals regurgitated the pellet, or the pellets became coated with calcium phosphate, thus reducing the release of Co into the rumen (Underwood, 1971; Judson *et al.*, 1995). Various methods of 'packaging' the Co pellet have been tried to alleviate these problems, including the use of a large grub screw as a grinder with the pellet (Judson *et al.*, 1995), or using 'tracerglass', a phosphate-based soluble-glass (Telfer *et al.*, 1984; Zervas *et al.*, 1988; Ellis *et al.*, 1987; Judson *et al.*, 1988).

2.9.3 Vitamin B₁₂ injections

2.9.3.1 *Short-acting injections*

Oral administration of vitamin B₁₂ has proved ineffective for ruminants since the vitamin is degraded by rumen micro-organisms. Therefore another way of ensuring adequate vitamin B₁₂ status in an animal is to administer the vitamin directly by intramuscular or subcutaneous injection. The amounts needed to replete deficient animals and the length of time the vitamin will last vary, depending on the type of animal, its degree of deficiency, the Co status of the pasture and the type of vitamin B₁₂ used. It is recommended to use OH-cbl in preference to CN-cbl because of its higher binding capacity to serum proteins (Shearman *et al.*, 1965.; Hedstrand, 1969). It has been found to give more lasting results than CN-cbl (Body *et al.*, 1968, in Judson *et al.*, 1989). Most studies have been done on lambs where 1 to 2 mg vitamin B₁₂ have been injected and shown to last between two weeks and two months (Sargison *et al.*, 1997). Hannam *et al.* (1980) found that 1 mg OH-cbl lasted lambs 14 weeks and weaner sheep 40 weeks. However, Grace *et al.* (1998) detected no significant difference in blood vitamin B₁₂ of treated (2 mg OH-cbl) and untreated lambs after 24 days, and after four weeks in their liver concentrations.

Smaller amounts at more frequent intervals are probably more efficacious than larger amounts less often. Weaned lambs and mature sheep require less frequent treatment. The advantage of the injections is that the farmer can be sure that the vitamin reaches the tissues, but this mode of administration is costly and labour intensive, and therefore usually reserved for individual animals only.

2.9.3.2 *Long-acting injections*

To combine the advantages of the injections with those of the long-acting Co bullets and to overcome the respective drawbacks, depot vitamin B₁₂ injections have been developed (Judson *et al.*, 1989 and 2000; Grace, 1998 and 1999c; Grace and Lewis, 1999; Grace and West, 2000). Two mg CN-cbl as a tannin complex, suspended in a sesame-oil aluminium-monostearate gel, has been used subcutaneously and shown to be effective in elevating plasma vitamin B₁₂

concentrations between 10 and 15 weeks after treatment (Judson *et al.*, 1989). Grace (1998 and 1999c) used 6 mg vitamin B₁₂ in a 95:5 lactide:glycolide polymer containing 15.6 % w:w vitamin B₁₂ in 1 ml of peanut oil. This significantly increased and maintained serum vitamin B₁₂ concentrations in lambs for nine months, which amounted to a daily release of the vitamin of 24 µg. Since the requirement of lambs has been calculated as 11 µg/d (Marston, 1970), the amount provided by the depot vitamin B₁₂ was not only sufficient in meeting the daily needs of the animals, but also to increase their liver concentrations. Judson *et al.* (2000) used a biodegradable pellet containing 2 mg CN-cbl subcutaneously and found serum and liver vitamin B₁₂ concentrations significantly greater than in the untreated group for at least 189 and 230 days, respectively.

Whatever the means of supplementing animals, once liver levels of vitamin B₁₂ exceed 220 nmol/kg fresh tissue, or pasture Co levels exceed 0.11 mg Co/kg DM for sheep and 0.07 mg Co/kg DM for cattle, it is unlikely that the animal will respond to further treatment. Any excess vitamin B₁₂ then is either stored in the tissues, predominantly the liver, or excreted in the faeces (Jones and Anthony, 1970).

1.9.4 Human supplementation

For monogastrics, vitamin B₁₂ can be given orally if it is lacking in the diet. Humans who are deficient in the vitamin (e.g. vegans) need to take supplements to meet their demands. However, if the deficiency is caused by failure to absorb vitamin B₁₂, e.g. due to lack of IF, megadoses of the vitamin need to be administered orally, sublingually (Delpre *et al.*, 1999) or as an intra-nasal gel (FDA, 1997).

However, injections are usually given when there is an absorption problem, generally due to antibodies against IF (as in PA) or gastric surgery. Since many factors can influence the absorption of the vitamin, injections are a safer way than oral supplementation to ensure adequate vitamin B₁₂ status.

Part 3: Assay and identification methods

2.10 Assay methods for vitamin B₁₂

2.10.1 Microbiological (bioautographic) method

When it became known that vitamin B₁₂ was produced by micro-organisms, it was a logical step to use this ability to assay the vitamin as a means of estimating its concentration. However, proteins in the sample to be analysed (liver, serum, milk) need to be inactivated first so the micro-organisms have access to the vitamin (Carlos, 1985). This has been achieved by boiling or steaming (Millar and Penrose, 1980). As OH-cbl (the form vitamin B₁₂ is present in after sample exposure to light) is unstable in heat cyanide ions have been added to the sample to convert the cbl to the more stable CN-cbl form. Following cyanide extraction, the sample needs to be incubated with the micro-organisms on a suitable growth medium. Optimum incubation periods have been estimated to be two days for *Lactobacillus leichmannii* and a minimum of seven days for *Ochromonas malhamensis* (Carlos, 1985).

Hine and Dawbarn (1954) tried various organisms, like *Escherichia coli*, *L. leichmannii*, and *O. malhamensis*. The results varied greatly, with vitamin B₁₂ activity measured by *E. coli* being ten to twenty times higher than those measured by *O. malhamensis*. The latter organism, however, produced an assay that was highly specific for vitamin B₁₂, whereas *E. coli* based assays also measure a number of vitamin B₁₂ analogues (Table 2.3).

Table 2.3: Specificity of micro-organisms used for microbiological assays of vitamin B₁₂ (from Mills, 1980)

Organism	Cobamides with benzimidazoles	Cobamides with other purine bases	Cobinamides
<i>Ochromonas malhamensis</i>	+	-	-
<i>Lactobacillus leichmannii</i>	+	+	-
<i>Euglena gracilis</i>	+	+	-
<i>Escherichia coli</i> mutants	+	+	+

Milk has also been assayed by microbiological methods. Gregory (1954) used *L. leichmannii* for assaying vitamin B₁₂ in milk. Comparisons with an *O. malhamensis* assay (Gregory, 1954) showed no difference between the results for the

two micro-organisms. Since the latter is specific for 'true' vitamin B₁₂, whereas *L. leichmannii* grows on analogues as well (Nexø and Olesen, 1982), milk does not seem to contain any vitamin B₁₂ analogues.

When Dawbarn and Hine (1955) measured vitamin B₁₂ activity in urine by the microbiological method they could achieve satisfactory results only when the sample vitamin B₁₂ concentration was high enough to allow for considerable dilution (~ 100-fold). *L. leichmannii* gave the best results, which could imply that cbl was broken down or altered in some way that 'true' vitamin B₁₂ activity was lost.

However, these methods are time-consuming and only small numbers of samples can be analysed at a time. Low concentrations of cbl are difficult to detect because of the low sensitivity of the assay (Lee and Griffiths, 1985). Besides, antibiotics interfere with the growth organisms, therefore no accurate results can be obtained after antibiotic treatment (Lau *et al.*, 1965). These problems could be overcome by radioisotope assays which became available in the 70's and 80's.

2.10.2 Radioisotope Dilution Assay

The Radio Isotope Dilution Assay (RIDA) for vitamin B₁₂ in serum or liver, based on the method developed by Lau *et al.* (1965), is the most commonly used assay nowadays. Figure 2.12 gives an overview over the steps involved in the process. The assay involves the conversion of the coenzymes and other forms of vitamin B₁₂ to cyanocobalamin. The unknown amount of tissue-bound vitamin B₁₂ is liberated by heat and acid before it is added to a known amount of radioactive vitamin B₁₂ [⁵⁷Co]CN-cbl. ('No boil' kits are also available but they do not denature anti-IF antibodies completely and require a long assay time.) A binder (IF or HC) is added which only binds approximately 80 % of the quantity of radioactive vitamin B₁₂. Radioactive and non-radioactive vitamin B₁₂ then compete for the binder. The B₁₂-binder complex is then separated from the free vitamin B₁₂ by adding albumin-coated charcoal which adsorbs the unbound fraction. Bound vitamin B₁₂ stays in the supernatant. Standards of CN-cbl are treated in the same manner. The radioactivity of the sample is compared to that of the standards. The amount of unknown vitamin B₁₂ that was in the sample can then be calculated from the standard curve.

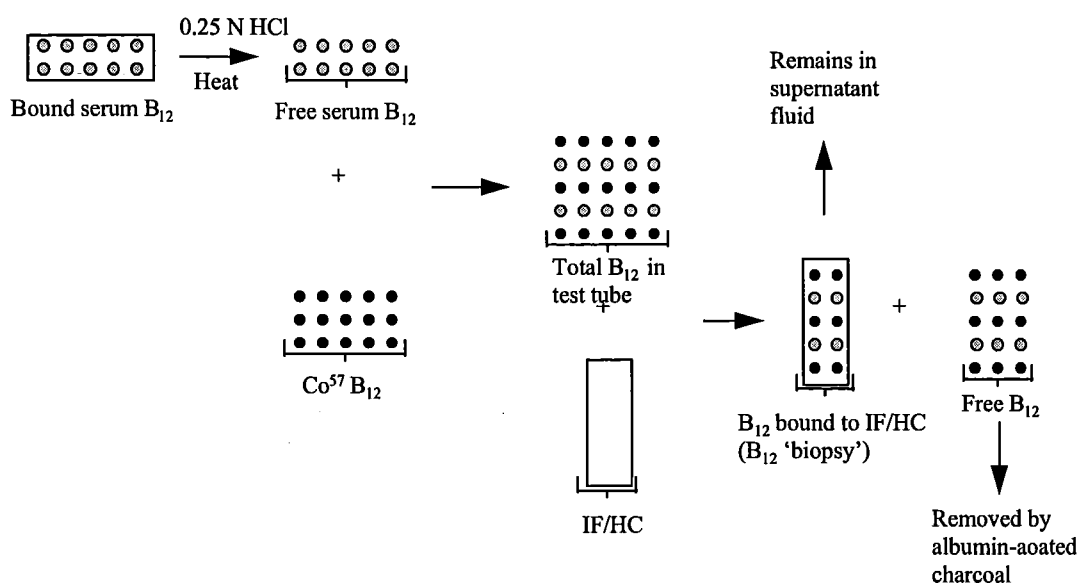


Figure 2.12: Vitamin B₁₂ assay using radioisotope dilution and albumin-coated charcoal (from Das *et al.*, 1991; Lau *et al.*, 1965)

Depending on the binder used, only 'true' vitamin B₁₂ or the vitamin plus its analogues are measured. When using HC both cbl and analogues are measured. This can give erroneously high results for vitamin B₁₂ and deficiency can be missed. In order to obtain information on the 'true' vitamin B₁₂ that is in the serum sample pure IF needs to be used as binder. However, commercially obtained IF is frequently contaminated with HC and therefore the assay gives falsely high results, thus masking deficiency (Lee and Griffiths, 1985; Kennedy *et al.*, 1992b). Kolhouse *et al.* (1978) found that up to 20 % of patients with cbl deficiency are not detected with current assays.

To overcome this problem, cobinamide has been included to saturate the HC, leaving only IF to bind with the vitamin B₁₂ in the sample to be analysed. But this process is not easily controllable. If the assay employs both haptocorrin and IF, the amount of total corrinoids ('true' vitamin B₁₂ and analogues) can be determined with haptocorrin and 'true' vitamin B₁₂ with IF. The difference of the two assays will reveal the amount of analogues present in the sample (Lau *et al.*, 1965; Das *et al.*, 1991). However, to calculate the amount of analogues by subtracting results obtained with IF from the ones obtained with HC can be misleading since the nature of the

analogues and hence their affinity for the respective binders is unknown (Gimsing and Beck, 1989).

Nowadays, either pure IF (Lau *et al.*, 1965) or chicken serum (Green *et al.*, 1974) are used in commercial assays. However, results between laboratories vary to such an extent that comparisons are difficult and serious errors in diagnosis are likely (Lee and Griffiths, 1985).

2.10.3 Radioimmunoassay

To overcome the problems associated with impure IF, Kennedy *et al.* (1990a and 1992b) developed two radioimmunoassays (RIA). The first one was specific for the upper ligand (β -face) in the vitamin B₁₂ molecule (Kennedy *et al.*, 1990a). Antisera against vitamin B₁₂ were raised in rabbits using a conjugate between 5'-O-succinyl CN-cbl and chicken serum albumin. The process then followed the procedure outlined in 2.10.2.

However, it is the α -face in vitamin B₁₂ that determines its biological activity in mammals. Therefore this assay would measure some of the analogues as well. To solve this problem, Kennedy *et al.* (1992b) developed a RIA that was specific for the lower ligand (DMB or α -face). Co deficient sheep were used to produce antisera by immunising them with a conjugate between Co- β carboxypropyl cbl and keyhole limpet hemocyanin. The antisera reacted with the β -face of the vitamin B₁₂ molecule but not with the α -face.

2.10.4 Chemiluminescence

In the last few years, the limitations (short reagent life, licensing requirements) and dangers (safety issues regarding the handling and disposal of radioactive materials) of using radioactive tracers, even in small amounts, have lead to the development of safer, non-isotopic alternatives. Lee and Griffiths (1985) predicted as early as 1985 the development of a fluorescent (i.e. non-radioactive) method for the determination of serum vitamin B₁₂. Zhou *et al.* (1991) developed a method where Co is used as a catalyst for the oxidation of luminol in alkaline

hydrogen peroxide solution. When the sample containing an unknown amount of vitamin B₁₂ is then acidified, Co²⁺ is released and can be detected photospectrometrically. Wentworth *et al.* (1994) used an acridium ester with magnetic particle separation to determine vitamin B₁₂ in serum. Increasing use of the luminol and acridinium ester methods are made of in laboratories today.

However, since the common vitamin B₁₂ assays measure 'true' vitamin B₁₂ as well as some or all of the analogues present, irrespective of the requirement or utilisation of different metabolic pathways in carbohydrate and protein metabolism, they lack specificity. Up to 50 % false positive (Savage *et al.*, 1994) and as much as 40 % false negative (Norman and Morrison, 1993) results have been reported. Besides, since it takes some time (several years for humans), after intake or absorption of cobalamin has stopped, to develop deficiency symptoms, early and reliable diagnostic tools are essential (Lindenbaum *et al.*, 1990).

Further, no information can be gained on the amount of the specific coenzymes or biologically active forms present in the body. The light sensitivity of the Co-α substituted forms is such that they convert within minutes to OH-cbl, or CN-cbl in the presence of cyanide ions (Nexø and Olesen, 1982; Toohey and Barker, 1961; unpublished data from our laboratory). However, the different forms of corrinoids can be determined by chromatography under the exclusion of light.

2.11 Separation and identification of coenzymes and analogues

2.12.1 Separation

Originally, Thin Layer Chromatography (TLC) was used to separate and identify cobalamins and their analogues. In the early 1980's, High Performance Capillary Electrophoresis (HPCE) and High Performance Liquid Chromatography (HPLC) became available and drastically changed research into vitamin B₁₂.

Scientists in the USA and later in Europe (Binder *et al.*, 1982; Jacobsen *et al.*, 1982; Gimsing and Beck, 1986) were quick to pick up the new methods to isolate

and try to identify the various analogues that had so far eluded them. With HPLC, a method had become available that was able not only to differentiate between the various upper axial ligands in 'true' vitamin B₁₂, but also to monitor their conversion from one coenzyme form to another or their distribution in the body (Binder *et al.*, 1982).

In all of these methods, corrinoids were prepared in very much the same way: the tissue to be analysed was first homogenised if it was not a liquid already. The supernatant or liquid was then incubated with cadmium acetate. Cbl was liberated from binding proteins by hot ethanol, and extraneous inorganic salts were removed by Amberlite XAD-2 chromatography. Identification of each fraction of cobalamin was achieved in TLC by comparison of their R_f values with those of the standards (Gimsing *et al.*, 1983), and in HPLC by comparing their retention times to that of known standards. All steps were carried out in dim red (photographic) safe light.

In HPCE, all the corrinoids were converted to the CN-form first by exposure to light and addition of KCN. As in HPLC, corrinoids were then identified by their retention times. Lambert *et al.* (1992) found that the conversion made the method more efficient and gave better discrimination between cbl and analogues. It was easier to perform than HPLC, provided sufficient material was available. However, when very small amounts of corrinoids need to be detected, as in biological material, HPLC is the method of choice. HPLC also allows the collection of the different corrinoid-containing fractions so that they can be further analysed and quantified by other methods (e.g. RIDA).

Discussion arose as to whether HPLC is best performed under isocratic or gradient conditions (Binder *et al.*, 1982; Jacobsen *et al.*, 1982 and 1986). Both groups of scientists agreed that the gradient method is more advantageous. Complex mixtures of corrinoids are better resolved, it is less dependent on small pH changes, and retention times for some of the analogues are shorter. The gradient method is also better suited for naturally occurring cbl in biological systems (Jacobsen *et al.*, 1982). However, when studying coenzyme and coenzyme analogue decomposition reactions, the isocratic system is preferred (Jacobsen *et al.*, 1982).

2.12.2 Recovery

Gimsing and Beck (1986) stated what they expected of an ideal assay method: all cobalamins should be recovered fully and equally, they should not be altered in the assay procedure, all interfering substances should be removed, and the different cobalamins should be well separated. HPLC comes close to satisfying these conditions. However, only mixtures containing known corrinoids can be separated that way. Unknown analogues could have the same retention time as known cobalamins, thus be hidden underneath that peak and hence be missed. Therefore, if a sample contains unknown corrinoids, at least two different analytical methods should be used (Gimsing and Beck, 1986).

There are several assumptions, though, that have to be made in order to obtain correct results, regardless of the method used, which are not always fulfilled (Gimsing *et al.*, 1983). Firstly, all corrinoids are recovered equally after extraction. Secondly, each of the cobalamins responds uniquely to the analytical method used. Thirdly, the same cobalamins are detected with different methods (eg. HPLC and TLC).

OH-cbl does not fulfill these criteria since it binds nonspecifically to histidine residues in the binding proteins. This can be inhibited by preincubation of the sample with excess cadmium ions (as cadmium acetate) or a thiol-blocking agent (such as *N*-ethyl-maleimide). Furthermore, OH-cbl is not recovered totally when desalting with Amberlite XAD-2 (Gimsing *et al.*, 1983; Gimsing and Beck, 1986). Therefore, adjustments have to be made to allow for losses during the extraction procedure.

Despite the drawbacks, HPLC in combination with one of the assay methods described in 2.10 would be the method of choice when analysing the different corrinoid components of a biological sample.

particularly in lambs and in cattle. Liver values (obtained through biopsy) reflect the vitamin B₁₂ ‘reserves’ (Millar *et al.*, 1984) and are considered to give a more accurate reading of the amount of vitamin B₁₂ available to the animal (Table 2.6). Yet biopsies are more expensive than serum samples and pose a greater risk to the animal.

From trials with ruminant lambs Marston (1970) estimated the minimum concentration of liver to be ~ 75 nmol/kg fresh tissue before symptoms of deficiency developed. Andrews *et al.* (1960), however, estimated the ‘critical level’ for liver vitamin B₁₂ concentrations to be twice this amount to assure absence of deficiency symptoms.

Table 2.6: Liver vitamin B₁₂ reference ranges of sheep (nmol/kg fresh tissue) (obtained by RIDA)

Condition of animal	Liver vitamin B ₁₂ concentrations					
	Andrews <i>et al.</i> (1960)	Judson <i>et al.</i> (1987)	Grace and Clark (1991)	Clark (1998)	Underwood and Suttle (1999)	Labnet Invermay
Deficiency	< 75	< 100	< 110	< 100	< 280	< 280
Marginal	75 - 140	100 - 200	110 - 220	100 - 200	280 - 340	280 - 375
Normal	> 140	> 200	> 220	> 200	> 340	> 375

Clark (1998) assumed that two to five months old lambs may require higher liver concentrations and gave a reference range of 282 to 375 nmol/kg fresh tissue. He was unsure, however, as to what would be a corresponding range for lambs under two months and above five months of age, and pregnant and lactating ewes. For cattle a reference range of < 75 nmol/kg fresh tissue as being deficient and > 220 nmol/kg fresh weight for adequate vitamin B₁₂ status has been suggested by Grace and Clark (1991).

Although, pragmatically, the above tests are used on the basis of cheapness and utility (Clark *et al.*, 1985), their limitations have lead to the search for assays based on the activities of the two coenzymes or the transport proteins. The following approaches have been suggested:

2.12.2 Enzymes

Increase in holo-mutase (mutase with ado-cbl bound to it) activity following oral Co or intramuscular OH-cbl was first described by Peters and Elliot (1984). Kennedy *et al.* (1990b) found that the activities of both holo-enzymes - mutase and synthase - fell during Co deficiency. In the case of the former, half-lives of the enzymes were measured and varied between 73 and 125 days in liver and lymphocyte, respectively. Total enzyme concentrations (holo and apo enzyme) behaved very differently - total mutase showing no change, but total synthase declining as a result of Co deficiency (Kennedy *et al.*, 1990b). Lowered PC values have also been found in vitamin B₁₂ deficient animals (Kennedy *et al.*, 1992a), and Gawthorne and Smith (1974) showed liver SAM concentrations being reduced by 50 % in vitamin B₁₂ deficient sheep. However, homocysteine and SAH concentrations remained unaffected.

As Kennedy *et al.* (1990b) discussed, these findings are difficult to interpret without data on enzyme turnover, since a common response to an 'enzyme under stress' is to upregulate either enzyme activity or enzyme concentration. We do not yet understand how vitamin B₁₂ participates in enzyme regulation sufficiently to allow their use in diagnosis. The assays are difficult and expensive.

2.12.3 Substrate clearance (Propionate loading)

The effects of propionate loading on uptake by the liver or on plasma clearance has been measured in vitamin B₁₂ depleted sheep. Peters *et al.* (1983) concluded that the ability of the liver to extract propionate is not affected by differences in liver vitamin B₁₂ in the range of 250 to 1640 nmol/kg fresh tissue (based on a microbiological assay). He observed greater capacity to remove propionate in depleted animals after propionate loading by intramesenteric infusion. They attributed this to the possible development of alternative pathways. Propionate half-life after propionate loading was significantly longer for vitamin B₁₂ deficient sheep (Kennedy *et al.*, 1991b), but only as a late manifestation of vitamin B₁₂ deficiency. Propionate loading therefore is unlikely to be a useful indicator of vitamin B₁₂ deficiency.

2.12.4 Accumulation of metabolic intermediates

2.12.4.1 Formiminoglutamic acid

The findings on the value of FIGLU excretion in urine are, to say the least, mixed. Russel *et al.*'s (1975) data suggest that the presence of FIGLU in urine of more than 20 % of animals may be a useful indicator of deficiency in the flock. Stebbings and Lewis (1986) could not find elevated concentrations of FIGLU in urine until vitamin B₁₂ deficient lambs developed weight loss and ill-thrift, some months after depletion of vitamin B₁₂. FIGLU and anorexia showed a significant correlation, but the correlation became apparent only very late in the development of Co deficiency. Therefore, these workers concluded that it would be of limited diagnostic value.

In contrast, Marsh and Turner (1988) claimed FIGLU to be a rapid, sensitive indicator in the early stages of vitamin B₁₂ deficiency. However, they did not measure vitamin B₁₂ status and were unable to show any relationship amongst properties between herbage Co and FIGLU concentration. Co supplementation increased the growth rate of lambs from 40 to 57 g/d ($p < 0.05$) by supplementation with Co on one property on which more than 70 % of animals had urinary FIGLU levels in excess of 0.05 mM.

Gawthorne (1968) found a 30 times greater concentration of FIGLU in severely vitamin B₁₂ deficient sheep than in pair-fed controls. Again, no attempt was made to measure vitamin B₁₂ status for comparison. He concluded that urinary FIGLU was more sensitive to early stages of deficiencies than urinary MMA, though it could be that the method used to detect MMA in these early studies lacked sensitivity.

Miller *et al.* (1988) described a five- to twelve-fold increase for urinary MMA and a 30+ fold increase in FIGLU when an animal was vitamin B₁₂ deficient. Although there is some controversy in the literature as to which of these two indicators is the more sensitive, O'Harte *et al.* (1989a), Price (1990a and 1990b) and Ulvund (1990a) are more inclined towards the MMA assay.

2.12.4.2 *Methylmalonic acid*

MMA has been determined by chromatography: colorimetric at first, then TLC, and later by Gas Chromatography with Flame Ionisation Detection (GC-FID), GC-Mass Spectrometry (MS) and HPLC. The concentration of MMA in urine is much greater than in serum, therefore the earlier analyses focused on measuring the former. With the advent of GC, in particular in combination with MS, the analytical methods for the determination of MMA in biological tissues and fluids have increased in accuracy and sensitivity.

The first quantification of MMA was carried out using colorimetric techniques (Giorgio and Plaut, 1965). Auray-Blais *et al.* (1979) described a TLC method for urinary MMA, whereas Millar and Lorentz (1974) had already developed a urinary GC method for MMA in 1974. Norman *et al.* (1982) and Marcell *et al.* (1985) outlined a GC-MS method (operated in the Selected Ion Monitoring - SIM - mode) for assaying MMA in human serum and urine. McMurray *et al.* (1986) developed a GC-FID method for the detection of MMA in sheep plasma. McGhie (1991) and Young *et al.* (1995) described methods for use with cattle serum, based on GC and GC-MS, respectively. For the GC detection of MMA, MMA has been derivatised into various esters (Marcell *et al.*, 1985; Montgomery and Mamer, 1988; Norman *et al.*, 1982; Straczek *et al.*, 1993; Millar and Lorentz, 1974; Stabler *et al.*, 1986; McMurray *et al.*, 1986; McGhie, 1991). Rasmussen (1989) developed a Stable Isotope Dilution method with Solid-Phase Extraction of the samples. Babidge and Babidge (1994) and Toyoshima *et al.* (1994) used HPLC to measure MMA. Schneede and Ueland (1993) created a liquid chromatography assay with fluorescence detection which allowed for faster, less laborious analysis of serum and urine samples.

O'Harte *et al.* (1989a, 1989b) suggested that plasma MMA concentrations > 5 $\mu\text{mol/l}$ were indicative of early Co deficiency in sheep. Inverse relationships between plasma MMA and vitamin B₁₂ were observed. However, whereas plasma vitamin B₁₂ concentrations fell rapidly after one week of feeding the deficient diet, MMA did not increase until six weeks of deficiency, probably reflecting utilisation of vitamin B₁₂ from the liver in the early stages. The major change in weight gain, however, occurred after week ten, when MMA levels exceeded 15 $\mu\text{mol/l}$.

McMurray *et al.* (1985) suggested upper and lower threshold levels of 5 $\mu\text{mol/l}$ and 220 pmol/l for plasma MMA and vitamin B₁₂, respectively (Table 2.7). Millar and Lorentz (1979) considered urinary MMA in excess of 250 $\mu\text{mol/l}$ to indicate a vitamin B₁₂ deficiency state, whereas Rice *et al.* (1987) suggested a threshold of 120 $\mu\text{mol/l}$. This, however, might need to be referenced against an internal marker such as creatinine to correct for variation in urine volume (Rice *et al.*, 1987).

Table 2.7: Plasma MMA in relation to vitamin B₁₂ status (from McMurray *et al.*, 1985)

Vitamin B ₁₂ pmol/l	MMA $\mu\text{mol/l}$		Comment	Deficiency Status
> 220	< 5	high B ₁₂	normal MMA	normal
< 220	< 5	low B ₁₂	normal MMA	normal
< 220	< 15	low B ₁₂	moderate MMA	subclinical
< 220	> 15	low B ₁₂	high MMA	clinical

However, O'Harte *et al.* (1989a) recognised, on the basis of this and previous studies (Rice *et al.*, 1987), that the threshold MMA concentration may vary with diet type and suggested approximately 10 $\mu\text{mol/l}$ for barley- compared to 5 $\mu\text{mol/l}$ for grass-fed lambs. Indeed, it seems reasonable to speculate that the type of rumen fermentation - propionate/acetate balance - may affect demand for vitamin B₁₂, given its importance in propionate metabolism. Other workers have produced comparable data. For example, Ulvund (1990a) defined the critical plasma MMA range from 5 to 15 $\mu\text{mol/l}$. Table 2.8 summarises these findings.

In both humans and animals, MMA is now increasingly used in research and routine diagnosis of vitamin B₁₂ deficiency. In human studies, elevated levels of MMA have been claimed to be the first sign of vitamin B₁₂ deficiency (Norman *et al.*, 1982) and specific for it (Ho *et al.*, 1987). Reference ranges for normal serum MMA in humans stretch from 53 - 160 nmol/l as the lower limit to 271 - 640 nmol/l as the upper limit (Stabler *et al.*, 1986; Rasmussen, 1989; Savage *et al.*, 1994). In normal human urine MMA ranges from 0.85 to 43 $\mu\text{mol/l}$ (Marcell *et al.*, 1985). Norman (1996) found MMA to be the most accurate test for vitamin B₁₂ deficiency with a sensitivity of 100 % and a specificity of 99 %. Given normal renal function, plasma and urinary MMA (adjusted to creatinine or collected over 24 h) correlate well for humans (Rasmussen *et al.*, 1989).

Table 2.8: Vitamin B₁₂ and metabolite assays in sheep from various trials reported in the literature

Source	Critical concentrations			Diet	Length of Trial	Deficiency Symptoms	Time of Onset of Deficiency	Notes
	Vit. B ₁₂	FIGLU	MMA*					
Stebbing & Lewis, 1986		1.6 µmol/l		semi-purified	3 years	anorexia, ill-thrift	FIGLU only occurred after 7 months in 3rd generation lambs born to deficient ewes	Rejected use of FIGLU
Marsh & Turner, 1988		0.05 mol/l		Co-def. soils	spot samples	liveweight response	very late	FIGLU reliable indicator
Gawthorne, 1968		30x controls	5-12x controls (u)	Co def. diet	8 months	reduced feed intake, body weight, haemoglobin	after 25 weeks	FIGLU reliable indicator
Price, 1990a	<185 pmol/l		5 µmol/l (p)	Low Co hay	32 wks	low B ₁₂ and high MMA	8 weeks for B ₁₂ , 6-16 weeks for MMA	MMA more sensitive than B ₁₂
Ulvund, 1990a	<150 pmol/l		5-15 µmol/l (p)	OWLD-pasture	6 years	reduced growth	symptoms appeared after 6-12 weeks	Critical MMA between 5-15 µmol/l
Millar & Lorentz, 1979	<370 pmol/l		254 µmol/l	Co def. pasture	4 months	reduced weight gain		Urine MMA >30 µg/ml in 10 animals indicates deficiency
O'Harte <i>et al.</i> , 1989a	<220 pmol/l		5 µmol/l (p)	Co def. ration	14 weeks	reduced weight gain	5 weeks for B ₁₂ , 6 weeks for MMA	
Rice <i>et al.</i> , 1987	<185 pmol/l		120 µmol/l (u), 5 µmol/l (p)	Co def. pasture	spot samples	reduced weight gain, poorer body condition		Important to express urine MMA/creatinine

* MMA measured either in plasma (p) or urine (u)

2.12.4.3 Homocysteine

Hcy has created much interest in the last decade as an independent risk factor for cardiovascular disease in humans (Ueland *et al.*, 1993). It is elevated in vitamin B₁₂ as well as in folate deficiency, therefore other tests need to be considered when hcy is to be used as a screening for cbl status. Savage *et al.* (1994) suggested the combined use of MMA and homocysteine: if both are normal, vitamin B₁₂ deficiency is ruled out with virtual certainty. Although they found MMA to be the better indicator, there are occasions when MMA appears normal despite deficiency, such as in renal dysfunction or disturbed bowel flora (e.g. after antibiotics). When Stabler *et al.* (1996) compared MMA and hcy in 60 normal patients, 60 with cbl deficiency and 60 with folate deficiency, all cbl deficient and all folate deficient patients had raised hcy ($> 15 \mu\text{mol/l}$), whereas only the cbl deficient patients had raised MMA. The two tests in conjunction were optimal in distinguishing between vitamin B₁₂ and folate deficiency (Stabler *et al.*, 1996; Allen *et al.*, 1990).

In ruminants, raised hcy is entirely due to cbl deficiency since their diet is rich in folate. Kennedy *et al.* (1992a) measured hcy in vitamin B₁₂ deficient sheep. Although their animals had become vitamin B₁₂ deficient by week 6, hcy did not rise until week 16. They found that serum values $> 20 \mu\text{mol/l}$ were indicative of deficiency. However, Kennedy *et al.* (1994a) observed hcy concentrations of up to $38 \mu\text{mol/l}$ in vitamin B₁₂ replete sheep. Babidge (1993) found significant differences in hcy between Co deficient and sufficient weaned lambs after 29 weeks of low Co intake, although MMA was already significantly elevated at week 13. Yet hcy in the untreated group was still below $20 \mu\text{mol/l}$ and MMA below $5 \mu\text{mol/l}$ at the end of the trial, and liveweight gain (LWG) had not differed between the groups. This would indicate that the animals were not truly deficient or were on the margin between deficiency and sufficiency. Vellema *et al.* (1999) found a significant difference in hcy between Co deficient and Co supplemented lambs. Yet hcy did not exceed $\sim 15 \mu\text{mol/l}$ although MMA had reached values of $\sim 80 \mu\text{mol/l}$ in Co deficient animals. In a recent study Stangl *et al.* (2000) employed both MMA and hcy as markers of deficiency in cattle and found that when both are normal Co deficiency is ruled out

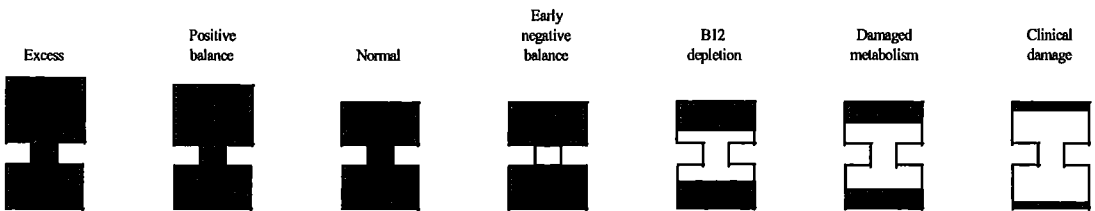
with virtual certainty. However, hcy has not yet become a routine diagnostic tool in ruminants.

The most common assays for hcy in mammalian tissue employs HPLC (Young *et al.*, 1994; Ueland *et al.*, 1993; Jacobsen *et al.*, 1989 and 1994; Araki and Sako, 1987; Vester and Rasmussen, 1991) which depends on derivatization so that hcy can be detected by fluorescence. TLC, GC, and ion exchange (amino acid analyser) have also been used (Ueland *et al.*, 1993) to determine hcy levels.

2.12.5 Transport proteins

Adaptive changes in transport proteins and their degree of saturation is being used in humans to assess vitamin B₁₂ status (Das *et al.*, 1991; Herbert *et al.*, 1990; Benhayoun *et al.*, 1993; van Kapel *et al.*, 1988). Since holo-TC II represents the metabolically active form in which tissues take up the vitamin, Herbert *et al.* (1990) investigated this and found that the earliest indication of a negative vitamin B₁₂ balance in people with PA and AIDS was low serum holo-TC II. It seems that vitamin B₁₂ appears to be depleted from TC II first in vitamin B₁₂ deficiency, therefore reduction in the degree of saturation of TC II could be the earliest sign of an impending deficiency (Figure 2.12) (Herzlich and Herbert, 1988; Herbert *et al.*, 1990). If vitamin B₁₂ deficiency is suspected but other markers, such as serum and liver vitamin B₁₂, MMA and homocysteine, are still within the normal range assaying holo-TC II becomes important (Kapel *et al.*, 1988).

Transport proteins have also been measured in sheep by Schultz and Judson (1985) and Babidge (1993). Babidge (1993) made similar observations to Herbert *et al.* (1990) in regard to decreased levels of holo-TC II in Co deficient sheep.



The boxed-in area denotes the amount of total TC II available for binding. The shaded area represents the amount of vitamin B₁₂ attached to TC II.

Figure 2.13: Vitamin B₁₂ depletion from TC II (adapted from Herbert *et al.*, 1990)

In the assay serum is treated with microfine precipitate of silica (Das *et al.*, 1991) or heparin sepharose (HS) (Benhayoun *et al.*, 1993) which adsorb TC II. Haptocorrin remains in the supernatant. To determine how much of the vitamin is bound in total and to haptocorrin, radioassays are performed on whole serum and on serum treated with silica, respectively. The difference reveals the amount bound to TC II (Das *et al.*, 1991). TC II can be released from HS by washing the precipitate with a glycine-K₂EDTA-KCN buffer and then assayed (van Kapel *et al.*, 1988).

2.13 Summary

This chapter outlined the current knowledge of the intricate mechanisms by which vitamin B₁₂ is absorbed and metabolised in the body. It further detailed deficiency states and supplementation regimes. Lastly, various analytical methods of assaying the vitamin, its components, metabolites and binders have been presented. Based on the current knowledge investigations have been made into various markers of vitamin B₁₂ deficiency. The results are presented in the following chapters.

CHAPTER 3

Materials and methods

3.1 Animals

The animals used in this research were lambs and sheep, either from the university's own experimental farm (Chapter 4) or from farms which had reported Co deficiency in the past (Chapters 5 to 7). Research for Chapters 4 and 5 was conducted in the university's own animal laboratory, the Johnstone Memorial Laboratory (JML), and the trials described in Chapters 6 and 7 took place on farms in the South Island of New Zealand.

3.2 Sample collection

3.2.1 Blood

Blood (~ 10 ml) was collected by jugular venipuncture into either 10 ml serum or plasma (140 USP Na heparin/10 ml) tubes (Baxter Health Care, Christchurch). Serum was taken when sampling was done on the farms and the blood needed to be sent to our laboratory. This was the case for the trials described in Chapters 7 (1998/99 sampling) and 8. Plasma was taken in all other trials and centrifuged at 3000 *g* for 10 min at 4°C within 3 h of sampling. Schultz (1987a) found plasma and serum equally suited for vitamin B₁₂ analysis.

3.2.2 Liver

Liver samples were obtained by biopsy. The technique of aspiration liver biopsy used for this research was first developed by Dick (1944) and has since been used by other researchers (Hogan *et al.*, 1971; Familton, 1985; Harrison, 1993). This technique, as described by Familton (1985), was modified for use in sheep.

All animals were prepared by shaving around the area of the right jugular vein. Pre-ruminant (PR) lambs were then given Pentothal (13 to 18 mg/kg b.w., 2 % thiopentone sodium, Virbac Laboratories NZ Ltd., Auckland, New Zealand) intravenously as a short-acting full anaesthetic. The animals were placed in left lateral recumbency on the operating table and a 100 mm² diameter area at the site of the incision was shaven and swabbed with 70 % v/v alcohol in preparation for the biopsy. Ewes and ruminant lambs then received lignocaine hydrochloride (2 %, 10 mg/kg b.w., Virbac Laboratories NZ Ltd., Auckland, New Zealand) as a local anaesthetic subcutaneously and into the underlying intercostal muscle at the site of incision.

A scalpel incision, approximately 5 mm in length, was made through the skin 2 to 3 cm ventral to the end of the last (12th) rib, in the intercostal space between the 11th and 12th rib. The biopsy needle (consisting of trocar, length: 250 mm, diameter: 3.15 mm, and cannula, length: 200 mm, diameter: o.d. 4.05 mm, i.d. 3.20 mm), was introduced vertically first through the incision and gently pushed through the intercostal muscles and the diaphragm towards the liver. Once inside the abdominal cavity the trocar was withdrawn and the cannula carefully advanced cranially at an angle of approximately 30° from the horizontal plane. Care had to be taken not to have the cannula more dorsal than the site of entry so the liver hilus or gall bladder would not be damaged.

Once the liver was reached (distinguishable from surrounding tissues by its high surface rigidity) the cannula was gently driven 2 to 3 cm into it by using rotating movements. A syringe was then attached to the cannula and a core of liver tissue drawn up by pulling the syringe plunger a short distance. Syringe and cannula were then withdrawn together from the body and the liver tissue expelled onto a gauze swab so the blood could drain off. Between 0.1 and 0.5 g of sample were obtained that way. Using forceps, the sample was immediately transferred into an aluminium foil wrapped tube to exclude light which could damage the coenzymes.

The PR lambs and the ewes received Penstrep LA (1 ml/10 kg b.w., 100,000 iu procaine penicillin, 100,000 iu benzathine penicillin and 250 mg dihydrostreptomycin per ml, Bomac Laboratories Ltd., Auckland, New Zealand) immediately after biopsy sampling, injected subcutaneously into the anterior half of the neck. The

wounds were dressed with Wound Powder (resorcinol 0.5 % w/w, camphor 2.5 % w/w, iodoform b vet c 1958 2.5 % w/w and activated charcoal 63.5 % w/w, Ethical Agents Ltd., Wiri, Auckland, New Zealand). The ruminant lambs used in Chapter 8 received Bomacillin LA (1 ml/10 kg liveweight, 150 mg procaine penicillin and 112.5 mg benzathine penicillin per ml, Bomac Laboratories Ltd., Auckland, New Zealand) instead of Penstrep LA, and their wounds were not dressed. The incision was too small to warrant suturing. The PR lambs were carried to their pens to recover. They were usually standing again within 10 min. On farm operations, the ewes and ruminant lambs were released into a small paddock and kept under observation until recovery was complete.

3.2.3 Milk

Milk samples from ewes were taken by hand milking, without hormonal induction of 'let down'.

3.2.4 Faeces

Faecal samples were obtained through rectal probing or, if the animal was in a metabolic cage, by 24 h collection.

3.2.5 Urine

Urine samples were obtained by partial smothering.

3.2.6 Pasture cobalt

Herbage samples were collected from the paddocks the animals grazed on for cobalt content determination. Care was taken to randomize samples to obtain a cross-section of herbage from the respective paddocks. This was done by walking the paddock in a 'W' shape and taking a grass sample every 40 paces. The sample, no more than half a handful at a time, was plucked with fingers and any soil or root

material was removed. Around 50 to 60 samples per paddock were collected that way and stored in a bag. The samples were lyophilised before being sent for Co analysis.

3.3 Drugs and diet

Prolaject B₁₂ (Bomac Laboratories Ltd.), which contained 1000 µg OH-cbl per ml was used as a vitamin B₁₂ supplement for lambs. Doses varied according to the trial (see respective chapters) but were generally between 0.5 and 2 mg. Unless otherwise stated, Prolaject was injected into the anterior half of the neck.

Cobalt bullets (PermaCo, Schering-Plough, Animal Health Ltd., Upper Hutt, New Zealand), 10 g pellets each containing 2 g Co as Co₃O₄, were used as Co supplement for ewes and for lambs after weaning. The daily release rate of the bullets was given as 0.6 to 1.2 % (12 to 24 mg/d) initially by the manufacturer.

The milk powder used in the trials described in Chapters 5, 6 and 7 was Anlamb, obtained from Anchor (N.Z. Dairy Board).

3.4 Assays

All chemicals, unless stated otherwise, were obtained from BDH (Auckland, New Zealand) and were AR or HPLC grade.

3.4.1 Vitamin B₁₂ in serum, milk, liver and faeces

A minimum of 0.5 ml of serum or plasma, 1 ml of milk, approximately 100 mg of liver, or 2 g of faeces was sent to the Animal Health Laboratory (Labnet) in Invermay, Mosgiel. Vitamin B₁₂ was determined by radio-isotope dilution assay (RIDA). The following method was used (from Labnet Invermay Diagnostic Chemistry Test Method Manual), based on the method by Green *et al.* (1974):

3.4.1.1 Test principle

Vitamin B₁₂ is extracted from its protein complex by boiling at pH 4. A known amount of ⁵⁷Co-B₁₂ is then allowed to compete with the extracted vitamin B₁₂

for binding to a limited amount of B₁₂-binding protein. Finally, bound and free portions of vitamin B₁₂ are separated. Measurements of the amount of ⁵⁷Co-B₁₂ that is bound allows determination of the degree of radioisotopic dilution, and hence the amount of sample vitamin B₁₂ present, by comparison to a standard curve.

3.4.1.2 *Reagents and solutions*

The vitamin B₁₂ standard was prepared by dissolving CN-cbl (10 mg, Sigma V 2876, St. Louis, USA) in 25 % (v/v) ethanol (500 ml) to obtain a 20 mg/l solution. This was stored in an amber bottle at 4°C. A 5 mg/l solution was made by diluting the above solution (25 ml) to 100 ml with 25 % (v/v) ethanol which was stored in the same way. From this, a 10 µg/l solution was prepared fresh as required by diluting the 5 mg/l solution to 500 ml with 0.9 % (w/v) saline and adding a 1 % (w/v) aqueous potassium cyanide solution (50 µl). Of this, 0, 1, 2, 4, 10 and 20 ml were pipetted into separate 100 ml volumetric flasks and diluted to volume with the saline. The resulting concentrations were 0 (A), 74 (B), 148 (C), 295 (D), 738 (E) and 1476 (F) pmol/l. Aliquots of these (0.5 ml) were frozen until needed.

To make the extraction buffer, sodium acetate (trihydrate) (0.4 M) was titrated to pH 4.0 with HCl (0.4 M). Glycine (0.8 M) was made up in NaCl (0.8 M) for the neutralising buffer. The pH was adjusted to 10.0 with NaOH (0.8 M). Chick serum (CS) binder (from serum from a local chicken farm) was made up in 0.9 % (w/v) saline and diluted to give 40 to 60 % binding (1:500, 1:1000, 1:1500, 1:2500, 1:3000, 1:3500, 1:4000).

For each dilution, the following were set up in duplicate: total count tubes, blank tubes and zero standards. The test procedure was carried out as usual (see below), however, the boiling stage was omitted. The total counts were read and the duplicates averaged. To calculate the binding of each dilution, the following equation was used:

$$\% \text{ Binding} = \frac{\text{Zero std counts} - \text{Blank counts}}{\text{Total counts}} \times 100$$

A 5 % (w/v) preparation of albumin-coated charcoal was made by adding activated charcoal (Sigma C5260) (40 g) to water (800 ml). It was stored at room temperature and thoroughly mixed before use. A 1 % (w/v) (bovine) albumin (Fraction 5, Boehringer, Mannheim, Germany) solution was made fresh each time by adding albumin (0.5 g) to water (50 ml). This mixture was stirred gently until the albumin had dissolved. For the albumin/charcoal suspension, equal volumes of albumin solution and the charcoal suspension were mixed with constant stirring.

A 5 % (w/v) dithiothreitol (DTT) (Boehringer, Mannheim, Germany) solution was prepared in water (100 ml). Aliquots (1.0 ml) were individually frozen until use. For use in the assay, one aliquot of DTT was diluted by the addition of neutralising buffer (50 ml).

The tracer solution was made by combining extraction buffer with distilled water in a 1:1 ratio. To each 10 ml of this, ^{57}Co -CN-cbl solution (10 μl , 10 $\mu\text{Ci/ml}$, Amersham, code CT2), a 1 % (w/v) KCN solution (10 μl) and the 5 % (w/v) DTT solution (100 μl) was added. This was prepared fresh daily as required.

3.4.1.3 *Procedure*

Duplicate test tubes were labeled with 'total', 'blank', and standards 'A' to 'E'. Further tubes were labeled according to the samples to be analysed. The 'blank' tubes received 200 μl of 0.9 % (w/v) saline; 200 μl of standards or sample were pipetted into the other tubes.

Liver samples were prepared by weighing exactly 0.2 g of sample, this was then homogenised with 0.9 % (w/v) saline and diluted to 4 ml. This solution (100 μl) was diluted again to 5 ml with 0.9 % (w/v) saline. This was used for the assay.

All test tubes received tracer solution (1000 μl) and vortexed. The test tubes were covered with foil and placed in a boiling water bath for 15 min. They were then cooled in cold water until the temperature was 20 to 25°C.

DTT solution (500 μl) was added to all the test tubes, followed by CS binder (200 μl) to all tubes, with the exception of the test tubes 'total' and 'blank' which received 0.9 % (w/v) saline instead (200 μl). The test tubes were vortexed and incubated for 1 h at room temperature.

Charcoal mixture (1000 µl) was added to each test tube except the 'total' and vortexed for 5 sec. The test tubes were left to stand for 10 min and then centrifuged at 1400 g for 10 min at 20°C.

The supernatant was decanted and radioactivity therein measured using a Gamma counter (LKB Wallace, 1470 Wizard, Finland). Readings were recorded to the nearest 10 pmol/l for serum and to the nearest 10 nmol/kg fresh weight for liver.

Faeces and milk samples were treated like liver: weighed and homogenised to make up a solution, which was then processed like serum.

3.4.1.4 *Detection and accuracy*

Detection limits for the assay were 57 pmol/l for serum and 70 nmol/kg for liver. The 95 % Confidence interval was ± 17 pmol/l for serum and ± 29 nmol/kg for liver.

3.4.2 **Methylmalonic acid in serum and urine**

For the analysis of methylmalonic acid (MMA) the method developed by McMurray *et al.* (1986) was adopted which uses gas chromatography with flame ionisation (GC-FID).

3.4.2.1 *Test principle*

MMA is extracted from serum following protein denaturation. It is then derivatised into its butyl-ester before being analysed using GC. Since ethylmalonic acid (EMA) behaves very similarly to MMA and serum contains only negligible amounts of it, EMA was chosen as an internal standard.

3.4.2.2 *Reagents and solutions*

Reagent A was made up by saturating 0.5 M sulphuric acid (M_r 98.08) with sodium chloride.

Reagent B, the internal standard - EMA (1 mmol/l, M_r 132.1) - was prepared by diluting a 10 mmol/l stock standard (1.32 g/l EMA in acetone) with acetone.

Reagent C, the MMA standard (0.5 mmol/l, M_r 118.1), was prepared by diluting a 10 mmol/l stock standard (1.18 g/l MMA in acetone) with acetone. Reagents A and B were stored at 4°C. MMA and EMA were purchased from Sigma (Auckland, New Zealand).

Reagent D was prepared fresh each day before use by adding one part of acetyl chloride slowly to ten parts of butan-1-ol and mixed well.

3.4.2.3 *Procedure*

Serum (250 µl) was placed into glass Quickfit tubes (3 ml), and reagent B (50 µl) was added as internal standard. Acetone (250 µl) was added while vortexing, followed by reagent A (1.0 ml) to denature and precipitate the serum proteins. This was followed by ethyl acetate (0.5 ml).

The vials were then stoppered and vortexed for 20 sec to extract both MMA and EMA into the ethyl acetate phase. The samples were centrifuged at 1200 g for 10 min at 4°C, the ethyl acetate layer pipetted off with glass Pasteur pipettes, transferred into Quickfit vials (1.5 ml) and stoppered. The ethyl acetate extraction was then repeated and the two extracts combined.

The samples were evaporated to dryness under oxygen-free nitrogen, using a multi-needle manifold. Reagent D (50 µl) was added, the samples vortexed and placed into a waterbath (70°C) for 20 min. After cooling, hexane (150 µl) and nanopure water (400 µl) were added, the samples were stoppered and vortexed. When the layers had separated, the top hexane layer was transferred into GC vials with inserts, sealed and kept at 4°C until analysis. All serum samples were prepared in duplicate.

Together with the preparation of the serum samples, two standards and a blank sample were prepared. For the standards, reagent C (MMA) (50 µl) and nanopure water (200 µl) were used instead of serum, and for the blank, nanopure water (250 µl) was used, and no reagent B (EMA) added.

3.4.2.4 *GC Analysis*

For the analysis on the GC-FID (HP 6890), a BP1 Megabore column (SGE International Pty. Ltd.) with the following set-up was used initially until the end of 1998. Sample (1 μ l) was injected onto the column from the autosampler. The injector was used in the splitless mode. The column was operated in constant flow mode (4.2 ml helium per min), and a nominal initial pressure of 30 kPa. The injector port temperature was 200°C and the flame ionisation detector temperature was 300°C. The oven temperature used for the chromatography was 120°C with an initial time of 2 min, followed by a temperature ramp of 5°C/min to 175°C. The post-run temperature was held at 300°C for twelve min to flush the column.

From early 1999 until the end of the trials a HP-5MS cross linked 5 % PH ME Siloxane capillary column (Hewlett Packard) was used. The GC set-up was as follows: Injection volume was 5 μ l, split ratio was 10:1. Flow rate was 4.2 ml/min in constant make-up flow mode. Nominal initial pressure was 147 kPa. The injector port temperature was 200°C and the detector temperature was 280°C. The initial oven temperature was 120°C with a temperature ramp of 5°C/min to 160°C. The post-run temperature was held at 280°C for 5 min.

3.4.2.5 *Quantification*

Each run was started by a blank sample, followed by a standard. The second standard was run at the end of the serum samples of that run. Peak areas were integrated by the GC's own integrator and the standards used to find a mean Relative Response Factor (RRF). The RRF was then used to calculate the amount of MMA in μ mol/l in each sample.

3.4.2.6 *Precision and linearity*

Repeatability of a serum sample (n=10) gave a mean of 1.45 μ mol/l with a standard deviation of 0.19. A linearity assay was done by adding MMA to serum samples increasing the endogenous MMA concentration by 0, 0.5, 1, 10, 20, 50, 100, 150, and 200 μ M. Linear regression analysis gave $y = 0.9314x + 0.2583$, with $R^2 =$

0.9994, where y is the ratio of the MMA peak area to internal standard peak area, and x is the added MMA (endogenous MMA was subtracted from the MMA measured).

3.4.3 Homocysteine

The determination of homocysteine (hcy) in serum was based on the method developed by Vester and Rasmussen (1991) for HPLC.

3.4.3.1 Test principle

Homocysteine, a thiol, was derivatised with ammonium 7-fluorobenz-2-oxa-1,3-diazole-4-sulphonate (ABD-F) to obtain a fluorescent compound (Figure 3.1). This could then be detected and quantified by HPLC with fluorescence detector. N-(2-mercaptopropionyl)-glycine was used as an internal standard.

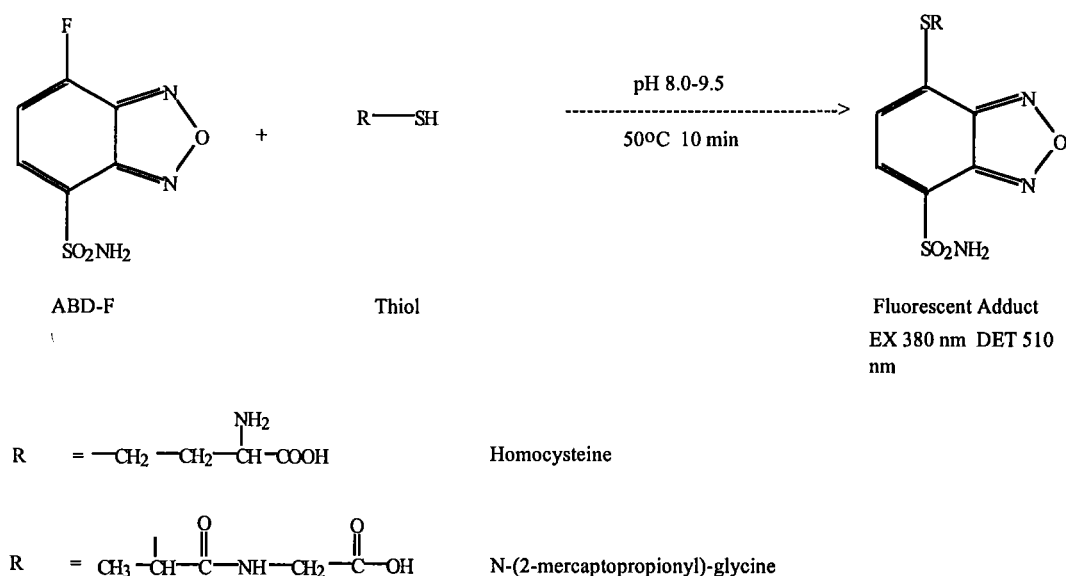


Figure 3.1: Derivatisation of homocysteine

3.4.3.2 Reagents and solutions

N-(2-mercaptopropionyl)-glycine (7 mmol/l, Mr 163.2) and DL-homocysteine (7 mmol/l, Mr 135.2) (both from Sigma, Auckland, New Zealand) were dissolved in HCl (0.1 mol/l) and stored in the freezer. They were prepared fresh

monthly. Tris-(2-cyanoethyl)phosphine (0.078 g/ml, M_r 193.19) (Molecular Probes, Oregon, USA) was dissolved in dimethyl formamide. A new batch was prepared fresh each day. Trichloro acetic acid (TCA) (10 %) containing ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) (1 mmol/l, M_r 372.2) was made up. Borate buffer containing Na_2EDTA (4 mmol/l) was prepared by mixing equal volumes of boric acid (0.1 mol/l, M_r 61.83) with borax (sodium tetraborate decahydrate, $\text{Na}_2\text{B}_4\text{O}_7 \times 10\text{H}_2\text{O}$) (0.1 mol/l, M_r 381.36) (Sigma, Auckland, New Zealand). The resulting solution was adjusted to a pH of 9.23.

ABD-F (1 mg/ml, M_r 217.17) (Molecular Probes, Oregon, USA) was dissolved in borate buffer. This compound is light sensitive when in solution so it needed to be foil-wrapped and stored under refrigeration. If the solution appeared yellow it was discarded. (The analysis was not affected if samples appeared yellow after derivatisation.)

HPLC solvent A consisted of phosphoric acid (0.15 mol/l, M_r 98) containing (50 mmol/l, M_r 101.19) triethylamine (TEA). HPLC solvent B was acetonitrile.

3.4.3.3 Procedure

A solution of DL-homocysteine and N-(2-mercaptopropionyl)-glycine (70 $\mu\text{mol/l}$) was prepared by taking 100 μl of each of the previously prepared stock solutions (7 mmol/l) and making it up to 10 ml in a volumetric flask with borate buffer. A solution of N-(2-mercaptopropionyl)-glycine (70 $\mu\text{mol/l}$) was prepared by taking 100 μl of the stock solution (7 mmol/l) and making it up to 10 ml with borate buffer.

Serum (125 μl) was pipetted into Eppendorf tubes. Six of these were used to make the two point calibration curve and needed to be from the same serum.

To the first serum sample to be used as a standard, the homocysteine/N-(2-mercaptopropionyl)-glycine standard (50 μl) was added. To the second serum standard, and to each of the other samples, the internal standard N-(2-mercaptopropionyl)-glycine (50 μl) was added. Tris-(2-cyanoethyl)phosphine (12.5 μl of 0.078 g/cm³) was added, the samples were vortexed and placed at 4°C for 30 min to reduce the disulfide bonds between homocysteine and other thiols or blood

proteins. To each sample chilled 10 % TCA (125 μ l) containing disodium EDTA (1mM) was added to precipitate the proteins. The samples were vortexed and then centrifuged at 1000 g for 5 min at 20°C.

The clear supernatant (25 μ l) was taken and borate buffer (50 μ l of 0.5 mol/l) added. ABD-F in borate buffer (25 μ l of 1 mg/cm³) was added to each sample. The samples were vortexed, then heated at 50°C for 10 min and afterwards cooled in crushed ice.

The samples were filtered into a limited volume HPLC vial insert, using a syringe (1 ml), a Phenomenex (AFO-3368) filter (0.45 μ m) and a syringe needle.

3.4.3.4 *HPLC Analysis*

HPLC columns and filters were bought from Phenomenex (Auckland, New Zealand). A Prodigy column ODS 250 mm x 4.3 mm, 5 μ m, was used, with a securiguard system, C18 (ODS) cartridge, as guard column. Flow rate was 1 ml/min with a pressure of 1440 $\pm$ 15 PSI. Solvent composition was 85 % A, 15 % B. The amount of sample injected was 15 μ l.

A fluorescence detector with excitation at 380 nm and detection at 510 nm was used. The column was conditioned prior to the running of samples at 85 % A and 15 % B until a stable pressure and detector signal were obtained.

3.4.3.5 *Quantification*

A two point internal standard method was used. The gradient of the graph was determined in which x is the ratio of the homocysteine peak to the internal standard and y is the increase in homocysteine concentration between the homocysteine spiked standard and the 'blank' standard (no added homocysteine). Addition of the homocysteine/N-(2-mercaptopropionyl)-glycine standard (50 μ l) gave a final concentration increase in homocysteine of 20 μ M.

The concentration of the sample was determined by dividing the ratio of the homocysteine peak to the internal standard peak by the gradient of the standard graph. This two point standard method allowed for the standards to be made of genuine serum samples and overcame matrix effects observed in methods using

simple homocysteine solutions as standards. The standards were run in duplicate at the start of the sample run and again at the end of the run.

3.4.3.6 *Precision and Linearity*

On multiple preparations of the same sample (n=10) a coefficient of variation of 3.5 % was obtained, with a confidence (95 % confidence interval) of 2.2 %, which is equivalent to the quoted 'within run imprecision' and compared favourably with similar assays reported in the literature (Vester and Rasmussen, 1991). The detection limit for a sample with a signal-to-noise ratio of 4:1 was 1 μ M.

Two linearity assays were done with different serum samples. Homocysteine was added to serum samples so as to increase its concentration by 0, 1, 10, 50, 100 and 500 μ M. Linear regression analysis of the response gave $y = 0.024x - 0.0824$, $R^2 = 0.9952$, for the first assay and $y = 0.0158x + 0.1564$, $R^2 = 0.9991$, for the second, where y is the ratio of the homocysteine peak area to internal standard peak area, and x is the added homocysteine.

3.4.4 **Vitamin B₁₂ on transcobalamin II and haptocorrin**

The method of Benhayoun *et al.* (1993) was used to separate vitamin B₁₂ bound to transcobalamin II (TC II) and to haptocorrin (HC).

3.4.4.1 *Test principle*

The basis of the separation of the binding proteins is that TC II is adsorbed onto heparin sepharose (HS), whereas HC is not. It stays in the supernatant which is removed. The TC II in the precipitate is then washed out. Both fractions are assayed for vitamin B₁₂.

3.4.4.2 *Reagents and solutions*

Buffer 1 was made up by adding K₂EDTA (4 mM, M_r 404.47) and KCN (0.15 mM, M_r 65.12) to sodium phosphate buffer (phosphoric acid, 88 %, M_r 111.4, and adjusted to pH 5.6 with NaOH) (25 mM).

For buffer 2, K₂EDTA (4 mM) and KCN (0.15 mM) were added to a glycine (0.2 M, M_r 75.07) solution and adjusted to pH 3.0.

Buffer 3 consisted of Tris(hydroxymethyl)-methylamine (Tris) (0.02 M, M_r 121.14) (Boehringer, Mannheim, Germany), to which NaCl (1 M) was added and the pH adjusted to 7.4.

3.4.4.3 *Procedure*

To assay haptocorrin-bound vitamin B₁₂, HS (Amersham Pharmacia Biotech, Sweden) (0.5 g) was washed twice with buffer 1 (100 ml) and suspended in buffer 1 (4 ml). Buffer 2 (0.5 ml) was added to serum (0.5 ml) and mixed with HS suspension in buffer 1 (0.3 ml). The mixture was incubated at room temperature for 2 h with stirring and then centrifuged at 2500 g for 10 min at 10°C. The supernatant was taken off and analysed for vitamin B₁₂.

To assay TC II-bound vitamin B₁₂, the pellet was homogenised with buffer 3 (1.3 ml) and stirred for 3.5 h at room temperature. After centrifugation at 2500 g for 10 min at 10°C the supernatant was taken off and analysed for vitamin B₁₂.

3.4.5 **Milk protein**

Milk protein content was determined by the method of Kjeldahl, as described in the manual of the autosampler used (Kjeltec Auto 1035/38 Sampler system, Tecator, Sweden, pages 63- 69).

3.4.5.1 *Reagents and solutions*

The catalyst consisted of potassium sulphate (Haisol-K Fertilisers and Chemicals, Haifa, Israel) and selenium at a ratio of 999:1. The solutions used by the autosampler were

- (1) 40 % (w/v) NaOH (ICI NZ Ltd.),
- (2) 1 % (w/v) boric acid (Unilab, Ajax Chemicals, Australia) solution (50 g boric acid in 5 l of water) with 1 % (w/v) bromocresol green/methyl red (Merck, Darmstadt, Germany) indicator (0.050 g bromocresol green in 50 ml methanol and 0.035 g methyl red in 35 ml methanol),
- (3) 0.2 M HCl (100 ml conc. HCl in 5 l of water) and
- (4) Ammonium ferrous sulphate (~ 0.5 g) as control.

3.4.5.2 *Procedure*

Milk (1-2 g) was weighed into tared large test tubes and the weights recorded. All samples were done in duplicate. Two tubes were left blank. To each tube, catalyst (~ 4 g) and concentrated sulphuric acid (12 ml) were added. The samples were then heated to 430°C for 30 min. The solution turning a clear yellow colour indicated the completion of the digestion process.

After cooling, the tubes were placed into the autosampler for analysis by distillation as ammonia and titration against boric acid. The factor for milk protein by which results were multiplied was 6.38.

3.4.5.3 *Quantification*

The percentage protein was obtained from the print-out of the autosampler. A mean was calculated from the duplicate samples. If there was more than 5 % discrepancy between the duplicates, the analysis was repeated.

3.4.6 **Faecal egg count**

The faecal egg count (FEC) was done by staff at JML. The method used was a modification of the McMaster method (Pomroy, 1995) developed by the Ministry of Agriculture and Fisheries (1973).

Faeces were made into a pellet to a standard volume which appropriated to a weight of 1.7 g ($\pm$ 5 % variation, depending on DM). The pellet was placed in a jar with water (5 ml) and soaked overnight to soften. The following day, saturated NaCl

(46 ml) was added and the sample mixed for 25 sec, either by hand or with an electrical stirrer, until the faeces pellet was completely broken up. A Pasteur pipette was used to fill both chambers of a moistened McMaster slide with the faecal suspension. Any eggs present on the slide would float to the surface of the salt solution and stick to the cover glass where they could be counted.

The number of eggs present in both chambers of the slide were counted under a microscope, totalled and multiplied by 100 to give the number of eggs per gram (epg) for that sample. Thus this method has a sensitivity of one egg counted representing one hundred epg in the sample.

3.4.7 Pasture cobalt content

For Chapter 7, analysis was done by RJ Hill Laboratories Ltd., Hamilton, New Zealand. Aliquots of plant tissue (1 g) were weighed into 30 ml digestion tubes. Nitric-Perchloric Digestion Acid (12 ml of 5:1 $\text{HNO}_3:\text{HClO}_4$), three to five anti-bumping granules and one drop of kerosene were added and the samples agitated to thoroughly wet the plant material. The tubes were then transferred to the digestion block where they were heated for 5 h using a temperature ramp up to 220°C (Figure 3.2). After the samples had cooled to room temperature 18.5 ml of water was added to each tube and then vortexed. Analysis of Co was then achieved by Inductively Coupled Plasma Source Mass Spectroscopy (ICP-MS). This method is based on the ones described by Clinton (1979) and the Analytical Methods Committee (1979).

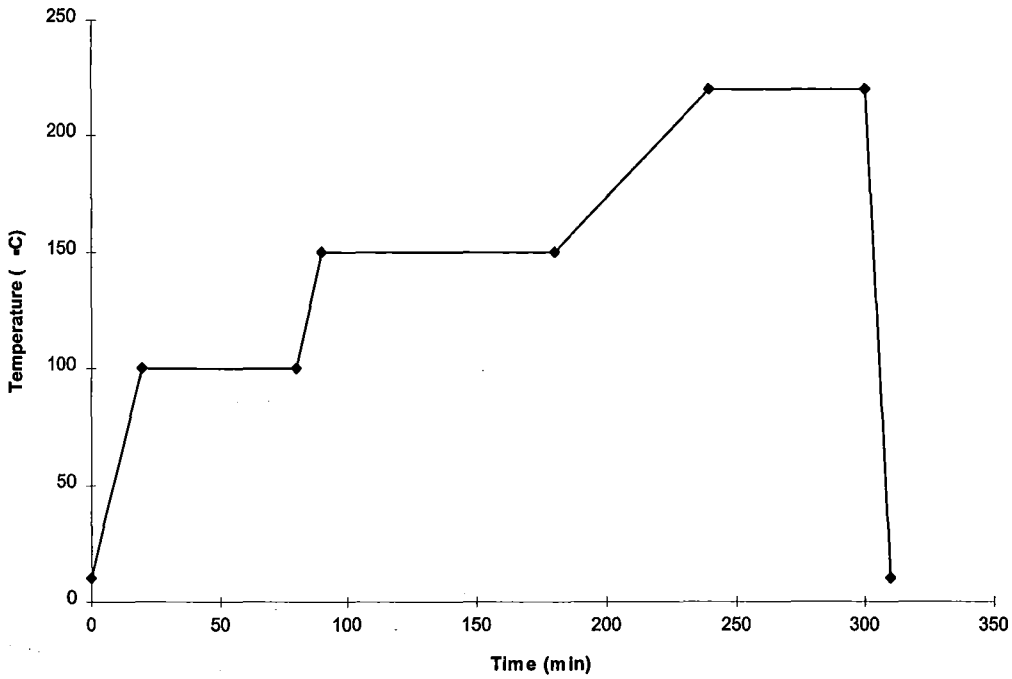


Figure 3.2: Temperature profile for Co digestion

For Chapter 8, Co analysis was performed by Soil Fertility Service at AgResearch, Hamilton, New Zealand. Levels of pasture Co were determined in the nitric-perchloric acid digests using graphite furnace atomic absorption spectrophotometry. Limit of detection was 0.01 ppm. The method is based on the ones by Poole (1980) and Simmons (1973 and 1975).

In both laboratories, iron (Fe) was also determined to gauge the level of soil contamination on the samples.

CHAPTER 4

The influence of propionate in the diet of pre-ruminant lambs on vitamin B₁₂ and methylmalonic acid status, and weight gain

4.1 Introduction

Ruminants rely almost entirely on gluconeogenesis, of which propionate is a major substrate, to meet their energy needs because they cannot utilise glucose and other carbohydrates in their feed for the direct production of ATP. The liver is the main site for the conversion of propionate to succinate (van Houtert, 1993); it uses more than 90 % of the absorbed propionate (Brockman, 1993). The suckling PR lamb, however, does not yet have a functional rumen and hence can obtain glucose directly from enzymatic digestion of milk, like monogastrics. Therefore, its vitamin B₁₂ requirement for the gluconeogenic pathway may be comparatively low.

Clinical evidence suggests that PR lambs do not store much vitamin B₁₂ in the liver. A critical time for the development of deficiency signs is soon after weaning at a time when the propionate - succinate pathway needs to increase activity for gluconeogenesis. It is possible that propionate is necessary to promote liver uptake and storage of vitamin B₁₂.

4.2 Aim

This trial was set up to find out if propionate stimulates the uptake and/or storage of vitamin B₁₂ in the liver, reflecting the need to deal with incoming propionate.

4.3 Experimental design

Sixteen new-born lambs were divided into two groups of eight, balanced by weight. One group (+Pr) received propionate in their diet, the other group (-Pr) received milk only. Half of each group, further balanced by weight, were then allocated to vitamin B₁₂ treatment (+B₁₂ or -B₁₂). This gave a factorial design with four animals in each group (Table 4.1). The trial lasted four weeks, from September 29 to October 25, 1997.

Table 4.1: Factorial design of the trial

	-B ₁₂	+B ₁₂
-Pr	4	4
+Pr	4	4

4.4 Materials and methods

4.4.1 Experimental animals

The lambs were Dorset Down/Coopworth cross-bred. They were separated from their dams at birth, weighed, and housed in heated pens at JML. Lambs belonging to the same sub-group shared the same pen.

4.4.2 Diet

The lambs were fed milk substitute (Anlamb) for eleven days initially so they would be strong enough at the start of the trial before being subjected to liver biopsies. They were offered 2100 ml/d in equal feeds at 0800 h, 1200 h and 1600 h. Each feed consisted of milk powder (120 g) dissolved in warm water (600 ml). One group continued to receive milk only, the other group received milk 7.5 % of which (w/w) had been replaced by sodium propionate, (9 g CH₃CH₂COONa, Datachem, Auckland, NZ) and milk powder (111 g) in warm water (600 ml). Refusals were weighed and recorded. Anlamb contained 2210 pmol/l vitamin B₁₂.

4.4.3 Vitamin B₁₂ treatment

The lambs allocated to vitamin B₁₂ treatment received Prolaject (250 µl of a 1 mg/ml solution of OH-cbl) subcutaneously at the start of the treatment and after each blood sampling. The total amount of vitamin B₁₂ administered was 2 mg (~ 1.5 µmol).

4.4.4 Sampling

The lambs were weighed before the start of the trial, after two weeks and at the end of the trial. The day before propionate addition commenced first a blood and then a liver biopsy sample was taken from all animals. Throughout the trial, blood samples were taken twice weekly (Tuesdays and Fridays after the morning feed) for vitamin B₁₂ and MMA analysis. After four weeks, at the conclusion of the trial, a final blood sample was taken and another liver biopsy performed. The liver biopsy technique was described in Chapter 3. However, it proved impossible to obtain liver samples by this technique from most of the lambs that had received the propionate supplement. Therefore, a laparotomy was performed on those animals. Nembutal (2 ml/5 kg b.w., Virbac Laboratories NZ Ltd.), administered into the jugular vein, was given as a general anaesthetic. A greatly distended, gas-filled rumen was revealed, obscuring the liver. There were difficulties with obtaining a liver biopsy sample from one lamb in group +B₁₂/-Pr on both occasions, so no liver data were available for that lamb.

4.4.5 Statistical analysis

Analysis of variance (ANOVA) was done using Genstat, calculating (where it was possible) means and overall standard errors of the means (SEM) for each group as well as the interaction between treatment x time of the various groups (Kenward, 1987). Degrees of freedom have been adjusted using the Greenhouse-Geisser epsilon (Greenhouse and Geisser, 1959) since not all measurements were independent of each other. Student's t-tests and standard errors of the difference (SED) have been used to find the least significant difference (LSD) for various p-

values ($p < 0.05$, < 0.01 and < 0.001). Orthogonal polynomial analysis of the data was performed to identify the components of the shapes of the curves and the relationship between the curves of the various groups.

4.5 Results

4.5.1 Plasma and liver vitamin B₁₂ and MMA concentrations

Table 4.1 and Figures 4.1 to 4.3 summarise the results obtained for plasma and liver vitamin B₁₂ and for plasma MMA. For plasma, the effect of vitamin B₁₂ treatment over time was highly significant ($p < 0.001$). The interactions between time and propionate treatment as well as between time, propionate and vitamin B₁₂ treatment were significant ($p < 0.05$). The shape of the curves was basically linear for all four groups. The difference was only in the slopes of the curves, with the steepest gradient for the +B₁₂/+Pr group, followed by the +B₁₂/-Pr group. Plasma vitamin B₁₂ in the -B₁₂ groups was virtually identical with a zero gradient. For the -B₁₂ groups plasma vitamin B₁₂ values stayed relatively constant between 280 and 400 pmol/l. For the +B₁₂ groups plasma values rose from 453 to 2355 pmol/l in the -Pr group and from 328 to 3323 pmol/l in the +Pr group.

For liver vitamin B₁₂ concentrations, the interaction between time and vitamin B₁₂ treatment was highly significant ($p < 0.001$). During the four weeks of the trial mean liver vitamin B₁₂ concentrations rose from 255 to 861 nmol/kg fresh tissue in the +B₁₂ groups, whereas for the -B₁₂ groups there was a non-significant drop from 296 to 268 nmol/kg fresh tissue.

Vitamin B₁₂ treatment had no significant effect on MMA. The only overall effects were due to time and propionate treatment and the interaction between these two ($p < 0.01$). Plasma MMA ranged from 0.5 to 1.6 $\mu\text{mol/l}$. It was consistently higher in the +Pr groups compared to the -Pr groups, except for the last sampling.

Table 4.2: Effect of vitamin B₁₂ and sodium propionate treatment on mean vitamin B₁₂ and MMA concentrations in milk-fed lambs during the trial period

	Plasma B ₁₂	Liver B ₁₂	MMA	Plasma B ₁₂	Liver B ₁₂	MMA
	pmol/l	nmol/kg	μmol/l	pmol/l	nmol/kg	μmol/l
SEM	± 278	± 69.8	± 0.112	± 278	± 69.8	± 0.112
Date	-B ₁₂ /-Pr			+B ₁₂ /-Pr		
29/9/1997	280	277	0.68	453	247	0.63
3/10/1997	251		0.95	1144		0.90
7/10/1997	275		1.05	1189		0.95
10/10/1997	430		0.50	1743		0.40
14/10/1997	367		0.73	1541		0.53
17/10/1997	305		0.60	1974		0.58
21/10/1997	350		0.88	2037		0.78
24/10/1997	298	300	0.65	2355	877	0.73
Date	-B ₁₂ /+Pr			+B ₁₂ /+Pr		
29/9/1997	395	315	0.68	328	263	0.65
3/10/1997	329		1.58	910		1.35
7/10/1997	375		1.38	1231		1.20
10/10/1997	390		0.65	2089		0.62
14/10/1997	397		1.00	2583		0.78
17/10/1997	375		0.85	4088		0.70
21/10/1997	355		1.40	3243		1.05
24/10/1997	365	235	0.65	3323	845	0.68

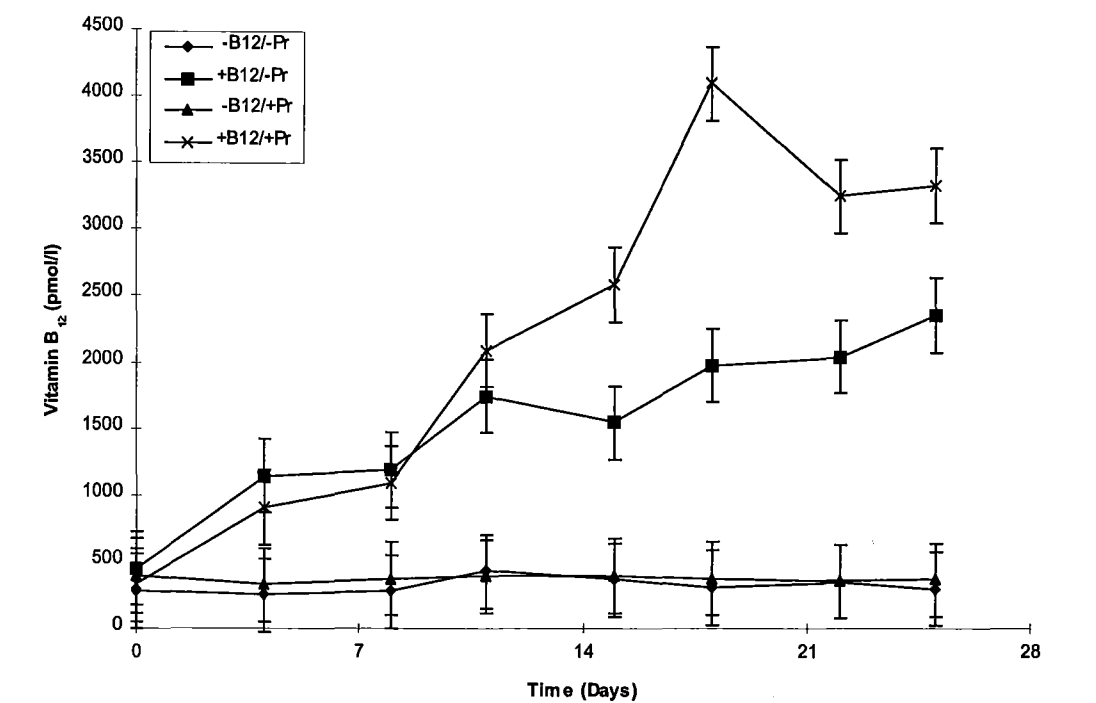


Figure 4.1: Effect of vitamin B₁₂ and sodium propionate treatment on mean plasma vitamin B₁₂ concentrations in milk-fed lambs (± SEM)

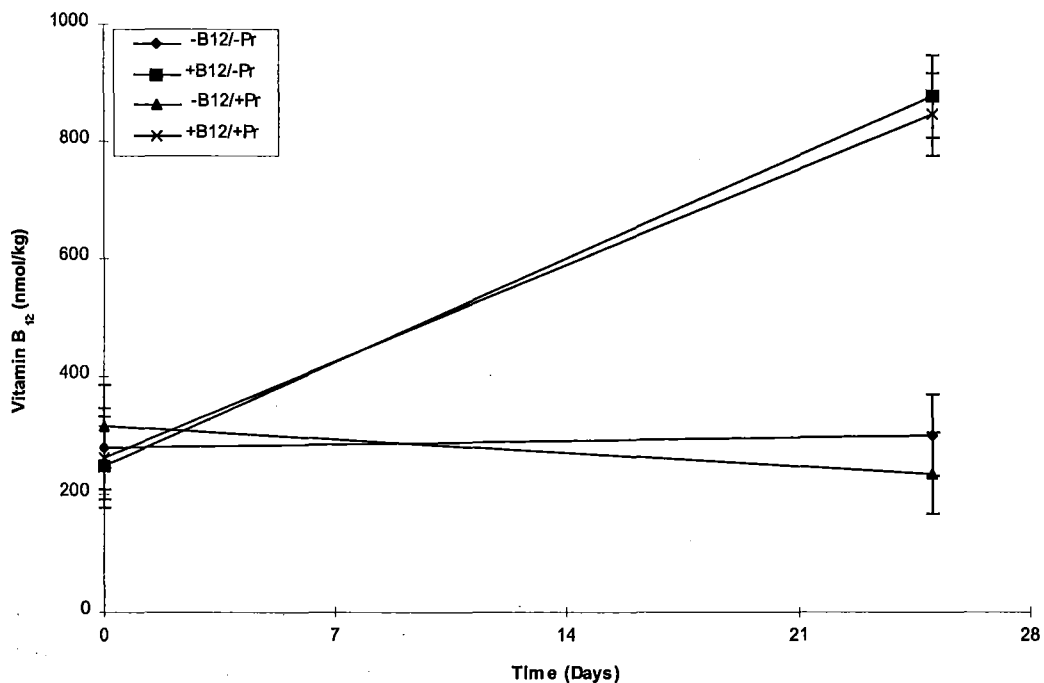


Figure 4.2: Effect of vitamin B₁₂ and sodium propionate treatment on mean liver vitamin B₁₂ concentrations in milk-fed lambs ($\pm$ SEM)

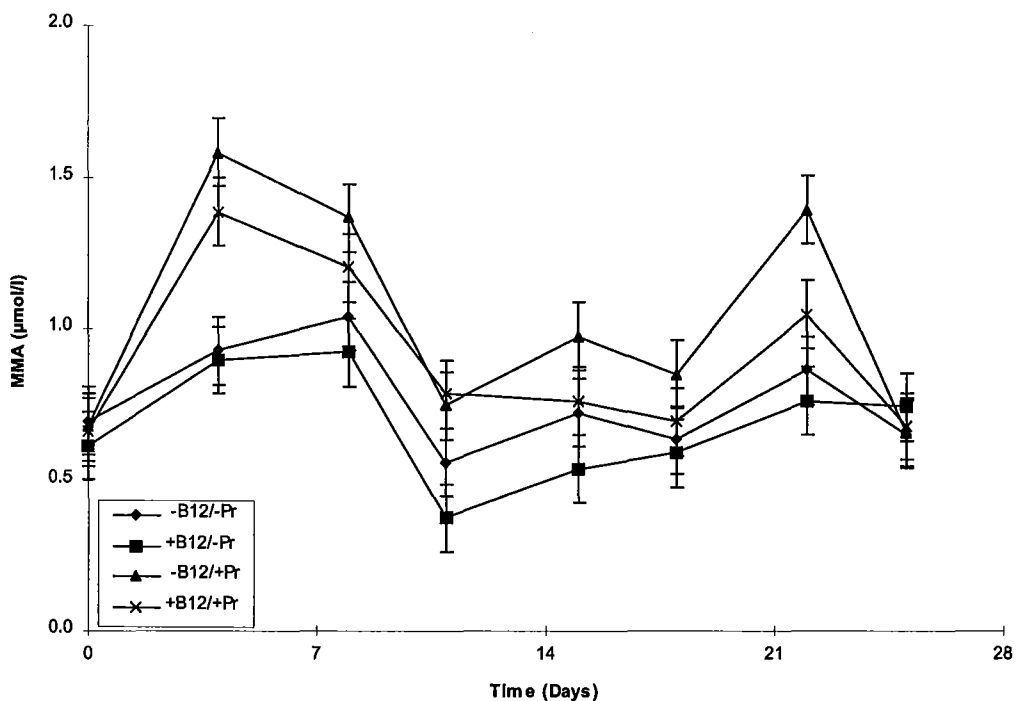


Figure 4.3: Effect of vitamin B₁₂ and sodium propionate treatment on mean plasma MMA concentrations in milk-fed lambs ($\pm$ SEM)

4.5.2 Milk intake and body weight gain

Table 4.3 shows the mean daily milk intake, LWG and milk intake to gain 1 g of weight of the four groups during the trial period. There was no significant difference in intake between the groups overall, although the +Pr group had a significantly lower milk intake ($p < 0.05$) on day twelve and the last three days of the trial compared to the -Pr group. The mean LWG of the four groups did not differ significantly, although the +B₁₂/+Pr group seemed to require more milk intake in relation to weight gain.

Table 4.3: Effect of vitamin B₁₂ and sodium propionate treatment on mean daily milk intake, LWG and milk intake to gain 1 g of weight ($\pm$ SEM)

		-B ₁₂	+B ₁₂	SEM
Mean daily milk intake (ml)	-Pr	1957	1857	± 119.9
	+Pr	1802	1693	± 119.9
Mean LWG (kg)	-Pr	0.29	0.27	± 0.02
	+Pr	0.27	0.20	± 0.02
Mean milk intake (ml) to gain 1 g of weight	-Pr	6.8	6.9	± 0.47
	+Pr	6.9	8.5	± 0.47

4.6 Discussion

4.6.1 Vitamin B₁₂ and MMA concentrations

Plasma and liver vitamin B₁₂ concentrations in the -B₁₂ groups were maintained throughout the trial, and LWG was the same for all groups. When multiplying the average amount of milk consumed each day (~ 1.8 l) with the amount of vitamin B₁₂ contained in the milk replacer (2210 pmol/l) the lambs had received ~ 4000 pmol/d of vitamin B₁₂. Marston (1970) found that sheep require 11 μ g (~ 8100 pmol) of vitamin B₁₂ per day. The lambs in his trial weighed 25 kg, in contrast to the PR lambs in this trial which weighed about 15 kg at the end. The lack of change in the plasma vitamin B₁₂ concentrations of the -B₁₂ groups throughout the trial indicated that the vitamin B₁₂ content of milk must have sufficed to meet the metabolic demands of these lambs. According to O'Halloran and Skerman (1961) milk vitamin B₁₂ is absorbed almost quantitatively. It is therefore reasonable to

assume that the metabolic requirements of 15 kg milk-fed lambs are around half of what Marston (1970) postulated for ruminant sheep.

During the trial period 2000 μg (~ 1500 nmol) vitamin B_{12} were injected, augmenting liver stores by ~ 600 nmol/kg to ~ 900 nmol/kg fresh tissue. Marston (1970) found that the maximum capacity of liver to store vitamin B_{12} was in the vicinity of 1.4 $\mu\text{g/g}$ (~ 1000 nmol/kg) fresh tissue. The amount of vitamin B_{12} injected may well have saturated liver concentrations in the lambs in this trial.

In order to obtain an estimate of their liver weights the figure of 1.5 % of total body weight was chosen, based on the findings of Wallace (1948) that livers of lambs weigh between 1.25 and 2 % of total body weight. With a liveweight of 15 kg at the end of the trial, the livers of these lambs would have weighed ~ 250 g. Since the increase in liver vitamin B_{12} concentration was ~ 600 nmol/kg fresh tissue, liver vitamin B_{12} increase in 250 g was ~ 150 nmol. The amount injected was 1.5 μmol , therefore ~ 10 % of injected vitamin B_{12} was taken up by the liver. (Whether a smaller dose could have led to the retention of a larger proportion of vitamin B_{12} would need to be explored in another trial.) To my knowledge such estimates have not yet been reported in the literature.

Although the administration of vitamin B_{12} lead to an accumulation of the vitamin in the liver, addition of propionate did not lead to an increase of vitamin B_{12} in this organ, as had been expected. The amount stored in the liver did not seem to be related to a functional need since there was no effect of propionate loading of the diet. On the other hand, plasma levels did respond positively to propionate. The $+\text{B}_{12}/+\text{Pr}$ group retained almost twice as much vitamin B_{12} in their blood as the $+\text{B}_{12}/-\text{Pr}$ group on the reasonable assumption that plasma volume was similar in the two groups. The production of blood transport but not intracellular binding proteins could have been stimulated by the incoming propionate, resulting in augmented blood but not liver concentrations.

Plasma MMA concentrations stayed low in all groups, well below the threshold of 5 $\mu\text{mol/l}$ set by McMurray *et al.* (1985). Hogan *et al.* (1973) observed a tenfold increase in urinary MMA in Co deficient weaned lambs after a single oral administration of 50 g sodium propionate whereas no such increase was found in their Co replete lambs. Although plasma and not urinary MMA was measured in this

trial, MMA in plasma and urine are reported to correlate well (Rice *et al.*, 1987; Rasmussen *et al.*, 1989). No changes in plasma MMA were seen in this trial, probably because the lambs were not deficient and did not receive such a large dose of propionate. In addition, MMA values were so low overall and, while close to the detection limit of the method, would be non-significant physiologically.

4.6.2 Effect of propionate on food intake

It has been reported (Marston *et al.*, 1961 and 1972; Smith and Marston, 1971; Farningham and White, 1993; Anil and Forbes, 1980) that one feature of vitamin B₁₂ deficiency is suppressed appetite, and therefore reduced food intake, due to propionate accumulation. Initial trials in our laboratory with new-born lambs showed reduced feed intake when given 10 % or 20 % propionate in milk. The propionate milk did taste somewhat salty and could have been unpalatable to the lambs. Alternately, the relatively high propionate loading could have suppressed their appetite (Anil and Forbes, 1980).

At 7.5 %, though, the propionate added to the milk did not seem to suppress appetite. The lambs would have received a daily average of 25 g or 0.6 mol of propionate which is in accordance with van Houtert and Leng (1993) who found with ruminant lambs that the maximum amount of propionate which the liver can process was between 0.52 and 0.78 mol/d. However, the lambs in this trial were smaller and therefore 0.6 mol/d may have already exceeded their liver capacity. Indeed, towards the end of the trial milk intake was significantly less in the +Pr groups than in the -Pr groups. Accumulation of propionate could well have suppressed their appetite at that stage.

One interesting aspect of this trial was the development of the rumen on propionate. This may be of further interest in cases where fast maturation of the rumen is desirable (e.g. for an earlier weaning time) and warrants additional research.

4.6.3 Transport proteins

The results obtained of the effect of propionate on plasma vitamin B₁₂ concentrations led to the decision to measure the distribution of plasma vitamin B₁₂

bound to either TC II or HC (Table 4.4 and Figure 4.4). Plasma from the 4th, 6th and 8th sampling time (i.e. after two, three and four weeks after treatment) from the +B₁₂ supplemented groups was used. Recovery of vitamin B₁₂ ranged from 79 to 122 % and the values given below have been adjusted for that. Unfortunately, the method (described in Chapter 3) did not allow analysis of samples with low vitamin B₁₂ concentrations, therefore no data are available on the -B₁₂ groups.

Table 4.4: Plasma vitamin B₁₂ bound to TC II and HC in milk-fed lambs receiving vitamin B₁₂ injections and soduim propionate

Group	Sampling	TC II-cbl pmol/l ± 462	HC-cbl pmol/l ± 114	TC II-cbl % ± 3.6	HC-cbl % ± 3.6
SEM					
+B ₁₂ /-Pr	4	1683	187	90	10
	6	1717	257	87	13
	8	2002	353	85	15
+B ₁₂ /+Pr	4	1753	335	84	16
	6	3474	613	85	15
	8	2791	532	84	16

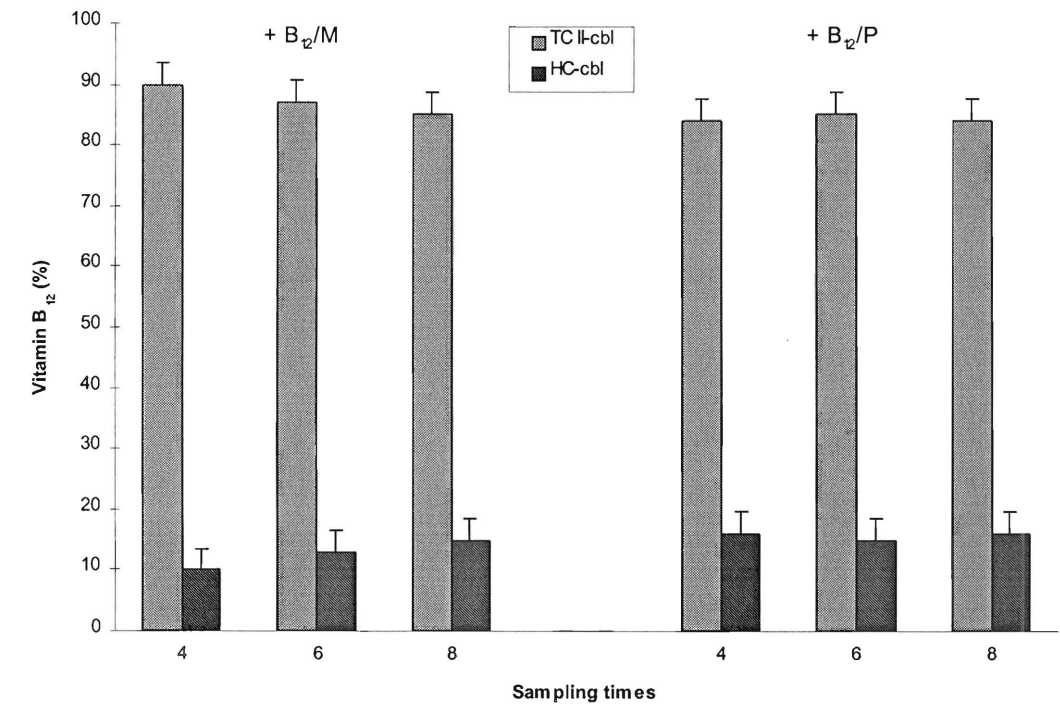


Figure 4.4: Proportion (%) of plasma vitamin B₁₂ bound to TC II and HC in milk-fed lambs receiving vitamin B₁₂ injections and sodium propionate

These results indicate that 80 to 90 % of vitamin B₁₂ in plasma of PR lambs is carried on TC II, regardless of dietary propionate. This is in agreement with Schultz and Judson (1985) who found that the major binder of injected vitamin B₁₂ in sheep blood was TC II. Babidge (1993) found a decrease in holo TC II to be an indicator of marginal Co deficiency in sheep. Unfortunately, this could not be verified due to lack of data from the -B₁₂ groups. As blood in this trial was taken in heparinised plasma tubes, competitive binding of this heparin with the HS in the assay binders (van Kapel *et al.*, 1988) was also likely. Further studies are warranted.

The increase in plasma vitamin B₁₂ concentrations as a result of propionate could not be explained by a preferential stimulation of one of the binders and further work will be required to elucidate the mechanism. The work does, however, highlight the possibility that dietary factors such as grass or grain feeding may be a source of variation in plasma vitamin B₁₂ concentrations and a source of confounding of clinical analysis. It remains to be seen, however, whether such effects operate under marginal vitamin B₁₂ status conditions.

CHAPTER 5

Absorption, assimilation and storage of vitamin B₁₂ in pre-ruminant lambs

5.1 Introduction

There are only a few trials that look at PR lambs and their vitamin B₁₂ requirement and utilisation. Lambs receive their first vitamin B₁₂ during gestation from maternal blood and after birth through milk. Yet no estimates on the availability of vitamin B₁₂ in milk have been reported. Supplementation with vitamin B₁₂ injections is short-lived (Sargison *et al.*, 1997; Grace *et al.*, 1998), suggesting that PR lambs do not readily store vitamin B₁₂ in the liver over longer periods. Although the studies outlined in Chapter 4 have shown that liver vitamin B₁₂ in lambs will respond to fairly large injections of vitamin B₁₂ there are no data on the ability of lambs to absorb vitamin B₁₂.

5.2 Aim

The aim of this trial was to investigate the availability of physiological amounts of vitamin B₁₂ to tissues (plasma and liver) and the ability of PR lambs to absorb vitamin B₁₂ by comparing the ability of injected and orally administered vitamin B₁₂ to raise blood and liver levels, and to gain an estimate of the requirement of young lambs for vitamin B₁₂ from an estimate of the amount required to raise tissue concentrations.

5.3 Experimental design

For the two-week trial, 27 Halfbred lambs were used. Three were kept as control and twelve assigned to each of two groups - oral and IM supplementation of vitamin B₁₂. The oral and IM groups were further divided into five sub-groups which received increasing quantities of vitamin B₁₂ by the designated route. The oral groups

received tenfold the amount of vitamin B₁₂ as the IM groups, on the assumption that all of the injected (IM) vitamin B₁₂ would be absorbed, but only 10 % of the oral dose. The amounts of vitamin B₁₂ in blood and liver of each group would then be similar. The precise ratio of the increases should reflect the proportion of vitamin B₁₂ absorbed. The experiment lasted two weeks, from October 28 to November 11, 1998.

In a follow-up experiment, 14 of these lambs were divided into three groups: 3 control, 6 oral and 5 IM vitamin B₁₂. They received the same amount of vitamin B₁₂ per 100 g liver weight that the highest supplementation groups received in the previous experiment and blood vitamin B₁₂ concentrations were monitored for 24 h. The appearance pattern in plasma would then reflect the relative amounts entering plasma. This experiment took place on November 18 to 19, 1998, when the lambs were three to four weeks old.

5.4 Materials and methods

5.4.1 Experimental animals

The 27 lambs were selected from a cobalt deficient farm near Methven (New Zealand) three to four days after birth and weighed on average 6.25 kg. They were ear-tagged and housed in pens at JML. Animals were allocated to three groups hierarchically according to body weight, comprising three, twelve and twelve lambs. Lambs belonging to the same sub-group in the first experiment shared the same pen. This pen allocation was maintained throughout the time the lambs were indoors (including the second experiment) to keep stress levels to a minimum. Any calculations made in the first part of the trial were based on an actual body weight of 6.67 kg, and estimates of a liver weight of 100 g and a plasma volume of 270 ml (1.5 % and 4 % of b.w., respectively - Wallace, 1948; Sykes, 1974). In the second part of the trial, calculations were based on an actual body weight of 10 kg, and estimates of a liver weight of 150 g and a plasma volume of 400 ml.

5.4.2 Diet

The lambs were fed exclusively on milk replacer throughout. In the two week trial, the lambs were fed by bottle at 0800 h and at 1600 h. The milk was made up freshly each feed and comprised 200 g of milk powder to one litre of water. The milk replacer used was Anlamb (Anchor, N. Z. Dairy Board, Auckland). The lambs were offered 300 g of milk per feed on day one and two, 500 g on day three to six, 600 g on day seven and eight, and 700 g until the end of the trial. Their individual bottles were weighed before and after feeding and intakes recorded. Some animals developed diarrhoea in the first week of the trial, therefore the milk replacer was diluted by 20 % (80:20; milk:water) from day ten to fourteen. Milk contained 2210 nmol/l vitamin B₁₂.

5.4.3 Vitamin B₁₂ treatment

In order to find the doses needed to raise liver vitamin B₁₂ in these lambs by administering physiological amounts, the following considerations were made: If a minimum increment of 0.1 µg vitamin B₁₂/g fresh liver is anticipated and the livers of the lambs in this trial weighed about 100 g, then absorption of at least 10 µg vitamin B₁₂ would be required. From the trial described in Chapter 4, liver stores increased by approximately 600 nmol/kg in a 28 day period. The daily increase amounted to roughly 25 nmol/kg or 35 µg/kg fresh liver. If this represents the maximum storage capacity this would represent 3.5 µg vitamin B₁₂/d for a liver weighing 100 g.

The outline of the two week trial is given in Table 5.1. Column three gives the dilution of Prolaject (1000 µg OH-cbl in 1 ml water) in physiological saline that was made up to bring the amounts of vitamin B₁₂ needed for treatment into a range so that it could be dispensed with ease. Columns four and five show the volumes administered, and columns six and seven give the amount of vitamin B₁₂ contained therein. (The actual amounts the lambs received were adjusted to their body weights.) The increments were chosen so that the highest would be close to the maximum liver storage ability of 35 µg/kg/d (or 3.5 µg/100 g/d). Lambs were treated each afternoon (between 1400 and 1500 h), before their second feed. Vitamin B₁₂ was either injected

IM (gluteus muscle) or administered orally via syringe.

Table 5.1: Treatment of PR lambs with either IM or oral administered vitamin B₁₂

Group	No. of lambs	Concentration (Prolaject in saline)	IM ml	Oral ml	B ₁₂ IM* µg/d	B ₁₂ oral* µg/d
0	3		0.0	0	0.0	0
1	3 IM / 3 oral	0.4 ml/l	0.5	5	0.2	2
2	2 IM / 2 oral	0.4 ml/l	1.0	10	0.4	4
3	2 IM / 2 oral	0.8 ml/l	1.0	10	0.8	8
4	2 IM / 2 oral	3.2 ml/l	0.5	5	1.6	16
5	3 IM / 3 oral	3.2 ml/l	1.0	10	3.2	32

* 1 µg ≅ 743 pmol

5.4.4 Sampling

The lambs were weighed at day 0, 7 and 14. Blood (5 ml in Na heparin) was taken twice a week from day 0 to day 14 of the trial. Liver biopsies (~ 100 mg) were obtained from all lambs on day 0 and 14. (For clinical and analytical procedures see Chapter 3.)

The 24 h trial started at 0800 h and finished the following morning at 0800 h. All the lambs were blood sampled (2 ml) twice just prior to treatment, treated and re-blood sampled (2 ml) immediately. Further blood samples (2 ml) were taken at intervals after the treatment, first half hourly, then every 2 h, every 4 h and the last sample after 8 h (Figure 5.1). The lambs were fed by bottle at 1030 h and 1630 h and their milk intake measured to determine the extra amount of vitamin B₁₂ derived from it.

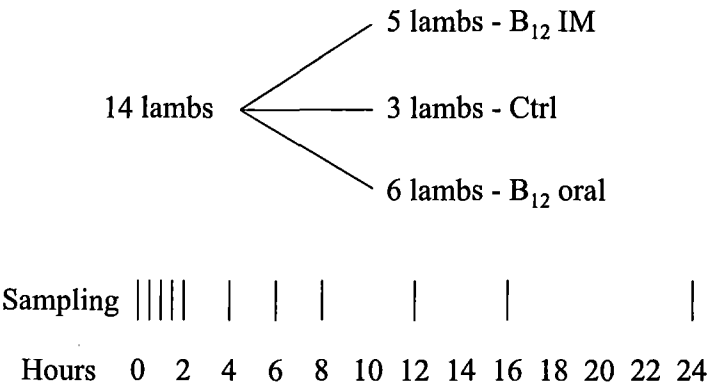


Figure 5.1: Outline of trial and sampling times

5.4.5 Statistical analysis

Means and SEM as well as differences in plasma and liver vitamin B₁₂, MMA and weight due to the varying levels of supplementation over time in the two week trial were calculated in Genstat (ANOVA). Standard errors (SE) for the vitamin B₁₂ intake from milk and supplementation was calculated in Excel. Comparisons within group five (oral, IM, Ctrl) in the two week trial and within the three groups at the last two samplings in the 24 h trial were done with Minitab, ANOVA, General Linear Model. The coefficients for the calculated curves in the 24 h trial were determined with Genstat.

5.5 Results

5.5.1 Two week trial

Table 5.2 summarises the mean daily milk intake of the lambs and the amount of vitamin B₁₂ they obtained from milk and treatment. No SE were calculated on the milk intake as some of the lambs drank all of their ration and would have drunk more had they been given the opportunity.

The mean weight gain in the two weeks was 1.32 ± 0.12 kg. There was no obvious pattern to suggest that weight gain was correlated with milk intake. There was no effect of treatment on weight gain.

Table 5.2: Mean milk intake and vitamin B₁₂ obtained from milk and oral/IM vitamin B₁₂ treatment (Trt) per day for each group

Group	Milk intake	B ₁₂ intake	Amount	Group	Milk intake	B ₁₂ intake
IM	(ml/d)	(milk + trt)	B ₁₂	oral	(ml/d)	(milk + trt)
		(pmol/d)				(pmol/d)
Ctrl	839	1854	0	Ctrl	839	1854
1	820	1970	1x	1	1046	3694
2	1052	2606	2x	2	925	4853
3	959	2679	4x	3	1107	8094
4	839	2971	8x	4	914	13193
5	1056	4592	16x	5	872	23799

The only sub-groups that showed any significant difference in type of treatment, time and dose of vitamin B₁₂ administered on plasma vitamin B₁₂ concentrations were subgroups 4 oral and 5 oral. From the third sampling until the

end of the trial 5 oral was highly significantly different from all the other sub-groups ($p < 0.001$).

Changes in plasma vitamin B₁₂ values are shown in Figures 5.2 and 5.3. Plasma vitamin B₁₂ values ranged from below 57 pmol/l to 240 pmol/l initially, then increased for all the sub-groups, including Ctrl, at the second sampling. Thereafter, plasma vitamin B₁₂ values declined in most cases, or the increase was only minimal. The only exception was sub-group 5 oral that showed consistently positive results. There was large variation between the lambs in the same sub-group as well as between the sub-groups and the different sampling dates. However, most lambs had plasma values below 500 pmol/l, i.e. low or marginal values, despite supplementation.

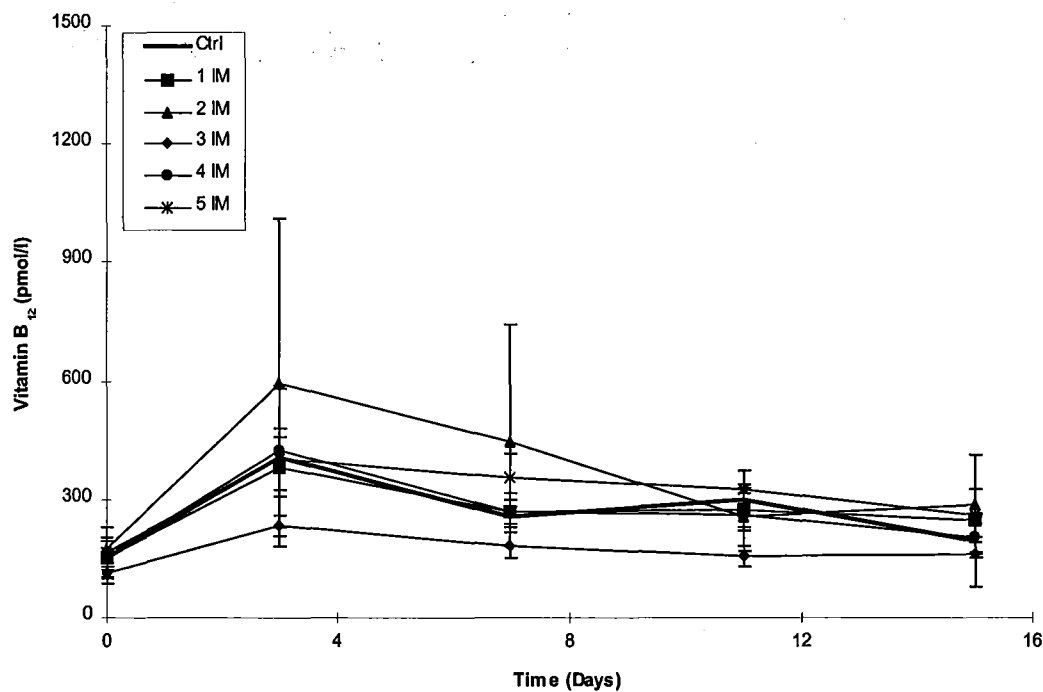


Figure 5.2: Changes in plasma vitamin B₁₂ for the IM vitamin B₁₂ supplemented group ($\pm$ SEM)

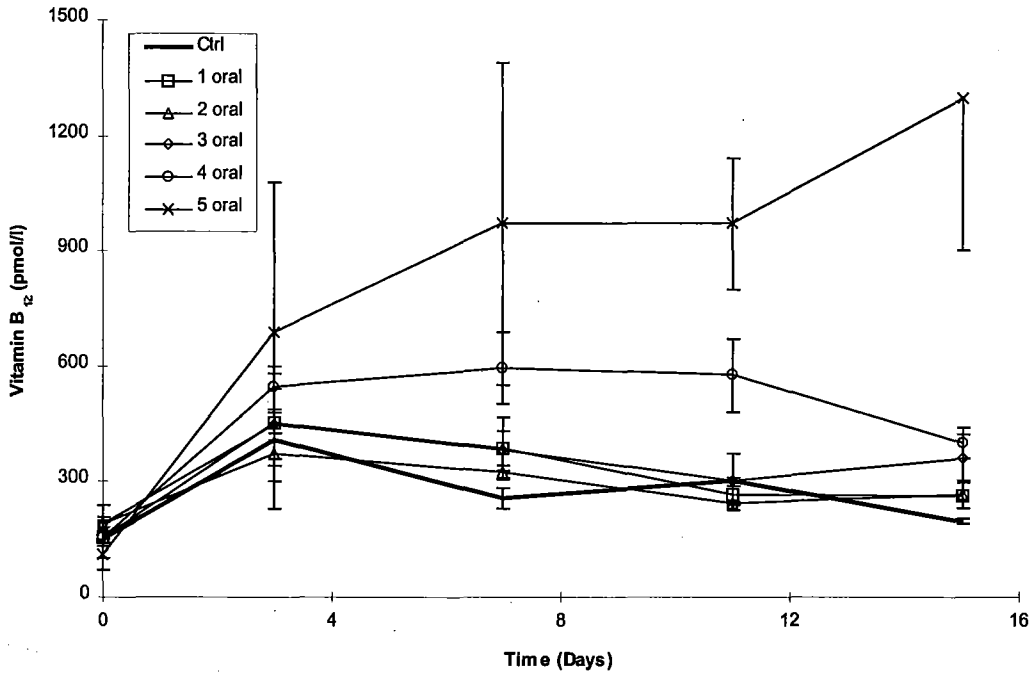


Figure 5.3: Changes in plasma vitamin B₁₂ for the oral vitamin B₁₂ supplemented group ($\pm$ SEM)

Figures 5.4 and 5.5 show the changes in liver vitamin B₁₂ during the trial. Liver vitamin B₁₂ did not show any consistent pattern. The only sub-group where all the lambs showed an increase in liver vitamin B₁₂ was 5 oral. This sub-group was also the one with the highest overall increase. However, it failed to reach statistical significance.

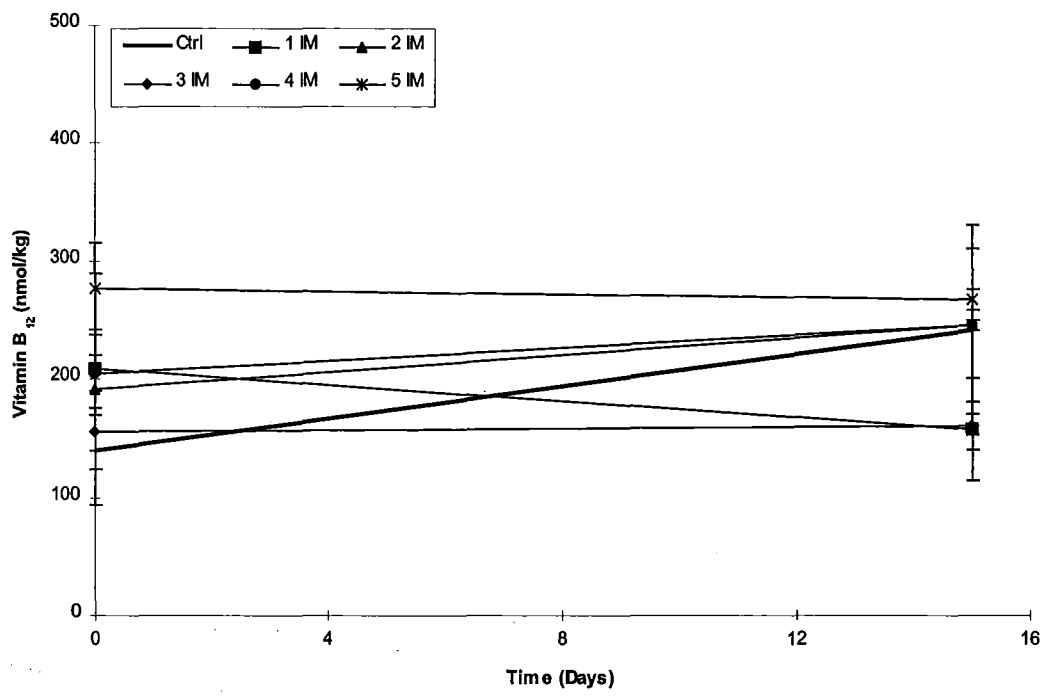


Figure 5.4: Changes in liver vitamin B₁₂ for the IM vitamin B₁₂ supplemented group (± SEM)

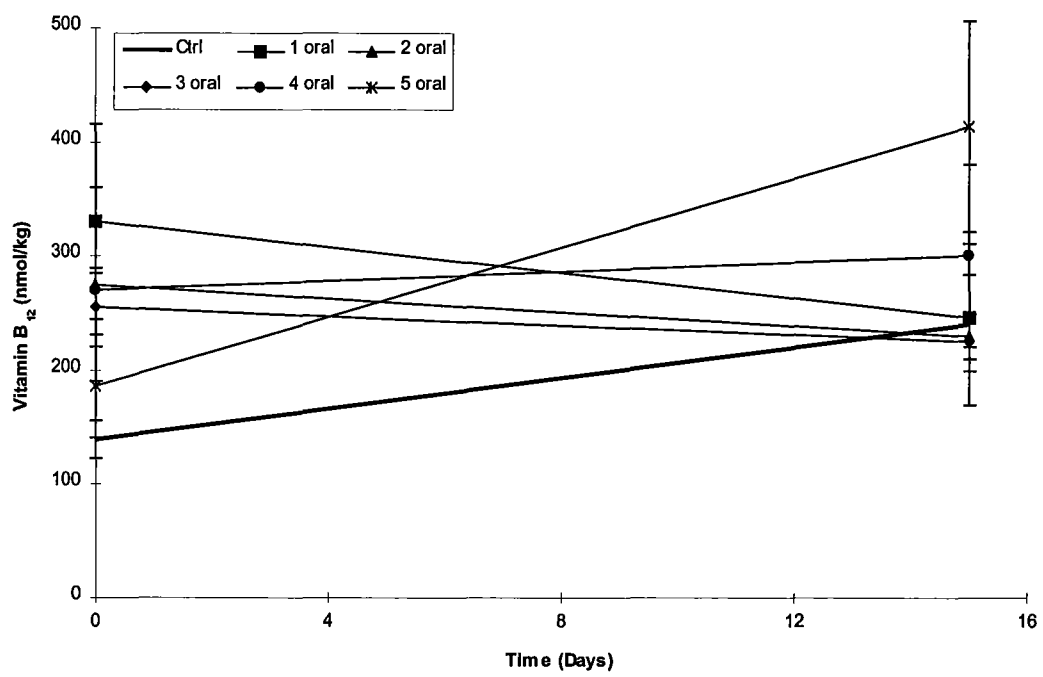


Figure 5.5: Changes in liver vitamin B₁₂ for the oral vitamin B₁₂ supplemented group (± SEM)

MMA ranged from 0.30 ± 0.19 to 1.24 ± 0.19 $\mu\text{mol/l}$. However, any statistically significant effects were biologically meaningless as these values were at the lower detection limit of the assay. Fluctuations were therefore more likely caused by the background noise of the assay rather than differences in the metabolically produced MMA.

5.5.2 24 hour trial

The amount of vitamin B₁₂ the animals received from milk in the two feeds offered was between 2400 and 3000 pmol, depending on how much the lambs drank (some did not finish their rations). Vitamin B₁₂ obtained from treatment added another 23770 pmol to the oral group and 2377 pmol to the IM group.

Figures 5.6 to 5.8 show the plasma vitamin B₁₂ levels in the three groups during the 24 h sampling period. The groups were three distinct, statistically highly significantly different, units ($p < 0.005$). The data ($\blacklozenge$) have been approximated by a best curve of fit (—). Equations for the calculated curves are given where x is the time in minutes.

For the control group, the curve was linear, expressed by

$$y = -0.0493x + 168.82$$

Vitamin B₁₂ stayed relatively steady throughout the 24 h trial period.

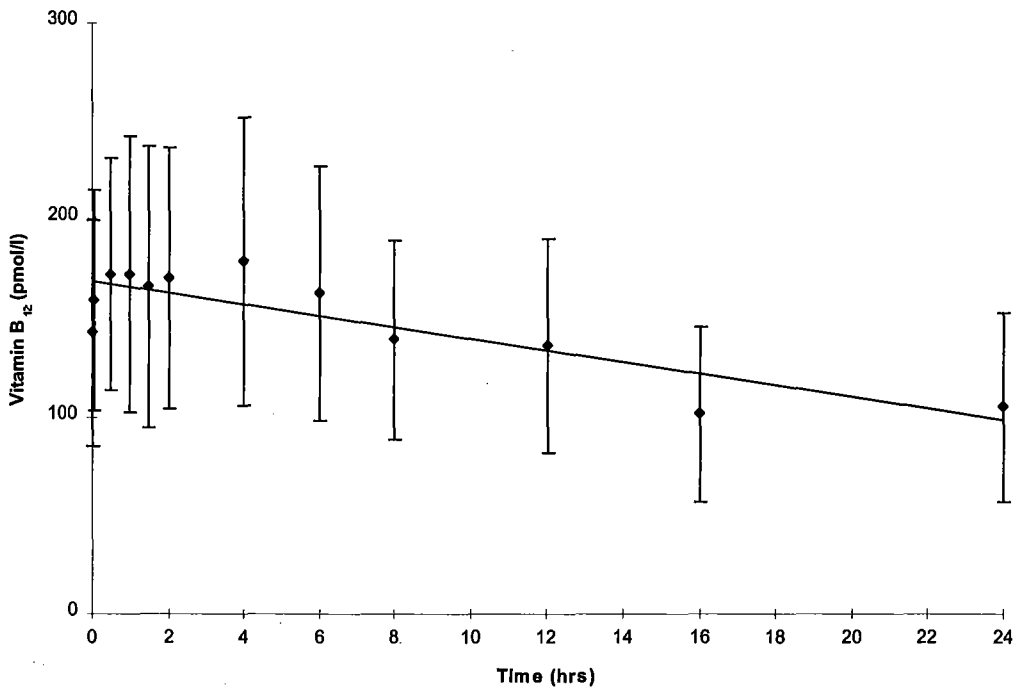


Figure 5.6: Plasma vitamin B₁₂ levels during the 24 h period for the unsupplemented (Ctrl) group ($\pm$ SEM)

For the IM group, the data could be approximated best by a double exponential curve:

$$y = 659 + (-6269 * 0.9536)^x + (6357 * 0.9968)^x$$

Plasma vitamin B₁₂ increased dramatically within 2 min of the IM injection and reached a peak after 1 h during which plasma values rose 5366 pmol/l. This was followed by a steep decline in plasma vitamin B₁₂ over the next 4 h where the loss from plasma far outweighed influx of the vitamin into plasma. This process slowed down somewhat during the last 8 h.

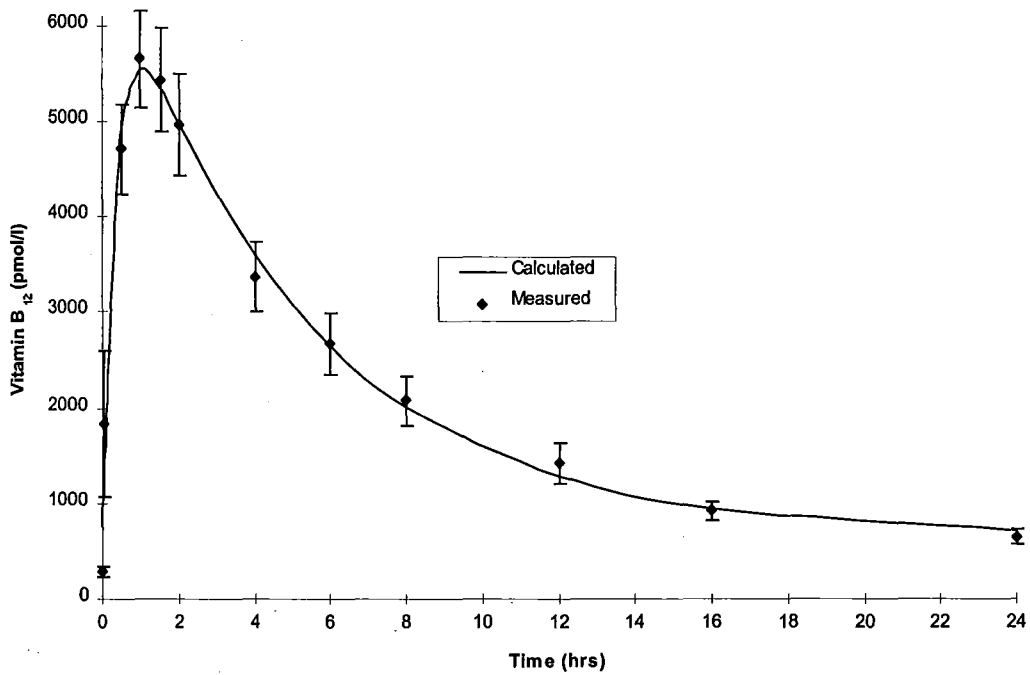


Figure 5.7: Plasma vitamin B₁₂ levels during the 24 h period for the IM vitamin B₁₂ supplemented group (± SEM)

The data for the oral group could be expressed best by a linear exponential curve:

$$y = 3803 + (-3742 * 0.99731)^x - 1.92x$$

Plasma values increased by 1677 pmol/l in the first 4 h of the trial. In that time, plasma uptake of oral vitamin B₁₂ was greater than losses due to metabolism. Maximum absorption of the vitamin into plasma occurred 2 to 6 h post-dosing. Between 6 and 12 h post-dosing an equilibrium between influx of the vitamin into blood and metabolism was established. In the last 12 h there was a gradual decline in plasma vitamin B₁₂ when metabolic losses exceeded plasma uptake.

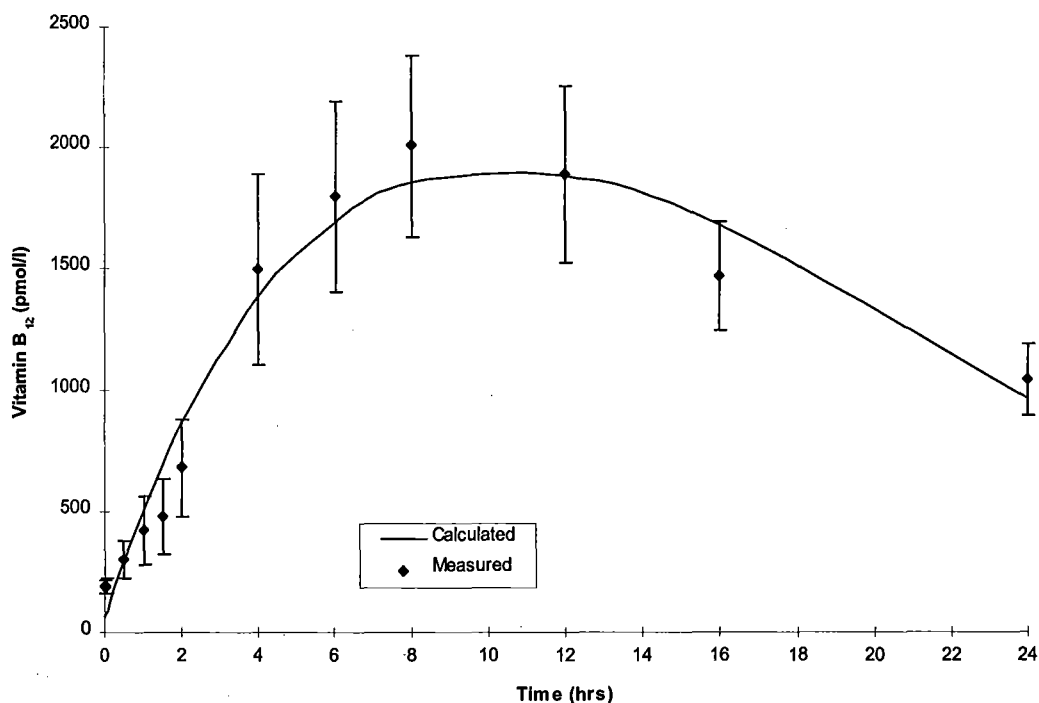


Figure 5.8: Plasma vitamin B₁₂ levels during the 24 h period for the oral vitamin B₁₂ supplemented group ($\pm$ SEM)

5.6 Discussion

5.6.1 Two-week trial

In this trial an estimate of the daily requirements of PR lambs for vitamin B₁₂ was to be found. However, even the highest rate of IM administration of vitamin B₁₂ was not enough to achieve a rate of entry sufficient to raise liver and plasma vitamin B₁₂ concentrations. Marston (1970), in a similar trial, determined that 11 μ g ($\sim$ 8100 pmol) was the minimum amount sheep needed for daily metabolism. The IM dose he used to achieve this result amounted to about half of what was injected as the highest dose in this trial on a body weight basis. Since his lambs were ruminants and required vitamin B₁₂ for gluconeogenesis, their needs for the vitamin would have been greater than the metabolic needs of the PR lambs used in this trial. An increase in tissue vitamin B₁₂ concentrations could therefore have been expected in this trial.

However, the 5 oral group did show the expected increase in liver and plasma vitamin B₁₂ concentrations. This could indicate that either oral absorption of the

vitamin is greater than 10 % of the injected dose in PR lambs or the vitamin is retained better in the body when administered orally. This could be due to a slower pattern of uptake or better coupling to transport proteins. Extending the trial by including higher doses of both IM and oral would clarify this further.

Marston's (1970) results were obtained with 25 kg lambs. Assuming a linear relationship of requirement for vitamin B₁₂ to body weight, the requirements would be 325 nmol/kg b.w. (8100 pmol/25 kg). The PR lambs in this trial would therefore require between 2000 and 3000 pmol/d (6 to 9 kg b.w. x 325 nmol/kg). This amount was amply provided by milk, assuming all of its vitamin B₁₂ could be utilised as was suggested by O'Halloran and Skerman (1961).

As was seen in the control groups in both experiments, the lambs showed little change in plasma vitamin B₁₂ so the amount of vitamin B₁₂ in the milk replacer seemed to have been metabolised at approximately the same rate as it was absorbed. All the treated lambs would therefore have had an extra supply of the vitamin that could have been used to increase blood and liver concentrations. That this was not the case may indicate that lambs use vitamin B₁₂ in a profligate way when more than their daily requirement is supplied and conserve it in times of need. Similarly, Smith and Marston (1970) found that the efficiency of conversion of Co into vitamin B₁₂ was higher in a Co deplete than replete animal. Determining urinary vitamin B₁₂ losses would have been an advantage. However, attempts to measure the vitamin in urine gave inconsistent results.

The onset of deficiency in Marston's (1970) trial in the six months old lambs started in week 18 (i.e. five weeks after the start of treatment); the lambs on higher dosages of the vitamin did not develop symptoms until week 60. Younger animals generally have less storage capacity and are less resilient and might be expected to develop signs of deficiency at an earlier stage. On the other hand, their needs are probably less. Nonetheless, in order to establish a link between vitamin B₁₂ and the onset of deficiency symptoms, this trial would have needed to be extended timewise. However, this is possible only to a limited extent since lambs generally progress from milk to forage far more quickly than deficiency can be induced. Besides, attempts in our laboratory at making up a vitamin B₁₂ deficient milk replacer that the lambs will accept is difficult, if not impossible (Glass, 1996).

Since the 5 oral group did show an increase in plasma and liver vitamin B₁₂ concentrations, the percentage of vitamin B₁₂ retained was calculated. These lambs received 309 nmol in 13 days from treatment. Of that, they had retained 22.6 nmol in their livers (100 g liver weight - Wallace, 1948) and 0.3 nmol in plasma (270 ml plasma volume; Sykes, 1974), i.e. ~ 23 nmol in total. This amounted to ~ 7.5 % of the dose administered. Given that some vitamin B₁₂ would have been metabolised this seems a conservative estimate of absorbability and suggests a value much higher than the 3 to 5 % calculated by Marston (1970) for ruminant lambs.

5.6.2 24 hour trial

When the vitamin is injected IM or subcutaneously, the first stage of binding to IF and absorption through the enterocyte is bypassed. Therefore, the vitamin would reach the blood in a very short time. In order to estimate the amount of IM vitamin B₁₂ absorbed into blood the intercept of the downward slope with the y-axis at 6500 pmol/l (Figure 5.9) was taken as the amount of vitamin B₁₂ in plasma after the injection if all of it had entered the blood immediately and none of it had already metabolised in that first hour. Subtracting the concentration of vitamin B₁₂ in plasma before the injection (~ 300 pmol/l) from this figure, the increase amounted to ~ 6200 pmol/l. Consequently, ~ 2500 pmol would have been in the 400 ml of plasma of these lambs. This roughly equals the amount injected (~ 2400 pmol). Hence, essentially all of the injected vitamin B₁₂ had appeared in plasma, i.e. uptake had been close to 100 %.

Babidge (1993) gave 2 mg of the different forms of 'true' vitamin B₁₂ subcutaneously to heifers and followed blood and liver vitamin B₁₂ concentrations over a period of two weeks. Despite the much larger dose administered by Babidge a similar pattern as that for the IM group in Figure 5.9 was seen. In Babidge's (1993) trial there were still marginal, non-significant decreases of the vitamin on day two and three, after which plasma vitamin B₁₂ concentrations had returned to near baseline.

For the oral group, appearance of vitamin B₁₂ in blood was much slower (Figure 5.9), reflecting the passage of vitamin B₁₂ through the ileal enterocyte which is considered to take about 4 to 8 h (Linnell and Matthews, 1984; Allen, 1976; el

Kholty *et al.*, 1991). The system would have had time to absorb and retain the incoming vitamin B₁₂, especially since the vitamin needs to pass through the liver first before it appears in systemic blood. At the end of the 24 h trial 6 % of the orally administered vitamin B₁₂ was still apparent in plasma.

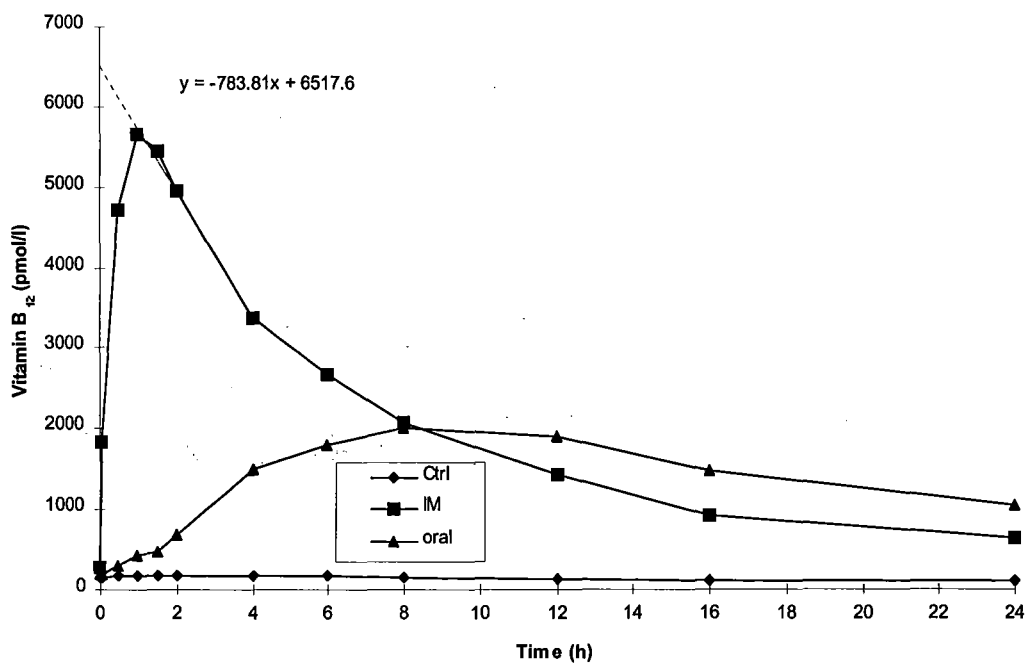


Figure 5.9: Plasma vitamin B₁₂ over a 24 h period following treatment for control, IM and oral groups, with trendline for IM group to indicate intercept with y-axis

To get an indication on the amount of vitamin B₁₂ that was available to the lamb the areas under the curves in the 24 h trial were compared. The areas between the treated and control groups' vitamin B₁₂ (Figure 5.9) were traced on paper and cut out. The area under the oral curve was 72 % of the area under the IM curve. Since the tenfold amount of vitamin B₁₂ was administered to the oral group compared to the IM group and assuming that all of the vitamin was taken up by the latter, it appears that uptake of the vitamin in the oral group was ~ 7 %. However, the body would have established some sort of equilibrium between metabolic reactions, storage and elimination over the 24 h so that this figure is a rather crude estimate and probably an underestimation.

The low oral absorption figure obtained here is in contrast to milk-derived vitamin B₁₂ of which virtually all was considered to be absorbed in these early stages of life (O'Halloran and Skerman, 1961). Perhaps the difference lies in milk vitamin B₁₂ being protein-bound, whereas the oral vitamin B₁₂ administered was in free (unbound) form.

In the control group no diurnal fluctuation was seen in the 24 h trial. This is in agreement with Millar *et al.* (1984) who found that plasma vitamin B₁₂ concentrations did not seem to vary over a 24 h period in sheep at pasture. However, Marston (1970) found a diurnal variation in serum vitamin B₁₂ concentrations in penned sheep in response to feeding. The difference between Millar *et al.* (1984) and Marston (1970) may well be that sheep at pasture have continual access to food and therefore a constant supply of the vitamin can be produced. On the other hand, the lambs in this trial were fed only twice a day, like in Marston's trial, but no diurnal variation was observed. It is possible that the different production and absorption rates of VFAs from grain/herbage and subsequent conversion to glucose in ruminants in comparison to glucose from lactose in PR lambs are responsible for this.

In this trial, increases in plasma and particularly liver vitamin B₁₂ concentrations from physiological doses has not been as effective as assumed. Other factors influencing the absorption, transport, and retention of the vitamin, such as the transport proteins TC II and haptocorrin or the intracellular binding proteins methylmalonyl-CoA mutase and methionine synthase, could well not be fully matured in a PR lamb. Further work is needed to understand the intricate metabolism of vitamin B₁₂, especially in PR lambs (and probably also in the young of other ruminant species).

CHAPTER 6

Vitamin B₁₂ and methylmalonic acid status of pregnant and lactating ewes and their lambs

6.1 Introduction

This chapter describes two supplementation trials of ewes and lambs on a property in Mid-Canterbury (43°47' latitude south, 171°20' longitude east). It was chosen on advice from the local veterinarian and an indication from the previous season of very high MMA levels (~ 150 µmol/l for serum MMA and 4000 µmol/l for urinary MMA) in suckling lambs. Responsiveness to Co and/or vitamin B₁₂ supplementation could therefore be anticipated. The pregnant and lactating ewes and their lambs grazed on acid orthic brown soil (Ruapuna stony silt loam) which has been recorded to be low to marginal in Co (Andrews, 1972). The pastures were traditional perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.).

6.2 Aim

The objective of the two trials described in this chapter was to define relationships between traditional markers of deficiency - serum and liver vitamin B₁₂ concentrations - and metabolic byproducts such as MMA and hcy concentrations and, if possible, define critical concentrations of the latter. By combining and comparing the results those markers may facilitate the establishment of more accurate reference values for Co deficiency in sheep and PR lambs.

6.3 Experimental Design

6.3.1 1997/1998

Fifty primiparous two-tooth pregnant halfbred ewes were selected from a large flock. Pregnancy was confirmed by scanning (Aloka 210 DXII, Aloka Co Ltd., Japan). The ewes were then divided randomly into two groups of 25. Group one received a Co bullet (PermaCo, 2 g Co) and a vitamin B₁₂ injection (1 ml of Prolaject, 1000 µg/ml) on day 0, and group two acted as control. The whole mob was grazed in the same paddock. Figure 6.1 gives an overview of the trial, the samples taken and their timing. The trial ran from 28/8/1997 until 9/2/1998 (= day 0 to day 165).

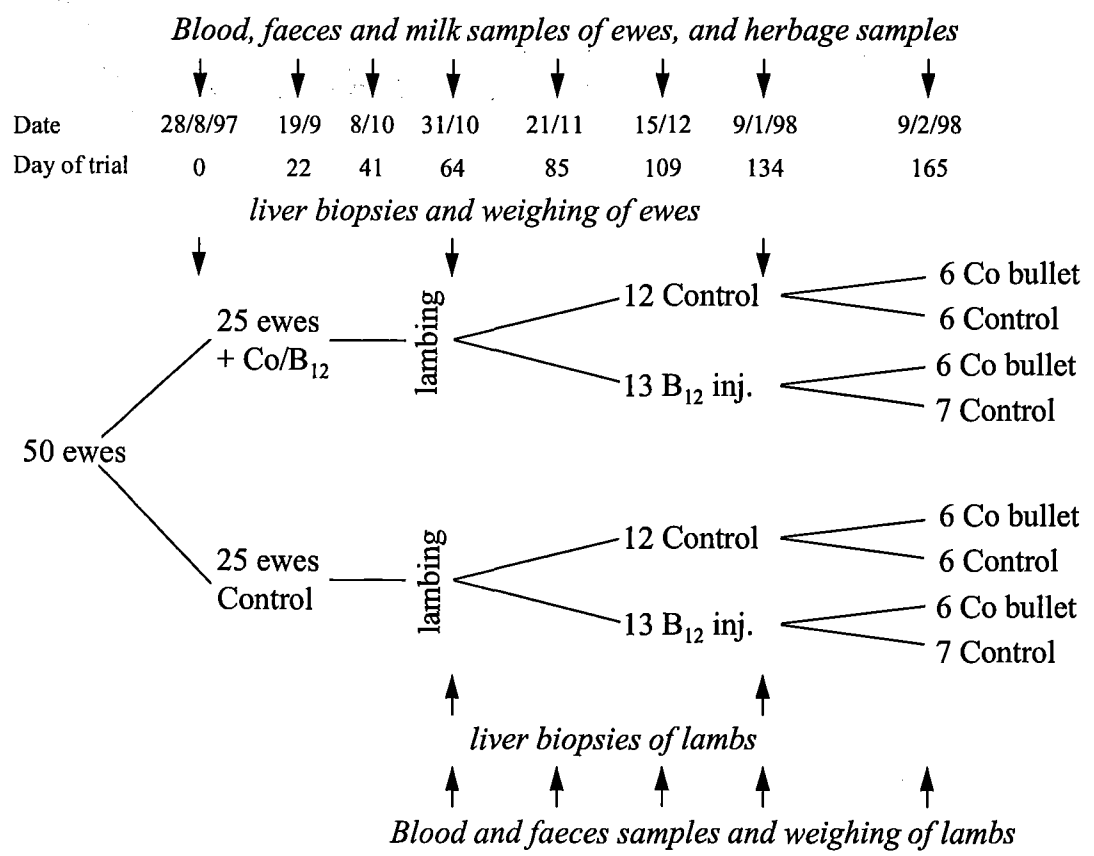


Figure 6.1: Trial design for 1997/1998

Lambing commenced on 29/9/1997 and was completed by 30/10/1997 (days 32 to 63). On 31/10/1997 the lambs from each group of ewes were subdivided into two groups, containing 12 or 13 lambs in each group. One group of lambs from each

ewe group received a vitamin B₁₂ injection (1 ml Prolaject) after each tissue sampling, and the second group of lambs served as control. At approximately ten weeks of age the groups of lambs were further subdivided and half of each group of lambs received a Co bullet (PermaCo) after tissue sampling. They were then followed up, together with their dams, for another four weeks.

6.3.2 1998/1999

The trial described above was repeated on the same farm one year later. The ewes from the previous year (three had died) were used again in this trial. Those that were supplemented in the 1997/1998 trial were supplemented again in the 1998/1999 trial, and unsupplemented ewes in 1997/1998 remained control ewes in the following year. They were augmented by pregnant (confirmed by scanning) two-tooth primiparous ewes from the next age cohort to make up ewe numbers to 76. The two-tooth ewes were randomly allocated to treatment or control. On day 0 all the treated ewes received two Co bullets (PermaCo) each to counteract any possible regurgitation or coating of the bullet. The whole mob was grazed in the same paddock as in the previous year. Figure 6.2 outlines the trial, which ran from 27/8/1998 to 7/1/1999 (= day 0 to day 133), and the samples taken. The following abbreviations have been used:

UE: Unsupplemented ewes (control)

SE: Supplemented ewes (Co bullet)

O: 'Old' ewes - from 1997/1998 trial

N: 'New' ewes - first used in 1998/1999 trial

UL: Unsupplemented lambs

SL: Supplemented lambs (vitamin B₁₂ injections)

e.g. OUE/SL: Supplemented lambs from unsupplemented ewes that were first used in the 1997/1998 trial.

Lambing started on 27/9/1998 and was completed by 8/11/1998 (days 31 to 73). On 29/10/1998 the lambs of each group of ewes that were born by that time (all but three) were divided and allocated to either treatment or control. Treatment of lambs (vitamin B₁₂ injection, 1 ml Prolaject) was given at each sampling until the

end of the trial after tissue samples had been taken. The two lambs born in the three days prior to the first occasion that liver biopsies were due to be taken were not biopsied at that stage due to their delicate nature. Three more lambs were born after group allocation. They were allocated to their respective groups to balance numbers at the next sampling on 19/11/1998. They too were not liver biopsied until the end of the trial.

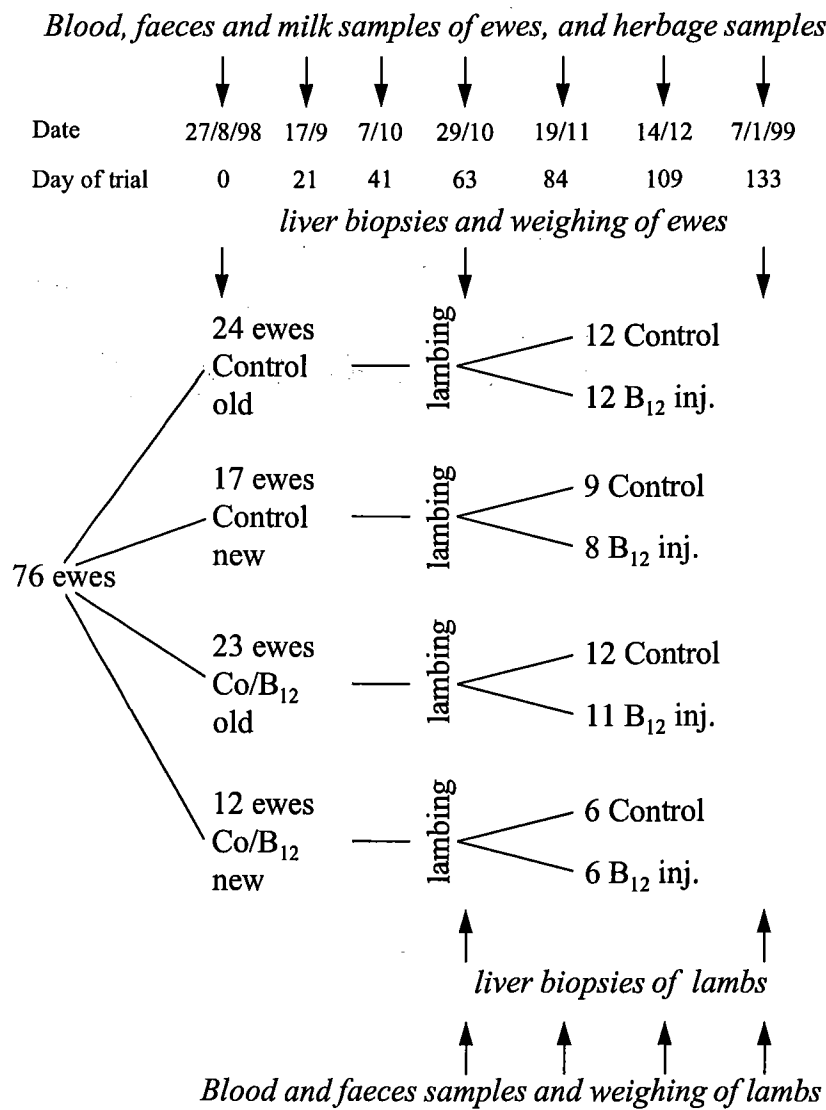


Figure 6.2: Trial design for 1998/1999

6.4 Materials and Methods

6.4.1 Sampling

The animals were yarded within 15 min of our arrival. First, blood (~ 10 ml) was collected by jugular venipuncture as the stress involved in prolonged yarding can elevate vitamin B₁₂ levels in blood (Millar *et al.*, 1984). In the 1997/1998 trial, blood was taken into Na heparin tubes and centrifuged at the farm (at 3000 g for 10 minutes at 20°C) within 2 h of sampling. In the 1998/1999 trial, blood was taken into plain tubes and the serum separated in the laboratory the following day.

Milk samples (10 to 20 ml), obtained by hand milking the ewes, were collected from the time the first lambs were born, i.e. from the third visit to the farm, until most ewes were dry at the seventh visit (October until January). Faecal samples from both ewes (only from the second sampling on in 1997/1998) and lambs were collected by rectal probing.

The animals were then weighed. In 1997/1998, ewes were weighed on the days liver biopsies were taken and again at the end of the trial. For the ewes in the 1998/1999 trial, weighing was done at the first sampling and at every sampling once they had lambed. Lambs were weighed at birth and at every sampling until the end of the trial.

Liver biopsies (as outlined in Chapter 3) were then performed on the farm by qualified veterinarians. Lastly, the animals to be treated received a vitamin B₁₂ injection or Co bullet, respectively. Herbage samples were taken at random from the paddock which the animals were grazing at the time of sampling. On return to the laboratory plasma, liver and milk samples were stored at -20°C and faeces samples at 4°C until analysis. Serum was pipetted off the following day and also stored at -20°C until analysis. A portion of the serum (~ 2 ml), liver (~ 50 mg) and milk (~ 2 ml) samples were sent to Labnet Invermay for vitamin B₁₂ analysis. Pasture samples were lyophilised before being sent for Co assay to Hill Laboratories, Hamilton, New Zealand.

6.4.2 Statistical analysis

Table 6.1 gives an overview of the structure of the groupings of the animals. The ewes were grouped according to treatment and, in the second year, also according to age. The lambs were grouped according to their dams' treatment and age, and their own treatment. For the last sampling in the first year, administration of a Co bullet after the previous sampling had also been taken into account for the grouping.

ANOVA was performed using Genstat, calculating where possible means and overall SEM for each group as well as the interaction between treatment x time, and treatment x 'age' x time (in 1998/1999) of the various groups (Kenward, 1987). Degrees of freedom have been adjusted using the Greenhouse-Geisser epsilon (Greenhouse and Geisser, 1959) since not all measurements were independent of each other. Student's t-tests and SED have been used to find the LSD for various p-values ($p < 0.05$, < 0.01 and < 0.001). Orthogonal polynomial analysis of the data was performed to identify the components of the shapes of the curves and the relationship between the curves of the various groups. Genstat has also been used to find any possible correlation between various traits of the ewes and their offspring.

In order to stabilise the error variance in data for the faecal egg count (FEC) of different animals, the data were transformed by calculating the cubed root, and then the conversion back was made by calculating $[\text{data}]^3$. Confidence intervals (CI) were calculated for each set of data rather than SEM.

Table 6.1: Grouping of animals for statistical analysis

Ewes	1997/1998		1998/1999	
	Lambs	Lambs (last sampling)	Ewes	Lambs
SE	SE/SL	SE/SL	OSE	OSE/SL
		SE/SL + Co		OSE/UL
	SE/UL	SE/UL	NSE	NSE/SL
UE	UE/SL	SE/UL + Co	OUE	NSE/UL
		UE/SL		OUE/SL
		UE/SL + Co		OUE/UL
	UE/UL	UE/UL	NUE	NUE/SL
		UE/UL + Co		NUE/UL

6.5 Results

This section has been set up in such a way that the results for each trait measured or analysed in each year are described in succession. The same scales, where possible, have been chosen for the figures to display the same parameters so that comparisons can be made more easily.

6.5.1 Pasture cobalt

Table 6.2 shows the pasture Co content during the 1997/1998 trial. The range was from 0.09 to 0.77 $\mu\text{g/g DM}$. In December, just prior to the sampling on 9/1/1998, the animals had grazed a different paddock (house paddock) for several days. Therefore a sample from both paddocks had been taken. The animals were returned to their usual paddock straight after that sampling.

Pasture Co for the 1998/1999 trial is given in Table 6.3. It ranged from 0.26 to 0.85 $\mu\text{g/g DM}$. For the first three sampling periods the animals had been shifted to a different paddock due to shortage of feed in their usual paddock. Whenever the animals grazed a different paddock from the one they usually occupy their usual paddock was also sampled and the Co values given in *italics*. The Co values for the second sampling seemed erroneously high (12.1 $\mu\text{g/g DM}$). As the farmer had not applied topical Co to his pastures these samples seem to have been contaminated.

Table 6.2: Cobalt content of pasture 1997/1998 ($\mu\text{g/g DM}$)

Date	28/08/1997	19/09/1997	8/10/1997	31/10/1997
Day	0	22	41	64
Co (usual paddock)	0.77	0.37	0.17	0.09

Date	21/11/1997	15/12/1997	9/01/1998	9/02/1998
Day	85	109	134	165
Co (usual paddock)	0.10	0.10	0.31	0.40
Co (house paddock)			0.41	

Table 6.3: Cobalt content of pasture 1998/1999 (µg/g DM)

Date	27/08/1998	17/09/1998	7/10/1998	29/10/1998
Day	0	21	41	63
Co (usual paddock)	0.35	21.90	0.41	0.49
Co (new paddock)	0.55	12.10	0.10	

Date	19/11/1998	14/12/1998	7/01/1999
Day	84	109	133
Co (usual paddock)	0.26	0.30	0.85

6.5.2 Plasma/serum vitamin B₁₂

6.5.2.1 Ewes

The mean plasma vitamin B₁₂ values of the 1997/1998 trial are given in Table 6.4 and Figure 6.3. There was a highly significant treatment x time interaction ($p < 0.001$) due to the difference between the treated and untreated ewes in all the samplings after the start of treatment.

In the untreated ewes the plasma vitamin B₁₂ concentration was 338 pmol/l at the start of the trial. It fell to 101 pmol/l at lambing and then rose to 949 pmol/l at the end of the trial. There was a highly significant increase ($p < 0.01$) between samplings six and seven, coinciding with an increase in pasture Co.

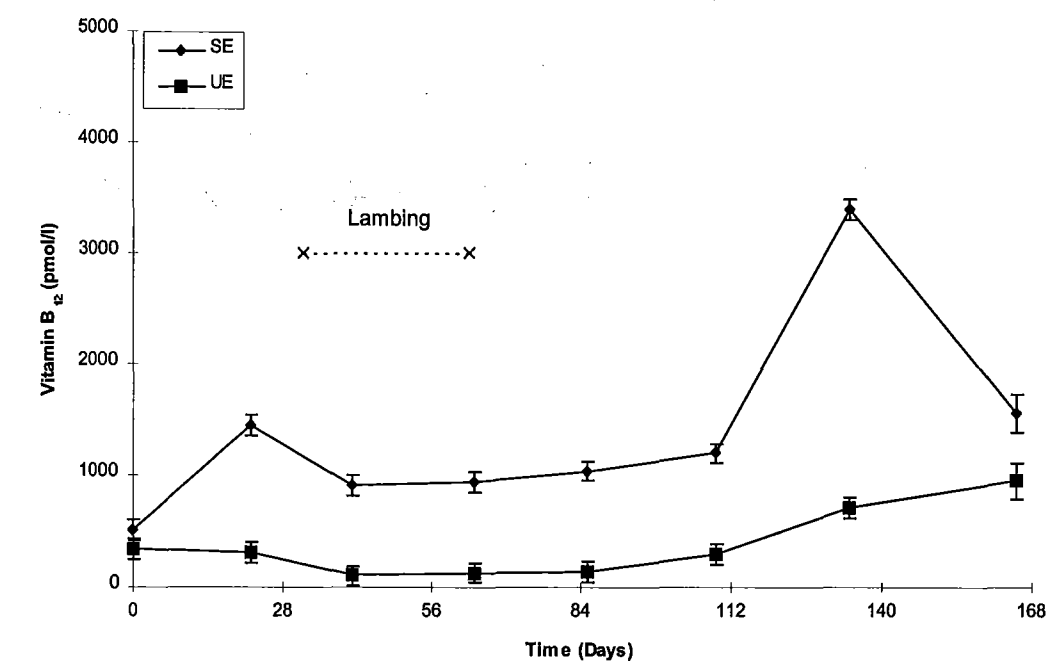
The treated ewes began with a mean plasma vitamin B₁₂ value of 506 pmol/l which rose to 1555 pmol/l at the end of the trial. In the second and seventh samplings there was a highly significant increase in plasma vitamin B₁₂ ($p < 0.001$). In both cases, this was followed by a highly significant drop ($p < 0.001$) in plasma values. By the second sampling, most of the treated ewes had plasma vitamin B₁₂ values above 1500 pmol/l, the upper limit of the assay at Invermay at the time. However, in samplings three to six only few ewes had values above this limit. From sampling seven onwards the assay was changed to allow for higher plasma vitamin B₁₂ values to be measured (hence the relatively large peak in comparison to the previous sampling). Therefore the mean for samplings two to six for the SE group was somewhat underestimated. The general trend, though, would still be reflected in Figure 6.3.

Table 6.4: Mean plasma vitamin B₁₂ (pmol/l) of Co bullet treated and control two-tooth ewes 1997/1998 (SEM = ± 90.5)

Date	28/08/1997	19/09/1997	8/10/1997	31/10/1997
Day	0	22	41	64
SE	506	1452	906	941
UE	338	311	101	130

Date	21/11/1997	15/12/1997	9/01/1998	9/02/1998
Day	85	109	134	165
SE	1037	1193	3384	1555
UE	140	288	702	949

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.3: Mean plasma vitamin B₁₂ of Co bullet treated and control two-tooth ewes 1997/1998 (± SEM)

For the 1998/1999 trial (Table 6.5 and Figure 6.4), there was a highly significant treatment x time x ‘age’ interaction ($p < 0.001$) due to the treated ewes having higher and increasing values compared to the untreated ewes and higher values in the OSE group than in the NSE group. The effect of treatment x time when the two age groups were combined was highly significant ($p < 0.001$) with predominately linear components.

The previously treated ewes (OSE) started with a mean serum vitamin B₁₂ level of 2764 pmol/l, whereas all the other groups started between 417 and 486 pmol/l. Mean serum vitamin B₁₂ of the untreated ewes fell to ~ 260 pmol/l when the lambs were born and increased again at the end of the trial to just above starting values (447 to 523 pmol/l). There was no significant change in vitamin B₁₂ concentration over time for the UE groups which essentially were similar.

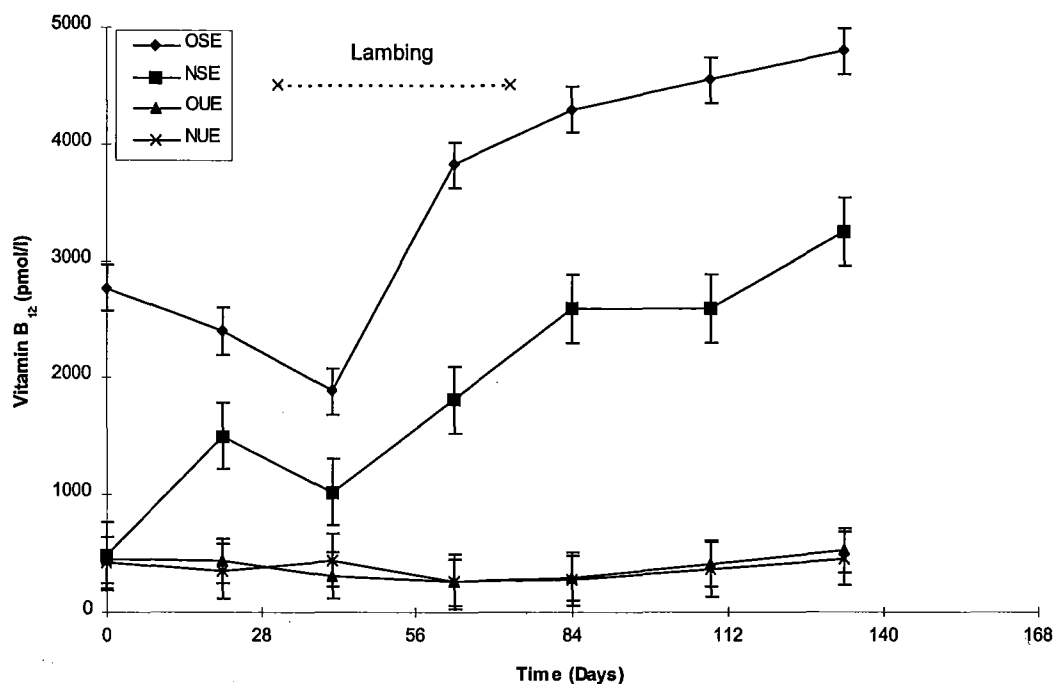
Supplementation in the NSE group brought vitamin B₁₂ levels up to 1500 pmol/l by the second sampling. However, by the third sampling, at the start of lambing, vitamin B₁₂ of both SE groups had dropped significantly ($p < 0.001$) by approximately one third. This was followed by a steep rise to 1814 pmol/l for the NSE group and to 3822 for the OSE group. This difference was highly significant ($p < 0.001$) throughout the trial.

Table 6.5: Mean serum vitamin B₁₂ (pmol/l) of Co bullet treated and control two-tooth and four-tooth ewes 1998/1999

Date	27/08/1998	17/09/1998	7/10/1998	29/10/1998
Day	0	21	41	63
OSE	2764	2398	1887	3822
NSE	486	1500	1023	1814
OUE	445	436	309	257
NUE	417	351	441	261

Date	19/11/1998	14/12/1998	7/01/1999	SEM
Day	84	109	133	
OSE	4291	4548	4794	± 196.8
NSE	2587	2587	3241	± 284.5
OUE	293	412	523	± 192.6
NUE	282	365	456	± 228.9

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.4: Mean serum vitamin B₁₂ of Co bullet treated and control two-tooth and four-tooth ewes 1998/1999 ($\pm$ SEM)

6.5.2.2 Lambs

The mean plasma vitamin B₁₂ values for the 1997/1998 lambs are given in Table 6.6 and Figure 6.5. There was a highly significant interaction between lamb supplementation and time which had linear ($p < 0.001$) and quadratic components ($p < 0.01$). Throughout the trial there was a significant difference in lamb plasma vitamin B₁₂ values due to supplementation of the ewe ($p < 0.05$).

The lambs of the SE groups had an initial mean plasma vitamin B₁₂ concentration of 324 pmol/l, whereas the lambs born to the UE groups had a significantly lower ($p < 0.05$) mean plasma vitamin B₁₂ value of 163 pmol/l. Plasma vitamin B₁₂ increased for all the groups in the course of the trial. The rise was slow initially, then steeper between samplings two and four, before the values declined somewhat at the last sampling.

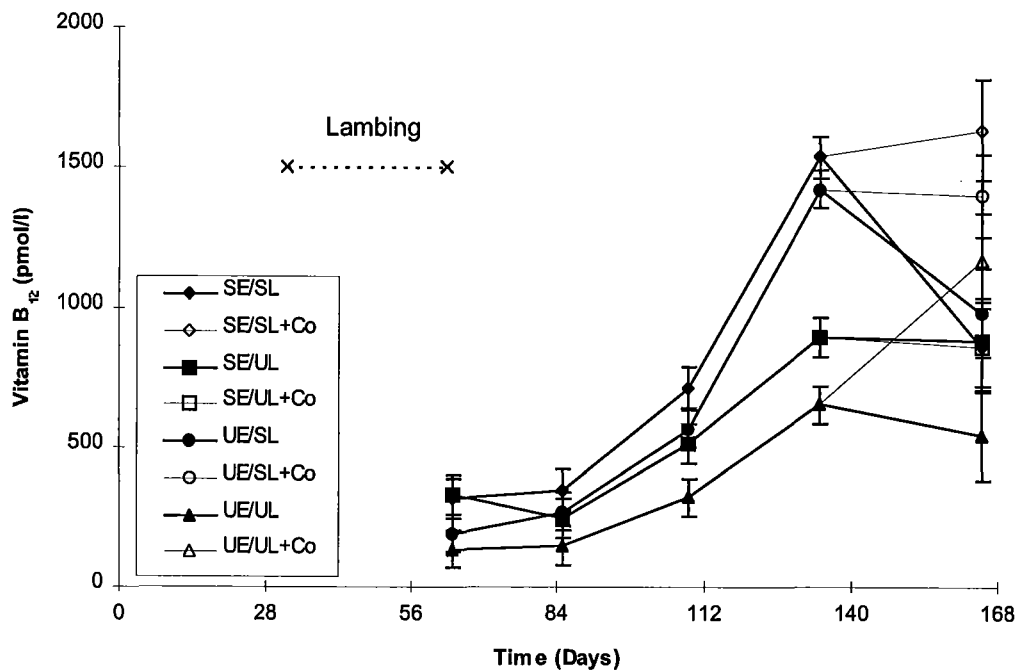
The treatment with Co bullets after the fourth sampling significantly ($p < 0.001$) elevated plasma vitamin B₁₂ concentrations in the fifth sampling in all the

groups except in SE/UL. Plasma vitamin B₁₂ in the UE/UL group nearly doubled on Co supplementation. In the non-Co supplemented groups plasma vitamin B₁₂ values fell between the fourth and fifth samplings except in the SE/UL group. In that group plasma vitamin B₁₂ stayed approximately the same between the fourth and last sampling, regardless of Co supplementation.

Table 6.6: Mean plasma vitamin B₁₂ (pmol/l) of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1997/1998 (± SEM)

Date Day	31/10/1997 64	21/11/1997 85	15/12/1997 109	9/01/1998 134	9/02/1998 165
SE/SL	319 ± 73.7	346 ± 73.7	709 ± 73.7	1536 ± 73.7	854 ± 160.3
+ Co					1630 ± 179.3
SE/UL	329 ± 70.1	247 ± 70.1	515 ± 70.1	893 ± 70.1	874 ± 160.3
+ Co					856 ± 160.3
UE/SL	191 ± 66.8	268 ± 66.8	562 ± 66.8	1420 ± 66.8	978 ± 160.3
+ Co					1398 ± 146.4
UE/UL	135 ± 66.8	147 ± 66.8	321 ± 66.8	650 ± 66.8	540 ± 160.3
+ Co					1166 ± 160.3

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.5: Mean plasma vitamin B₁₂ of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1997/1998 (± SEM)

Mean serum vitamin B₁₂ values for the lambs in the 1998/1999 trial are given in Table 6.7 and Figure 6.6. No significant effect due to the ‘age’ of dams was observed, therefore comparisons were made only between supplemented and unsupplemented ewes and lambs.

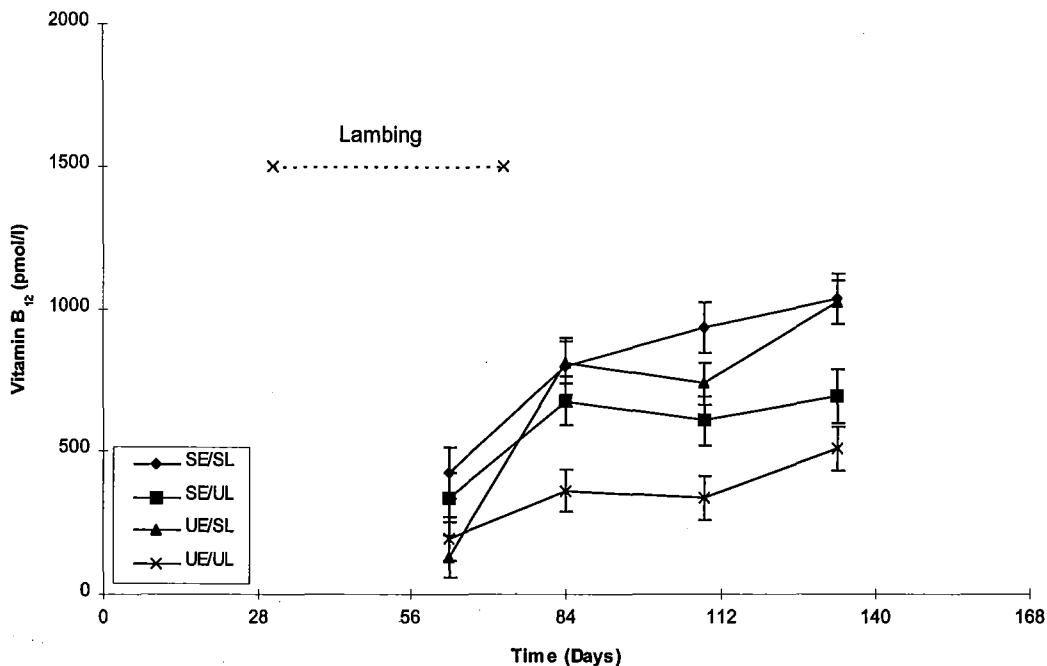
There were highly significant linear and cubic effects ($p < 0.001$ for both) of time for all the curves due to the initial increase in serum vitamin B₁₂ in all groups which was followed by a slight drop for all groups except SE/SL before rising again. The initial rise was highly significant ($p < 0.001$) for all the groups except for the UE/UL group. The interaction of lamb treatment x time had mainly linear components ($p < 0.001$).

The lambs from treated ewes had a significantly higher ($p < 0.01$) initial serum vitamin B₁₂ concentration (~ 10 days after birth) than the ones from untreated ewes (382 and 163 pmol/l, respectively). That difference lessened with time and by the fourth sampling there was no significant difference between those two groups.

Table 6.7: Mean serum vitamin B₁₂ (pmol/l) of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1998/1999 (± SEM)

Date Day	29/10/1998 63	19/11/1998 84	14/12/1998 109	7/01/1999 133
SE/SL	425 ± 88.0	798 ± 99.6	934 ± 88.0	1030 ± 88.0
SE/UL	339 ± 86.6	675 ± 86.7	605 ± 86.6	690 ± 93.1
UE/SL	132 ± 75.6	809 ± 74.2	735 ± 74.2	1021 ± 74.2
UE/UL	194 ± 75.3	362 ± 73.9	335 ± 75.5	507 ± 75.5

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.6: Mean serum vitamin B₁₂ of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1998/1999 ($\pm$ SEM)

6.5.3 Liver vitamin B₁₂

6.5.3.1 Ewes

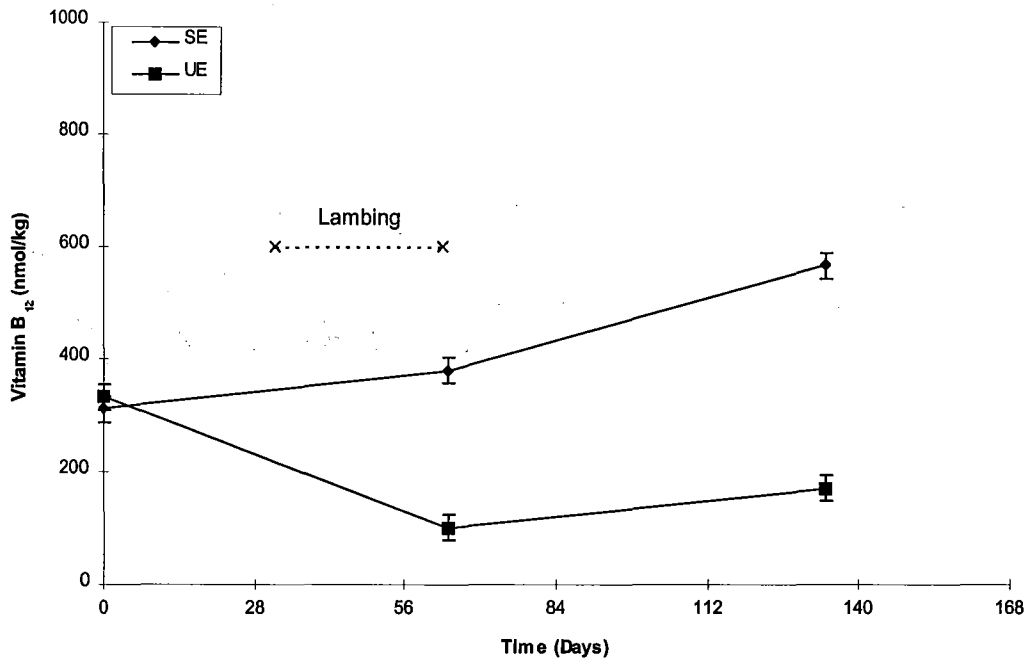
The mean liver vitamin B₁₂ concentrations of ewes in 1997/1998 are given in Table 6.8 and Figure 6.7. The treatment x time interaction was highly significant with linear ($p < 0.001$) and quadratic ($p < 0.05$) components.

The ewes had mean liver vitamin B₁₂ values of around 320 nmol/kg fresh tissue at the start of the trial. In the UE group liver vitamin B₁₂ fell to 100 nmol/kg fresh tissue at lambing time (sampling two) before reaching a mean of 170 nmol/kg fresh tissue at weaning (sampling three). The initial drop and the subsequent rise were significant ($p < 0.001$ and $p < 0.05$, respectively). There was a steady rise to 566 nmol/kg fresh tissue at the end of the trial for the SE group.

Table 6.8: Mean liver vitamin B₁₂ (nmol/kg) of Co bullet treated and control two-tooth ewes 1997/1998 (SEM = ± 22.8)

Date	28/08/1997	31/10/1997	9/01/1998
Day	0	64	134
SE	312	379	566
UE	332	100	170

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.7: Mean liver vitamin B₁₂ of Co bullet treated and control two-tooth ewes 1997/1998 (± SEM)

The mean liver vitamin B₁₂ concentrations of ewes in 1998/1999 are given in Table 6.9 and Figure 6.8. The ewes that were supplemented in the previous year started off with a higher mean liver vitamin B₁₂ value than the rest of the ewes (677 versus 421 nmol/kg fresh tissue), a highly significant difference ($p < 0.001$). There was a highly significant treatment x time interaction with linear components ($p < 0.001$) but no effect due to time x treatment x 'age'. However, the OSE group maintained its initial advantage, so that the rise in liver vitamin B₁₂ for the SE groups

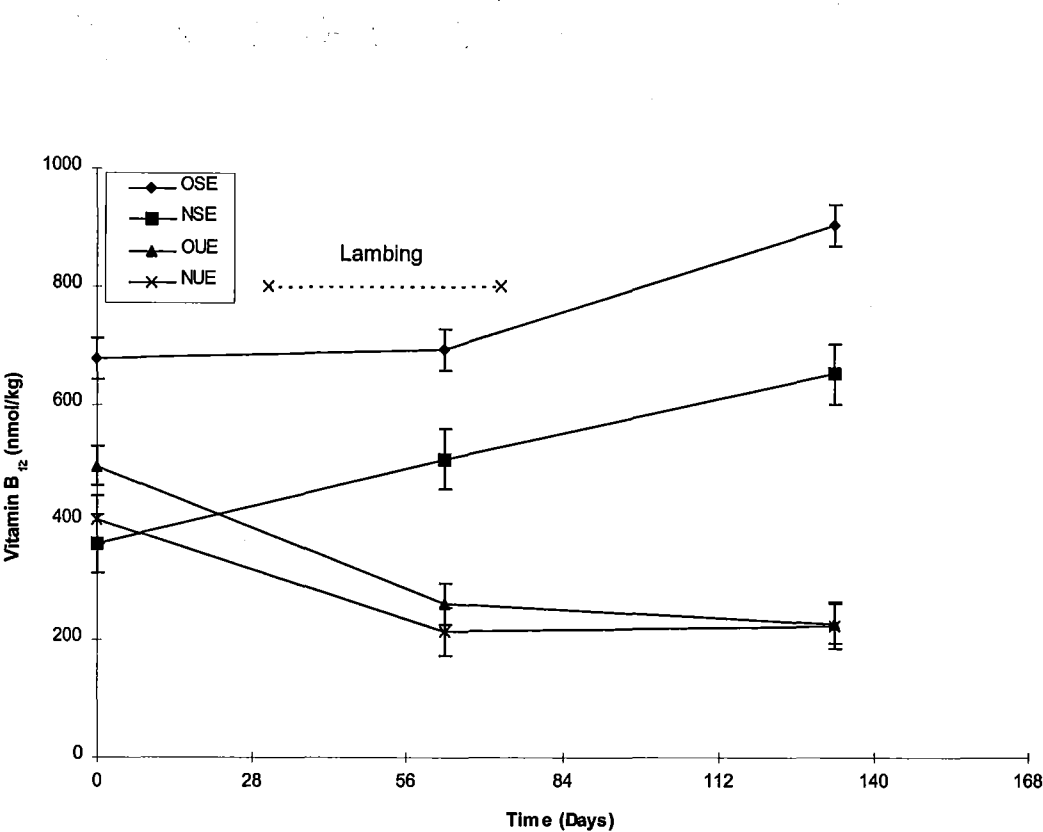
showed a parallel trend (the difference between OSE and NSE was 313 nmol/kg initially and 256 nmol/kg fresh tissue at the end of the trial).

In the UE groups liver vitamin B₁₂ fell significantly ($p < 0.001$) between the first and second sampling and stayed steady after that. Although the OUE group had started with a higher mean liver vitamin B₁₂ value than the NE groups there was no difference due to ‘age’ in the untreated groups at the end of the trial.

Table 6.9: Mean liver vitamin B₁₂ (nmol/kg fresh tissue) of Co bullet treated and control two-tooth and four-tooth ewes 1998/1999

Date Day	27/8/1998 0	29/10/1998 63	7/1/1999 133	SEM
OSE	677	693	901	± 34.6
NSE	364	507	650	± 50.0
OUE	495	262	228	± 33.9
NUE	404	216	225	± 40.2

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.8: Mean liver vitamin B₁₂ of Co bullet treated and control two-tooth and four-tooth ewes 1998/1999 (± SEM)

6.5.3.2 *Lambs*

Mean lamb liver vitamin B₁₂ values for the 1997/1998 trial are given in Table 6.10 and Figure 6.9. There was a significant difference between the lambs born to treated ewes compared to those born to untreated ewes ($p < 0.05$). The interaction between lamb treatment and time was highly significant ($p < 0.001$).

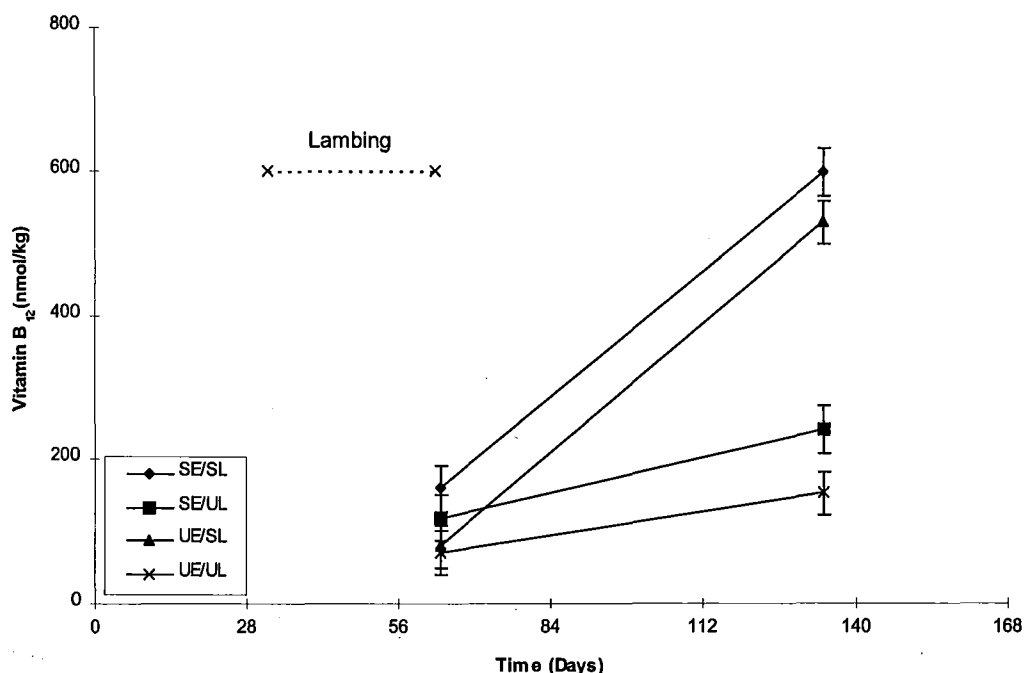
At the first sampling, the SE/SL group had the highest liver value (160 nmol/kg fresh tissue), followed by SE/UL, UE/SL and UE/UL (119, 80 and 71 nmol/kg fresh tissue, respectively), but there was no significant difference between these groups.

At the second liver sampling vitamin B₁₂ concentrations in the SE/SL group reached 597 nmol/kg fresh tissue. The UE/SL group followed this trend in a parallel way to reach a mean liver concentration of 528 nmol/kg fresh tissue. Similarly, liver vitamin B₁₂ values in the SE/UL and UE/UL groups rose parallel to 241 and 153 nmol/kg fresh tissue, respectively. Some lambs in the SE/SL and UE/SL groups reached levels close to 900 nmol/kg fresh tissue, but concentrations in the SE/UL lambs stayed below 500 and in the UE/UL lambs below 400 nmol/kg fresh tissue. The difference between treated and untreated lambs had become highly significant ($p < 0.001$). Even the SE/UL group had a significantly ($p < 0.05$) higher liver vitamin B₁₂ value than the UE/UL group.

Table 6.10: Mean liver vitamin B₁₂ (nmol/kg fresh tissue) of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1997/1998 ($\pm$ SEM)

Date Day	31/10/1997 64	9/01/1998 134
SE/SL	160 $\pm$ 31.4	597 $\pm$ 33.1
SE/UL	119 $\pm$ 31.4	241 $\pm$ 33.1
UE/SL	80 $\pm$ 29.9	528 $\pm$ 29.9
UE/UL	71 $\pm$ 29.9	153 $\pm$ 29.9

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.9: Mean liver vitamin B₁₂ of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1997/1998 ($\pm$ SEM)

Mean liver vitamin B₁₂ values for the 1998/1999 lambs are given in Table 6.11 and Figure 6.10. There was a highly significant difference ($p < 0.001$) between the lambs born to ewes in the SE and to those in the UE groups. The lamb treatment x time interaction was also highly significant ($p < 0.001$).

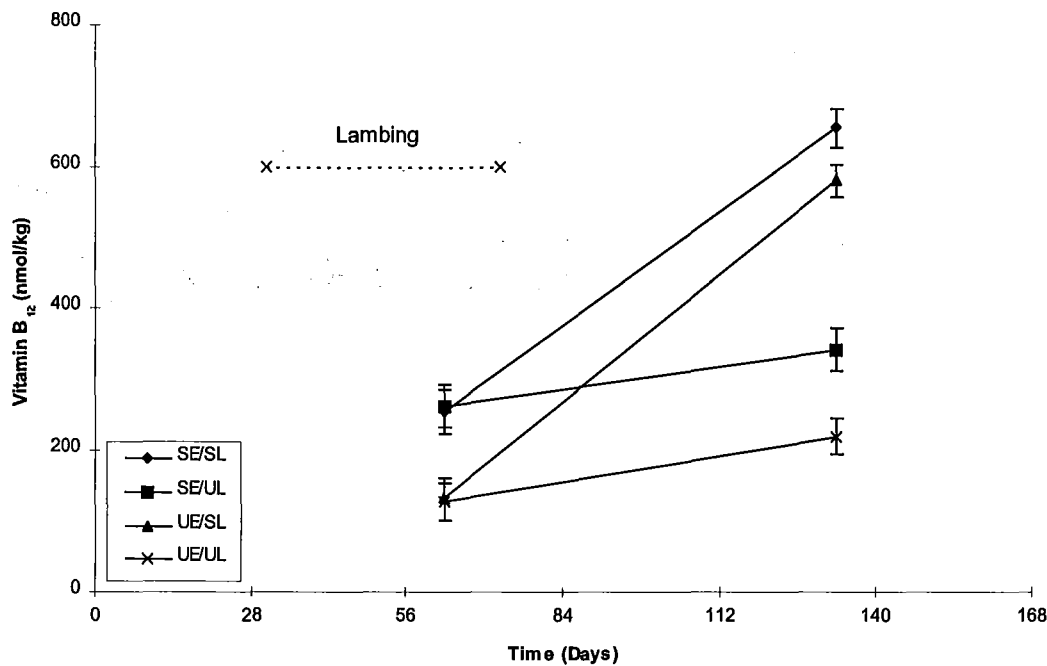
Liver vitamin B₁₂ concentrations were slightly higher than in the previous year. The effect of 'age' of the ewe was barely significant so groups were combined into ewe and lamb treatments only. Lambs from treated ewes had initial mean liver vitamin B₁₂ concentrations of 258 and final ones of 497 nmol/kg fresh tissue, whereas those of untreated ewes had initially 129 and at the end 399 nmol/kg fresh tissue.

Treated and untreated lambs had similar values at the first sampling (mean 194 nmol/kg fresh tissue). Liver vitamin B₁₂ concentrations in both SL and UL groups had increased by the second sampling to 616 and 280 nmol/kg fresh tissue, respectively. The increase in the SL groups and the UL groups followed a parallel trend, similar to that of the previous year.

Table 6.11: Mean liver vitamin B₁₂ (nmol/kg) of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1998/1999 (± SEM)

Date Day	29/10/1998 63	7/1/1999 133
SE/SL	254 ± 31.5	643 ± 28.3
SE/UL	262 ± 30.4	341 ± 30.1
UE/SL	131 ± 30.3	579 ± 23.3
UE/UL	127 ± 25.5	219 ± 24.4

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.10: Mean liver vitamin B₁₂ of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1998/1999 (± SEM)

6.5.4 Methylmalonic acid

6.5.4.1 Ewes

The mean plasma MMA values for the ewes in 1997/1998 are given in Table 6.12 and Figure 6.11. There was a highly significant interaction between treatment and time which had quadratic components ($p < 0.001$) due to the rise in MMA to

14.2 $\mu\text{mol/l}$ on day 64 and subsequent fall while supplemented ewes maintained relatively constant values in the range of 1 to 3 $\mu\text{mol/l}$.

The UE group started with a mean MMA concentration of 2.3 $\mu\text{mol/l}$. Mean MMA concentrations reached 14.2 $\mu\text{mol/l}$ immediately after lambing, with individual ewes in the UE group reaching up to 65 $\mu\text{mol/l}$. (One high MMA value of 187 $\mu\text{mol/l}$ was excluded from the mean and SEM of sampling six in the UE group.) Plasma MMA values then decreased to 3.4 $\mu\text{mol/l}$ at the end of the trial.

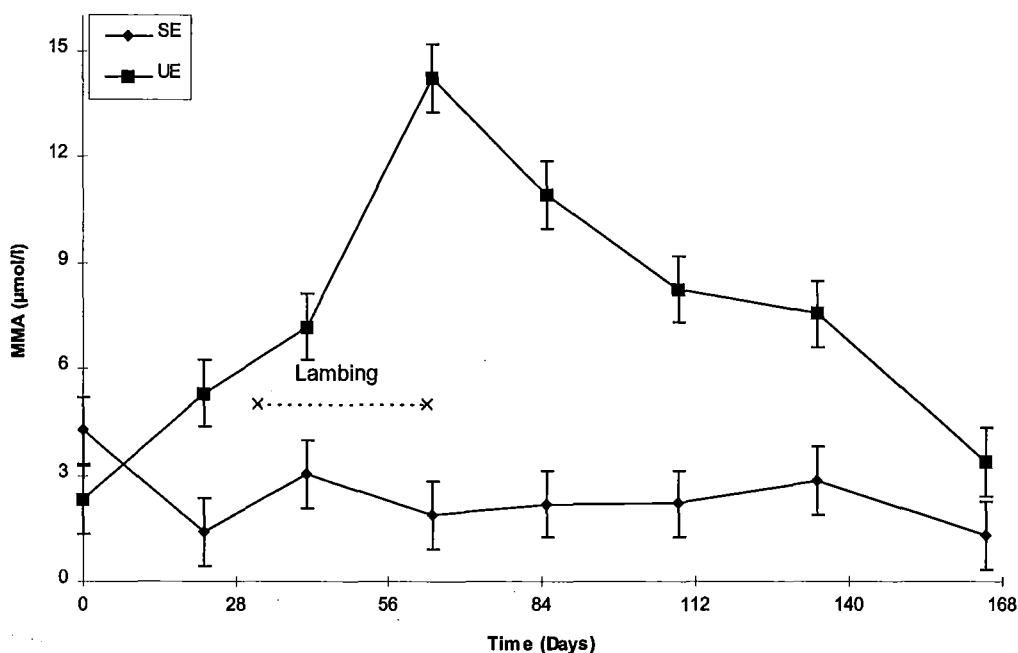
At the start of the trial the SE group had a mean MMA of 4.3 $\mu\text{mol/l}$. MMA fluctuated somewhat between 1.4 and 3.0 $\mu\text{mol/l}$ in samplings two to seven and dropped to 1.3 $\mu\text{mol/l}$ at the end of the trial. Only the initial drop in MMA at sampling two, after the ewes had been supplemented, was statistically significant ($p < 0.01$).

Table 6.12: Mean plasma MMA ($\mu\text{mol/l}$) of Co bullet treated and control two-tooth ewes 1997/1998 (SEM = ± 0.945)

Date	28/08/1997	19/09/1997	8/10/1997	31/10/1997
Day	0	22	41	64
SE	4.3	1.4	3.0	1.9
UE	2.3	5.3	7.2	14.2

Date	21/11/1997	15/12/1997	9/01/1998	9/02/1998
Day	85	109	134	165
SE	2.2	2.2	2.8	1.3
UE	10.9	8.2	7.5	3.4

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.11: Mean plasma MMA of Co bullet treated and control two-tooth ewes 1997/1998 ($\pm$ SEM)

The mean MMA values for the ewes in 1998/1999 are given in Table 6.13 and Figure 6.12. There was no significant difference due to the effect of the ewes' 'age', therefore Figure 6.12 shows the graphs for the treated and untreated ewes only. There was a highly significant treatment x time interaction with mainly quadratic and some small quartic components ($p < 0.001$ for both) due to an initial slight drop in mean MMA, followed by a steep rise to around $7 \mu\text{mol/l}$ (with individual values reaching around $25 \mu\text{mol/l}$) in samplings four and five (during lactation) in the UE groups. From then on MMA values fell to around $2 \mu\text{mol/l}$ at the end of the trial.

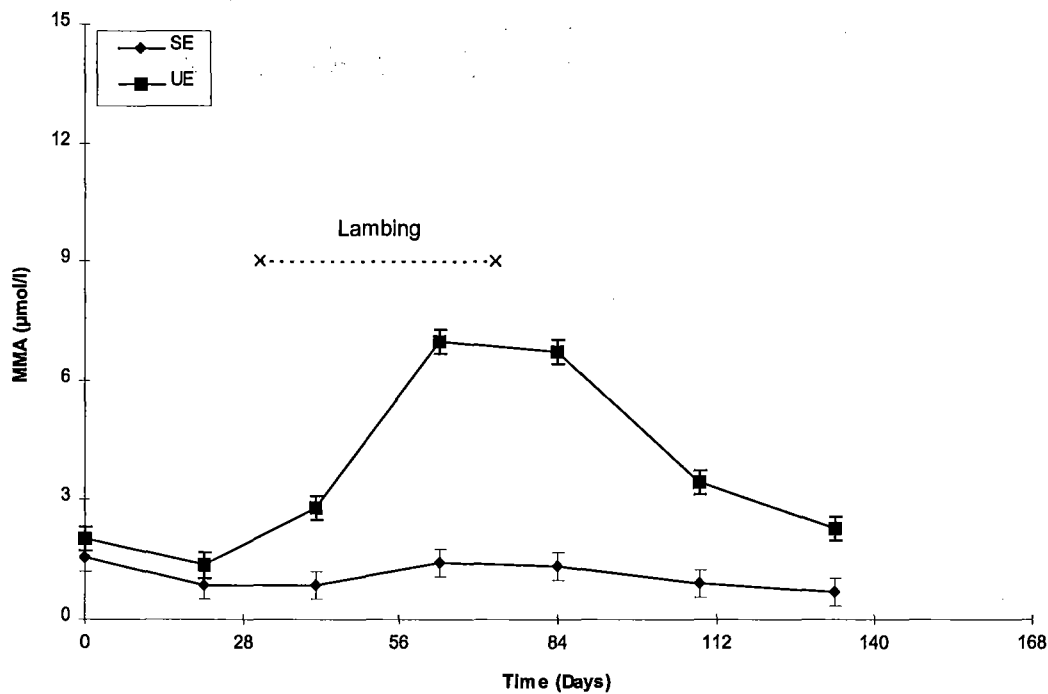
MMA for the SE groups stayed relatively constant. The OSE group started with a mean MMA value of $1.2 \mu\text{mol/l}$, whereas the other groups started with values around $2 \mu\text{mol/l}$. In the OSE group, MMA dropped to $0.7 \mu\text{mol/l}$ in samplings two and three, then doubled for the next two samplings around and shortly after lambing. MMA then dropped to $0.6 \mu\text{mol/l}$ at the end of the trial. There was a slow decline in MMA in the NSE group to $0.9 \mu\text{mol/l}$ at the end of the trial. None of those changes were significant, though.

Table 6.13: Mean serum MMA ($\mu\text{mol/l}$) of Co bullet treated and control two-tooth and four-tooth ewes 1998/1999

Date	27/8/1998	17/9/1998	7/10/1998	29/10/1998
Day	0	21	41	63
OSE	1.2	0.7	0.7	1.4
NSE	2.1	1.2	1.1	1.4
OUE	2.1	1.3	3.2	7.3
NUE	2.0	1.4	2.3	6.5

Date	19/11/1998	14/12/1998	7/1/1999	SEM
Day	84	109	133	
OSE	1.4	1.0	0.6	± 0.41
NSE	1.3	0.8	0.8	± 0.59
OUE	7.6	4.0	2.5	± 0.40
NUE	5.4	2.5	1.9	± 0.48

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.12: Mean serum MMA of Co bullet treated and control two-tooth and four-tooth ewes 1998/1999 ($\pm$ SEM)

6.5.4.2 *Lambs*

The mean plasma MMA concentrations for the 1997/1998 trial are given in Table 6.14 and Figure 6.13. There was a highly significant effect in the quadratic component of the lamb treatment x time interaction ($p < 0.001$). This was due to MMA rising for the second and the third sampling to around 9 $\mu\text{mol/l}$ in the SE/UL group and to nearly 20 $\mu\text{mol/l}$ in the UE/UL group (with individual lambs reaching MMA values up to 80 $\mu\text{mol/l}$) before dropping to below 5 $\mu\text{mol/l}$ in the SE/UL group and to below 7 $\mu\text{mol/l}$ in the UE/UL group. The only significant difference ($p < 0.01$) between individual groups when analysed by individual t-tests occurred between the SL groups and the UE/UL group in samplings two and three. There was no significant effect due to ewe treatment.

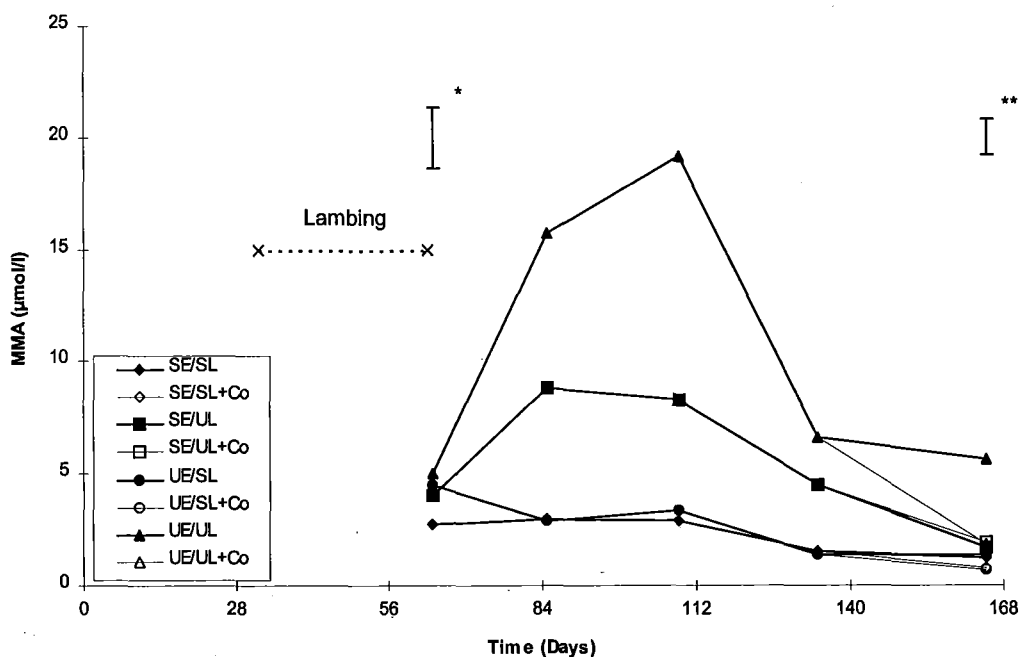
The SE/SL group started off with a lower MMA value (2.7 $\mu\text{mol/l}$) than the other three groups (4.1 to 5.0 $\mu\text{mol/l}$) though the difference was not significant. The SL groups showed little variation in MMA concentrations over time and finished below 2 $\mu\text{mol/l}$ at the end of the trial.

The addition of a Co bullet lowered MMA in all groups: to below 1 $\mu\text{mol/l}$ in the SL groups and to below 2 $\mu\text{mol/l}$ in the UL groups. MMA had fallen to below 2 $\mu\text{mol/l}$ in the groups that had not received a Co bullet, except for the UE/UL group which had a mean MMA of 5.6 $\mu\text{mol/l}$ at the end of the trial. There was no more significant difference between any of the groups at samplings four and five.

Table 6.14: Mean plasma MMA ($\mu\text{mol/l}$) of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1997/1998 ($\pm$ SEM)

Date Day	31/10/1997 64	21/11/1997 85	15/12/1997 109	9/1/1998 134	9/2/1998 165
SE/SL	2.7 $\pm$ 2.76	2.9 $\pm$ 2.90	2.9 $\pm$ 2.90	1.5 $\pm$ 2.90	1.2 $\pm$ 1.60
+ Co					0.8 $\pm$ 0.18
SE/UL	4.1 $\pm$ 2.76	8.8 $\pm$ 2.76	8.3 $\pm$ 2.76	4.5 $\pm$ 2.76	1.6 $\pm$ 1.60
+ Co					1.9 $\pm$ 1.60
UE/SL	4.5 $\pm$ 2.63	2.9 $\pm$ 2.63	3.4 $\pm$ 2.63	1.4 $\pm$ 2.63	1.4 $\pm$ 1.60
+ Co					0.7 $\pm$ 1.46
UE/UL	5.0 $\pm$ 2.63	15.8 $\pm$ 2.63	19.2 $\pm$ 2.63	6.6 $\pm$ 2.63	5.6 $\pm$ 1.60
+ Co					1.8 $\pm$ 1.60

For abbreviations see 6.3.2



* mean SEM (2.7 μmol/l) for treatment x time for sampling days 64 to 134

** mean SEM (1.6 μmol/l) for treatment for sampling day 165

For abbreviations see 6.3.2

Figure 6.13: Mean plasma MMA values of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1997/1998 (± SEM)

Mean serum MMA values for the 1998/1999 lambs are given in Table 6.15 and Figure 6.14. There was no influence of ewe 'age' on MMA, therefore the lambs have been grouped according to ewe and lamb treatment only. There were significant linear components of the interaction between lamb treatment and time ($p < 0.001$).

Treating the lambs had a highly significant effect ($p < 0.01$) on MMA concentrations from sampling two to the end of the trial. There was a significant difference ($p < 0.01$) between the UE/UL group and the other three groups from sampling two to the end of the trial, and between the SE/UL group and the other three groups ($p < 0.05$) from sampling three onwards.

Lambs from treated ewes had serum MMA concentrations about 50 % lower than those of lambs from untreated ewes at the first sampling (0.7 versus 1.6 μmol/l), a highly significant difference ($p < 0.01$). Although MMA values increased slightly for both the SE and UE groups, to 1.6 and 2.0 μmol/l respectively, at the end of the

trial the difference between groups was not significant. The overall effect of ewe treatment on lamb MMA was marginal ($p = 0.04$).

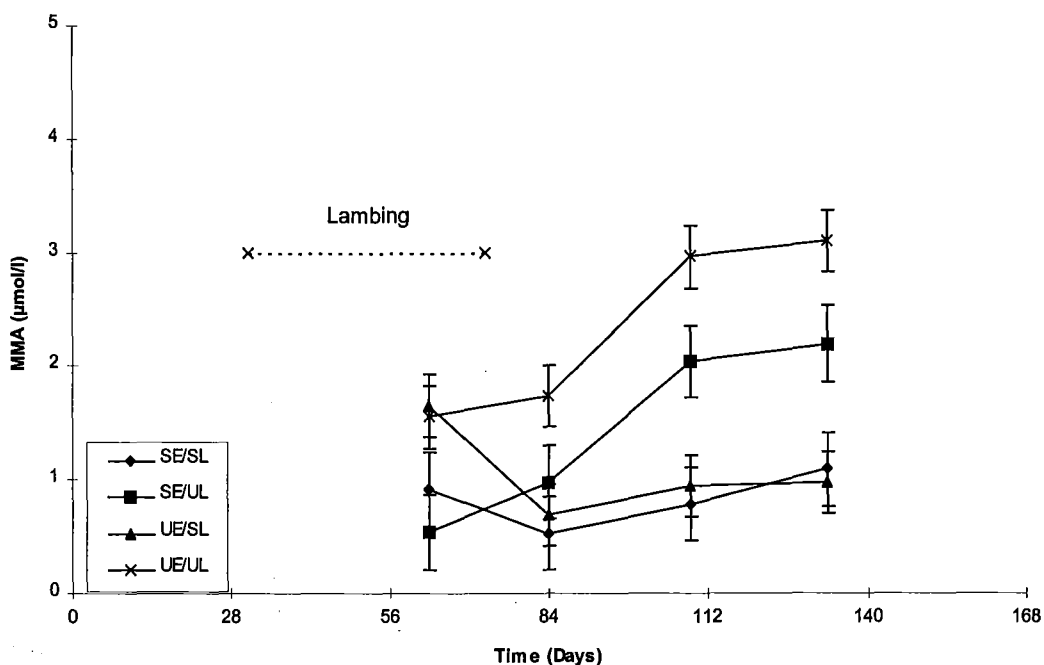
Supplementing the lambs of untreated ewes resulted in a drop in MMA to about 1 $\mu\text{mol/l}$ at the end of the trial, to about the same value as the SE/SL lambs. By that time MMA concentrations in the SE/UL group were around twice as high, and in the UE/UL group three times higher than in the SL groups (2.2 and 3.1 $\mu\text{mol/l}$, respectively). The highest individual MMA values, up to 6.7 $\mu\text{mol/l}$, occurred in the UE/UL group.

MMA for the SL groups dropped initially ($p < 0.001$ for the UE/SL group) before rising non-significantly. For the UL groups MMA rose throughout the trial, significantly ($p < 0.001$) between sampling two and three.

Table 6.15: Mean serum MMA ($\mu\text{mol/l}$) of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1998/1999

Date Day	29/10/1998 63	19/11/1998 84	14/12/1998 109	7/01/1999 133	SEM
SE/SL	0.9	0.5	0.8	1.1	± 0.33
SE/UL	0.5	1.0	2.0	2.2	± 0.33
UE/SL	1.7	0.7	0.9	1.0	± 0.27
UE/UL	1.6	1.7	3.0	3.1	± 0.27

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.14: Mean plasma MMA values of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1998/1999 (± SEM)

6.5.5 Homocysteine

Homocysteine analysis was only done on some of those animals that had MMA values above 5 μmol/l at some stage in the trials. In 1997/1998, two ewes from the SE group and three ewes from the UE group were chosen and five of their plasma samples were analysed for homocysteine. In the lambs, one of each of the UE/SL, SE/SL and SE/UL groups, and three of the UE/UL group were chosen under the same criteria as the ewes and all their plasma samples analysed. In 1998/1999, homocysteine was assayed on all the plasma samples of ten untreated ewes (three NUE and seven OUE) and four untreated lambs (three from UE/UL and one from SE/UL). Homocysteine levels ranged from 0 to 24 μmol/l. No statistical analysis was performed due to the small number of samples and because the samples were not representative of the whole mob. There were no correlations between homocysteine and MMA.

6.5.6 Weight

6.5.6.1 Ewes

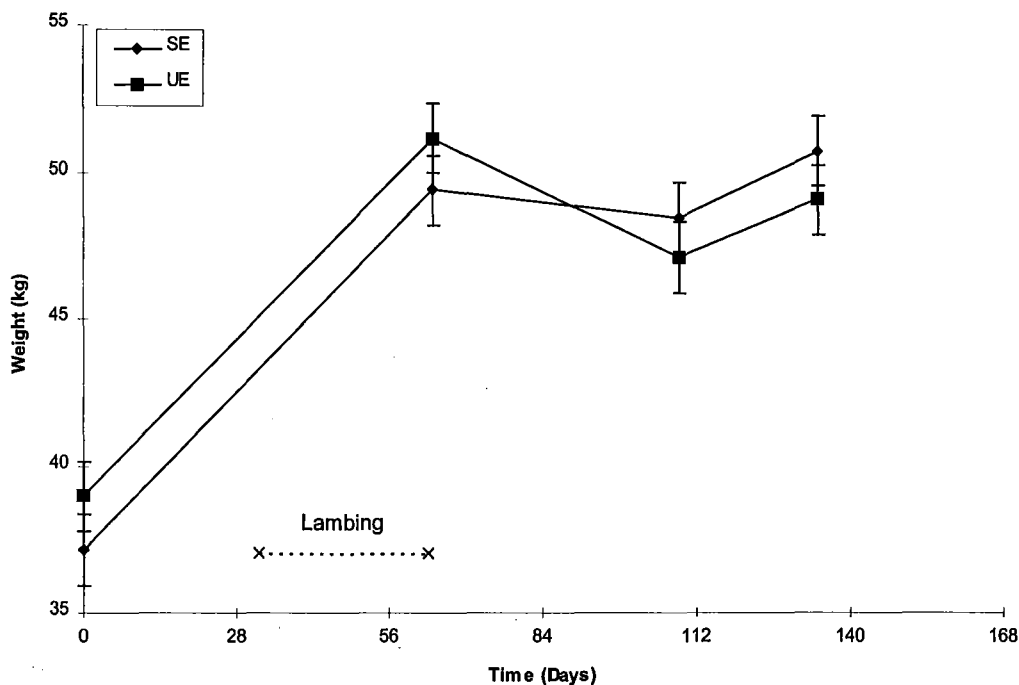
The mean liveweights of ewes in the 1997/1998 trial are given in Table 6.16 and Figure 6.15. There was a highly significant increase ($p < 0.001$) in liveweight in both groups during the trial. Weight increased from mid-pregnancy to after lambing, followed by a slight decline during lactation and a slight rise at weaning.

Although there was no significant difference between treated and untreated ewes at each sampling, there was a significant treatment x time interaction ($p < 0.01$) due to the difference in weight gain from mid-pregnancy to weaning between the SE and UE groups.

Table 6.16: Mean liveweight (kg) of Co bullet treated and control two-tooth ewes 1997/1998 (SEM = ± 1.21)

Date	28/8/1997	31/10/1997	9/1/1998	9/2/1998	Increase
Day	0	64	109	134	
SE	37.1	48.4	48.4	50.7	13.6 ± 1.01
UE	39.0	51.2	47.1	49.0	10.0 ± 0.97

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.15: Mean liveweight of Co bullet treated and control two-tooth ewes 1997/1998 ($\pm$ SEM)

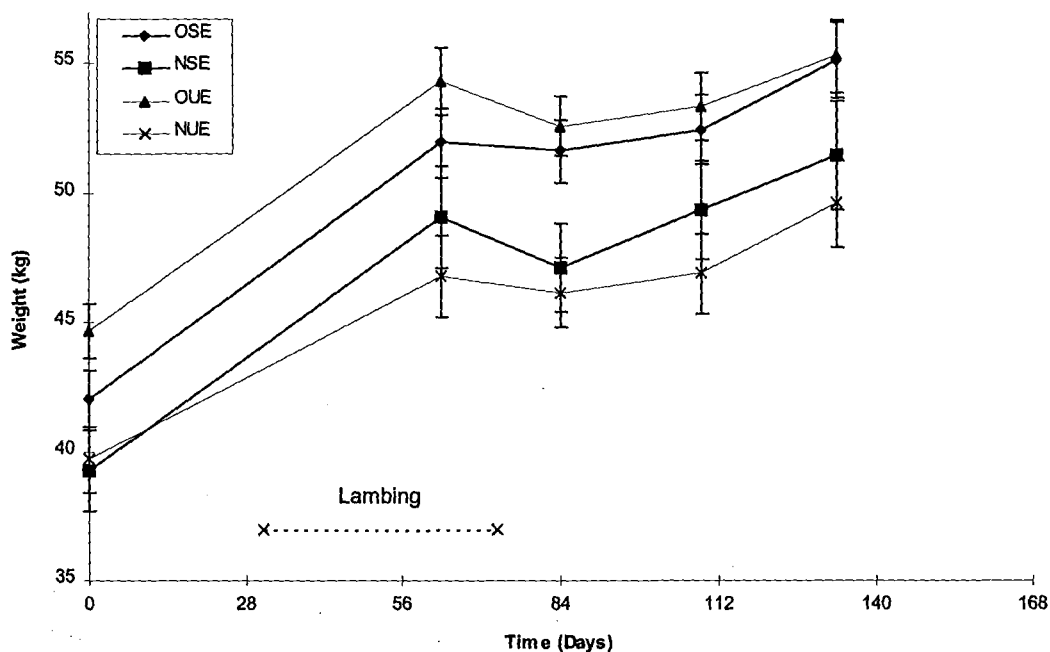
The mean liveweights of the 1998/1999 ewes are given in Table 6.17 and Figure 6.16. There was a significant treatment $\times$ time interaction, the difference being in the linear response to time ($p < 0.05$). There was a significant difference ($p < 0.001$) due to the 'age' of the ewes when averaged over time.

There was a highly significant response to time ($p < 0.001$) which was, as in the previous year, due to the weight increase between mid-pregnancy and after lambing of around 10 kg. Another increase of about 2 to 3 kg occurred around weaning time.

Table 6.17: Mean liveweight (kg) of Co bullet treated and control two-tooth and four-tooth ewes 1998/1999

Date	27/08/98	29/10/98	19/11/98	14/12/98	7/01/99	Increase	SEM
Day	0	63	84	109	133		
OSE	42.0	51.9	51.7	52.6	55.3	13.3	± 1.29
NSE	39.3	49.1	47.1	49.3	51.4	12.1	± 1.85
OUE	44.7	54.3	52.6	53.3	55.3	10.6	± 1.25
NUE	39.7	46.8	46.1	46.9	49.6	9.9	± 1.49

For abbreviations see 6.3.2



For abbreviations see 6.3.2.

Figure 6.16: Mean liveweight of Co bullet treated and control two-tooth and four-tooth ewes 1998/1999 ($\pm$ SEM)

6.5.6.2 Lambs

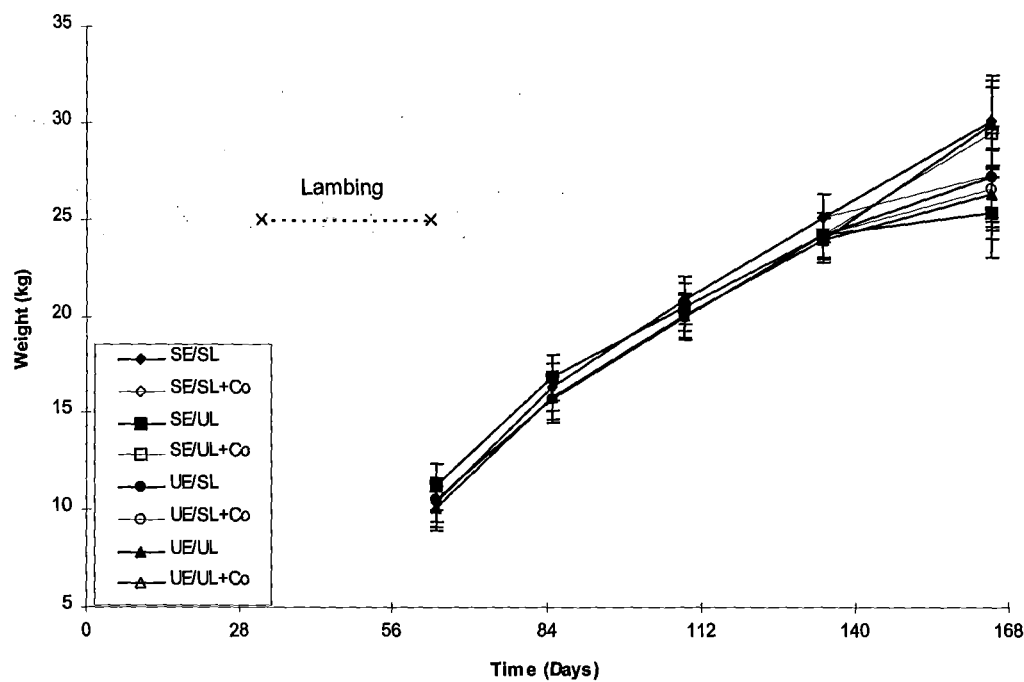
The LWG for the lambs in the 1997/1998 trial is given in Table 6.18 and the mean weight increase is given in Figure 6.17. The weight increase over time was highly significant with mainly linear and small quadratic components ($p < 0.001$ for both). There was no significant difference in either weight or LWG between any of the groups. The lambs weighed on average 4.4 kg at birth. They reached 24.3 kg at weaning time in January and 27.7 kg at the end of the trial one month later.

Initially, the lambs gained around 270 g per day which had slowed down to about 110 g per day at the end of the trial. This decrease over time was highly significant ($p < 0.001$). The addition of a Co bullet after sampling four led to a non-significantly higher LWG in the treated lambs compared to the untreated lambs.

Table 6.18: Mean liveweight gain (LWG in kg/d) of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1997/1998

Days	Birth to 64	65 to 85	86 to 109	110 to 134	135 to 165
SE/SL	0.27 ± 0.019	0.21 ± 0.036	0.17 ± 0.023	0.15 ± 0.016	0.10 ± 0.067
+ Co					0.11 ± 0.083
SE/UL	0.28 ± 0.019	0.27 ± 0.036	0.15 ± 0.023	0.15 ± 0.016	0.08 ± 0.074
+ Co					0.13 ± 0.074
UE/SL	0.27 ± 0.018	0.24 ± 0.035	0.18 ± 0.022	0.17 ± 0.016	0.08 ± 0.074
+ Co					0.09 ± 0.067
UE/UL	0.27 ± 0.018	0.27 ± 0.035	0.18 ± 0.022	0.16 ± 0.016	0.13 ± 0.074
+ Co					0.15 ± 0.067

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.17: Mean weight of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1997/1998 (± SEM)

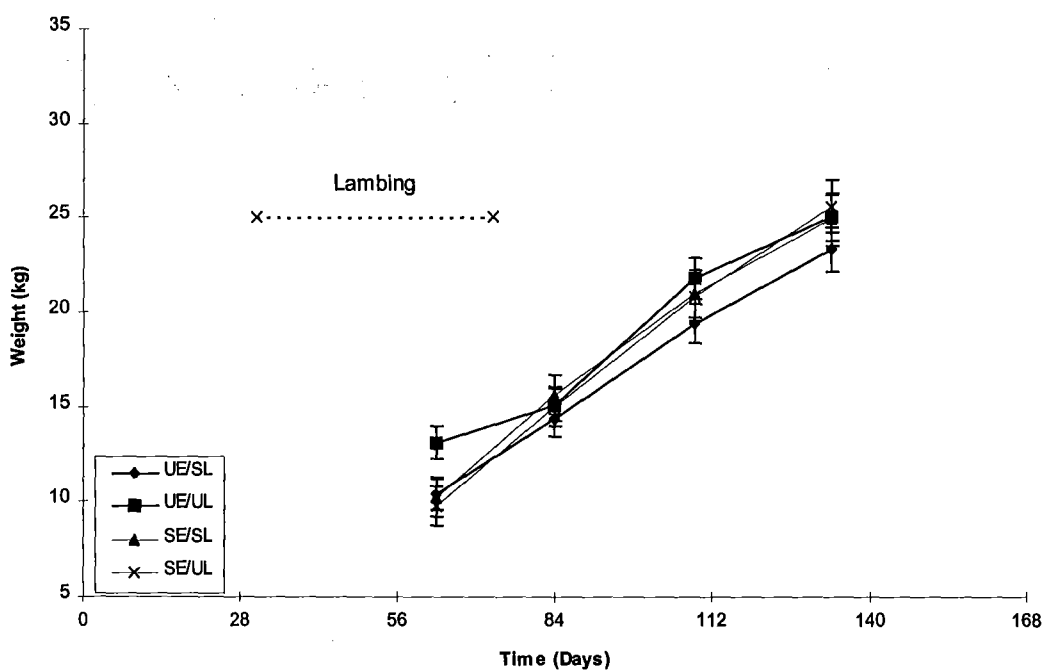
The LWG for the lambs in the 1998/1999 trial is given in Table 6.19 and the mean weight increase is shown in Figure 6.18. The weight increase over time was highly significant ($p < 0.001$) with mainly linear ($p < 0.001$) and small quadratic ($p < 0.01$) and cubic ($p < 0.05$) components. There was no significant difference in either weight or LWG amongst the groups.

Lambs weighed 4.8 kg at birth. They reached 24.6 kg at weaning time in January, the end of the trial. Initial LWG was 270 g and reduced to 110 g at the end of the trial.

Table 6.19: Mean liveweight gain (LWG in kg/d) of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1998/1999 (± SEM)

Days	Birth to 63	64 to 84	85 to 109	110 to 133
SE/SL	0.22 ± 0.035	0.24 ± 0.035	0.25 ± 0.035	0.15 ± 0.035
SE/UL	0.29 ± 0.034	0.25 ± 0.034	0.22 ± 0.034	0.21 ± 0.034
UE/SL	0.28 ± 0.030	0.20 ± 0.030	0.20 ± 0.030	0.17 ± 0.030
UE/UL	0.34 ± 0.030	0.14 ± 0.030	0.25 ± 0.030	0.13 ± 0.030

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.18: Mean weight of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1998/1999 (± SEM)

6.5.7 Faecal egg count

6.5.7.1 Ewes

Faecal sampling was not planned initially. However, the condition of the ewes and the clinical appearance suggested a parasite problem. Therefore, samples were taken from 19/9/1997 in the first year and throughout the trial in 1998/99.

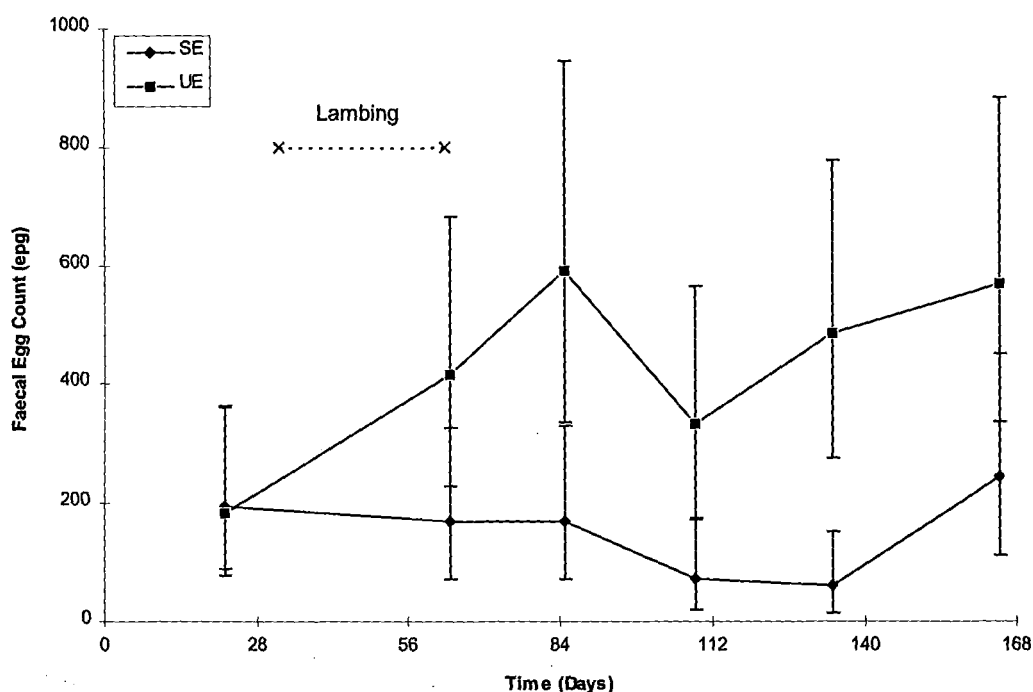
The FEC for the ewes in the 1997/1998 trial is given in Table 6.20 and Figure 6.19). There were differences in the linear and quadratic components ($p < 0.05$ for both) of the treatment x time interaction due to an initial rise in the UE group to 589 epg until after lambing whereas FEC in the SE group remained relatively constant. FEC for both groups then decreased (to 331 epg in the UE group and to 72 epg in the SE group) before rising again until the end of the trial (to 567 epg in the UE group and to 244 epg in the SE group).

Initially, SE and UE groups had similar egg counts (194 and 185 epg, respectively). However, there was a significant difference ($p < 0.05$) in FEC between treated and untreated ewes after the first sampling which became highly significant ($p < 0.001$) by the fifth sampling.

Table 6.20: Mean faecal egg count (epg) with upper and lower Confidence Interval of Co bullet treated and control two-tooth ewes 1997/1998

Date Sampling Day	8/10/1997 41	31/10/1997 64	21/11/1997 85	15/12/1997 109	9/01/1998 134	9/02/1998 165
SE	193.6	168.2	169.7	71.9	59.5	243.7
upper CI	361.8	326.4	329.1	170.8	150.6	449.6
lower CI	87.8	71.7	72.4	21.2	15.4	112.8
UE	184.7	416.0	588.9	331.4	483.0	566.6
upper CI	362.5	682.8	946.2	563.8	775.3	883.7
lower CI	77.3	229.7	335.2	173.6	275.3	336.2

For abbreviations see 6.3.2



For abbreviations see 6.3.2.

Figure 6.19: Mean faecal egg count of Co bullet treated and control two-tooth ewes 1997/1998 ($\pm$ Confidence Interval)

The data for the 1998/1999 ewes is displayed in Table 6.21 and Figure 6.20. There was no significant difference due to ewe treatment but a highly significant ewe 'age' $\times$ time effect ($p < 0.001$). Therefore comparisons have been made between the OE and NE groups only.

The difference in FEC between OE and NE was significant ($p < 0.05$) at every sampling except for sampling four. The curves were mainly linear with small quadratic components ($p < 0.001$ for both). However, there was no difference in the linear components between the curves, indicating that the peaks in each curve were displaced in relation to each other.

The NE groups had a significantly higher FEC than the OE groups (mean 16 and 1.1 epg, respectively) at the start of the trial ($p < 0.05$). FEC for the NE groups rose steeply ($p < 0.001$) between the second and third sampling to 355 epg (prior to lambing). It then dropped to just above 100 epg by sampling five ($p < 0.05$) and to 85 epg at the end of the trial.

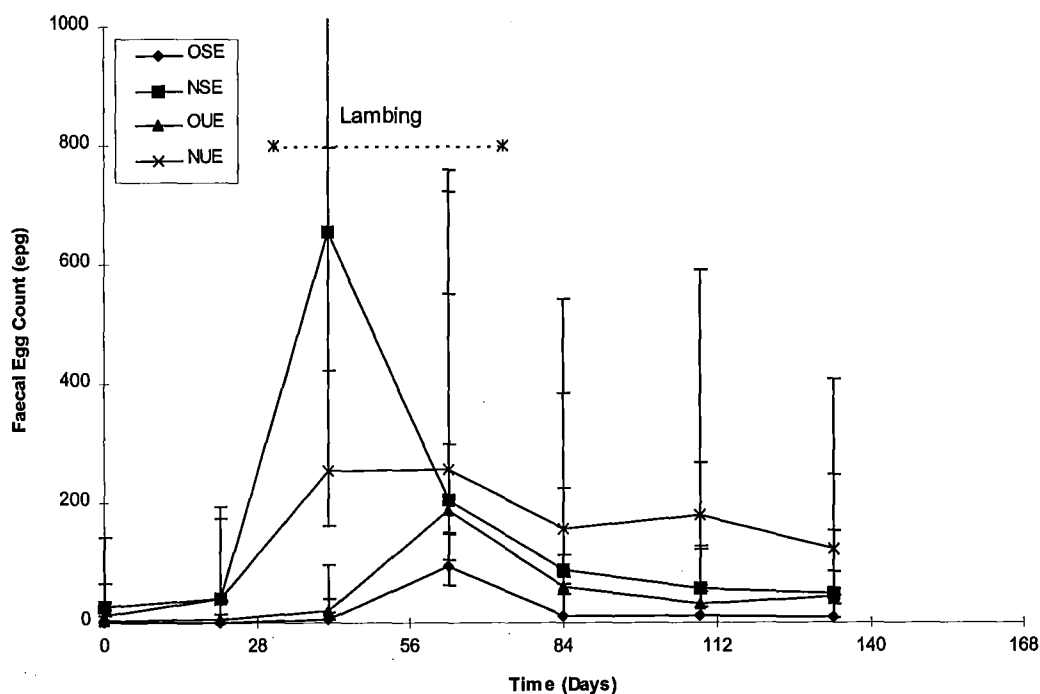
FEC in the OE groups did not rise until sampling four when it reached 140 epg ($p < 0.001$). This was followed by a drop to 32 epg by the next sampling ($p < 0.001$). FEC remained relatively constant after that, with 25 epg at the end of the trial.

Table 6.21: Mean faecal egg count (epg) with upper and lower Confidence Interval of Co bullet treated and control two-tooth and four-tooth ewes 1998/1999

Date	27/08/1998	17/09/1998	7/10/1998	29/10/1998
Sampling Day	0	21	41	63
OSE	0.5	1.0	5.7	93.4
Upper CI	10.0	14.0	33.7	207.8
Lower CI	-0.2	-0.1	0.0	31.3
NSE	24.8	39.6	654.4	204.8
Upper CI	116.7	155.4	1414.6	518.0
Lower CI	0.9	3.0	231.3	53.0
OUE	2.3	5.8	19.0	189.4
Upper CI	19.0	30.8	78.9	361.4
Lower CI	0.0	0.1	1.2	83.1
NUE	11.1	41.2	254.2	257.6
Upper CI	55.5	134.2	543.9	501.8
Lower CI	0.3	5.7	91.6	109.2

Date	19/11/1998	14/12/1998	7/01/1999
Sampling Day	84	109	133
OSE	12.3	10.9	10.0
Upper CI	52.8	51.5	47.5
Lower CI	0.7	0.4	0.3
NSE	87.4	56.4	47.4
Upper CI	298.6	212.9	202.1
Lower CI	10.5	4.9	2.6
OUE	61.2	32.7	42.7
Upper CI	164.3	96.0	112.6
Lower CI	13.9	6.0	10.1
NUE	156.2	180.9	122.5
Upper CI	386.3	409.2	286.1
Lower CI	42.5	58.8	37.4

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.20: Mean faecal egg count of Co bullet treated and control two-tooth and four-tooth ewes 1998/1999 ($\pm$ Confidence Interval)

6.5.7.2 *Lambs*

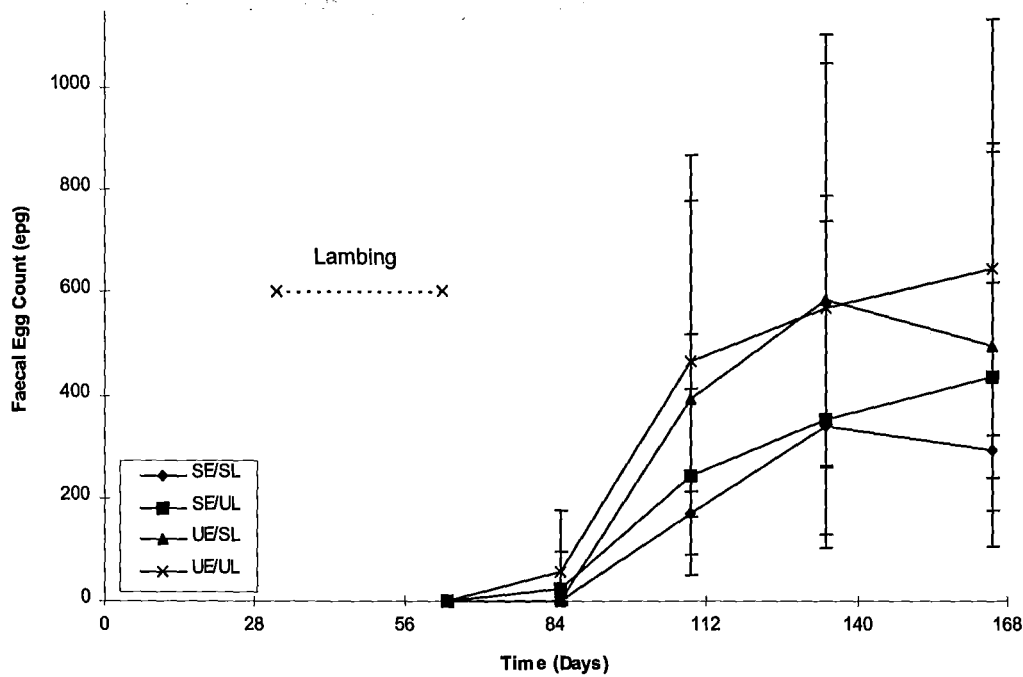
The mean FEC of the lambs in the 1997/1998 trial is shown in Table 6.22 and Figure 6.21. At no stage was there any significant difference due to ewe or lamb treatment. However, there was a trend for lambs from unsupplemented ewes to have higher FEC than those from supplemented ewes.

Straight after birth and while still fully suckled, none of the lambs had any parasitic eggs. At sampling two the SL groups still had hardly any eggs, whereas the FEC had tended to increase non-significantly to 22 and 56 epg in the SE/UL and UE/UL groups, respectively. This was followed by a steep and highly significant ($p < 0.001$) rise in all groups to between 171 and 465 epg (for the SE/SL and UE/UL groups, respectively). The curve flattened from then on, and there was no more significant difference between any of the groups, although supplementing the lambs through vitamin B₁₂ injections or a Co bullet tended to have a lowering effect on FEC.

Table 6.22: Mean faecal egg count (epg) with upper and lower Confidence Interval of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1997/1998

Date Sampling Day	31/10/1997 64	21/11/1997 85	15/12/1997 109	9/01/1998 134	9/02/1998 165
SE/SL	0.00	0.38	170.86	338.17	291.71
upper CI	0.00	22.95	412.00	787.86	619.36
lower CI	0.00	-2.70	48.91	103.69	106.50
SE/UL	0.00	22.24	243.67	351.75	434.66
upper CI	0.00	97.78	519.91	738.76	872.63
lower CI	0.00	1.05	88.18	130.94	175.05
UE/SL	0.00	0.00	393.03	584.28	495.48
upper CI	0.00	8.11	778.69	1104.87	891.67
lower CI	0.00	-7.38	161.88	259.94	238.44
UE/UL	0.00	56.09	465.48	567.25	645.64
upper CI	0.00	177.03	867.43	1045.99	1134.63
lower CI	0.00	8.50	212.03	262.76	322.26

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.21: Mean faecal egg count of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1997/1998 ($\pm$ Confidence Interval)

The results for the mean FEC in the 1998/1999 lambs are shown in Table 6.23 and Figure 6.22. Neither supplementing the ewes nor the lambs had any

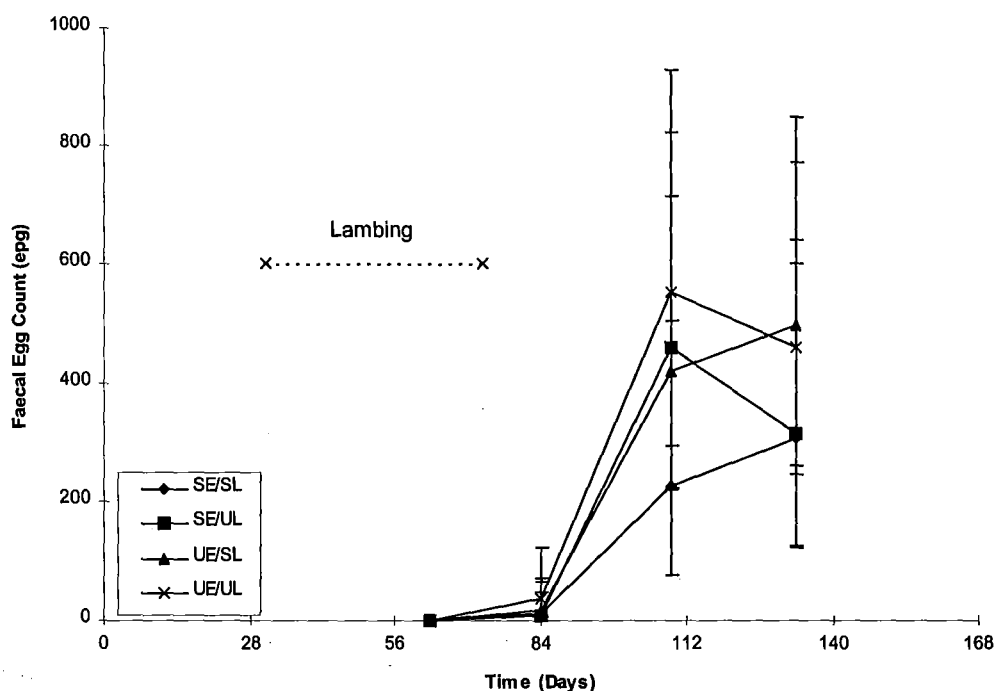
significant effect on FEC, nor had the ‘age’ of the ewe, but the trend was again for lambs from supplemented ewes to have lower FEC.

The lambs had no parasitic eggs straight after birth and while still fully suckled. However, there was a steep rise in FEC between sampling two and three, from below 40 epg to between 226 and 553 epg (for the SE/SL and UE/UL groups, respectively). This increase was highly significant ($p < 0.001$) for all groups. Between the third and fourth sampling FEC still increased for the SL groups, whereas FEC for the UL groups declined, but those changes were not significant.

Table 6.23: Mean faecal egg count (epg) with upper and lower Confidence Interval of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1998/1999

Date Sampling Day	29/10/1998 63	19/11/1998 84	14/12/1998 109	7/01/1999 133
SE/SL	0.00	11.35	226.20	304.28
upper CI	0.00	65.93	503.67	600.95
lower CI	0.00	0.09	75.67	125.99
SE/UL	0.00	7.69	459.56	313.60
upper CI	0.00	47.90	823.77	642.23
lower CI	0.00	0.03	222.52	122.04
UE/SL	0.00	17.03	420.02	496.98
upper CI	0.00	72.15	714.40	849.16
lower CI	0.00	0.95	220.18	258.77
UE/UL	0.00	36.53	552.78	459.21
upper CI	0.00	121.11	929.18	771.41
lower CI	0.00	4.81	294.86	245.17

For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.22: Mean faecal egg count of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1998/1999 ($\pm$ Confidence Interval)

6.5.8 Milk

Milk collection was achieved from early October until early January.

6.5.8.1 Milk protein

The results for mean milk protein (%) for the 1997/1998 trial are given in Table 6.24. Milk protein in the first sample ranged from 6.5 to 7.9 % and dropped to ~ 3.9 to 5.7 % thereafter. Although milk protein in the SE group was slightly higher than in the UE group, this difference was not significant. However, for both groups there was a significant difference ($p < 0.05$ for UE and $p < 0.01$ for SE) between the first sampling and the other four sampling times. There was no significant effect of treatment over time.

Table 6.24: Mean milk protein content (w:w) (%) of Co bullet treated and control two-tooth ewes 1997/1998 ($\pm$ SEM)

Day	n	8/10/1997 41	n	31/10/1997 64	n	21/11/1997 85	n	15/12/1997 109	n	9/01/1998 134
SE	6	7.9 $\pm$ 0.71	16	5.3 $\pm$ 0.47	15	3.9 $\pm$ 0.49	16	5.4 $\pm$ 0.48	17	5.7 $\pm$ 0.47
UE	5	6.5 $\pm$ 0.79	16	4.9 $\pm$ 0.47	18	4.9 $\pm$ 0.45	21	4.8 $\pm$ 0.42	16	5.1 $\pm$ 0.47

n = Number of samples
For abbreviations see 6.3.2

The results for mean milk protein (%) in 1998/1999 are given in Table 6.25. Except for the OUE group which had only 5.7 % protein, values for the first sampling ranged from 8.3 to 9.9 %. This was significantly higher ($p < 0.001$) than during the rest of the trial where protein values ranged from 4.9 to 5.9 %. There was no significant effect of treatment or treatment over time.

Table 6.25: Mean milk protein (w:w) (%) of Co bullet treated and control two-tooth and four-tooth ewes 1998/1999 ($\pm$ SEM)

Day	n	7/10/1998 41	n	29/10/1998 63	n	19/11/1998 84	n	14/12/1998 109	n	7/1/1999 133
OSE	7	8.3 $\pm$ 1.10	17	5.9 $\pm$ 0.28	17	5.4 $\pm$ 0.28	20	5.6 $\pm$ 0.27	19	5.9 $\pm$ 0.27
NSE	5	9.9 $\pm$ 1.55	8	5.1 $\pm$ 0.42	7	4.9 $\pm$ 0.43	7	5.3 $\pm$ 0.43	8	5.3 $\pm$ 0.42
OUE	5	5.7 $\pm$ 1.05	16	5.6 $\pm$ 0.28	21	5.3 $\pm$ 0.26	17	5.5 $\pm$ 0.27	16	5.7 $\pm$ 0.28
NUE	6	9.0 $\pm$ 1.25	13	4.9 $\pm$ 0.32	10	4.8 $\pm$ 0.35	12	5.4 $\pm$ 0.33	13	5.4 $\pm$ 0.33

n = Number of samples
For abbreviations see 6.3.2

6.5.8.2 Milk vitamin B₁₂

Table 6.26 and Figure 6.23 show the mean milk vitamin B₁₂ values for the 1997/1998 trial. The response to time had significant linear and quadratic components ($p < 0.001$ for both). There was a highly significant difference ($p < 0.001$) between the two groups from the second sampling, the time when all the lambs were born, until the last sampling. However, there was no significant interaction between the groups and time, i.e. the curves were parallel.

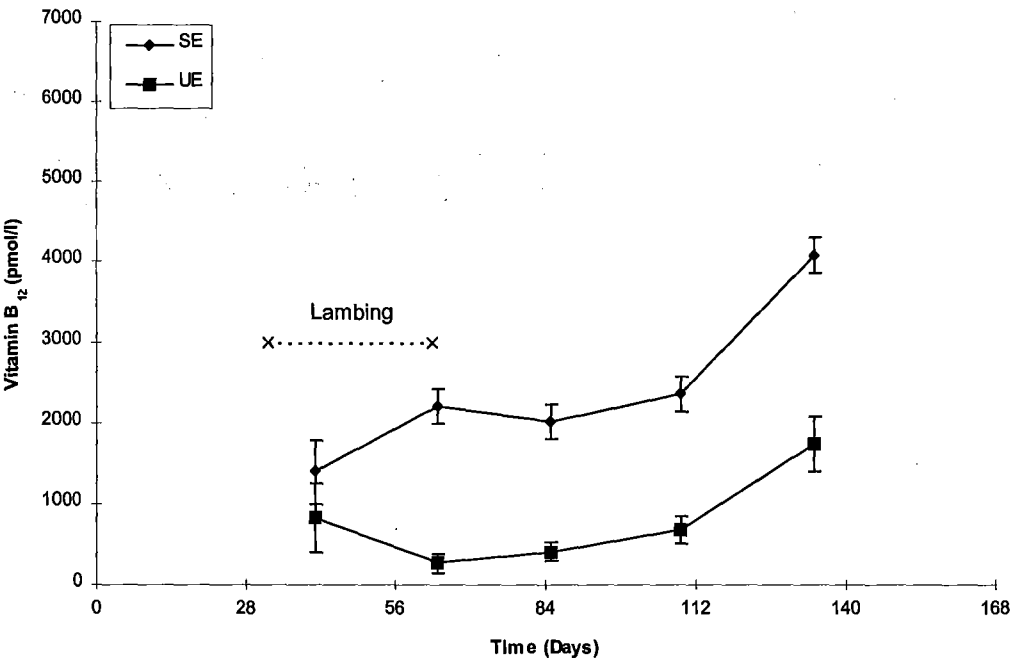
Milk vitamin B₁₂ in the UE group started at 819 pmol/l, then dropped to 272 pmol/l before rising to 1747 pmol/l at the last sampling. The differences between samplings two to four and the last sampling were highly significant ($p < 0.001$). In the SE group milk vitamin B₁₂ values started at 1394 pmol/l and rose to 4078 pmol/l

at the last sampling. There was a highly significant difference ($p < 0.001$) between the first four samplings and the last one.

Table 6.26: Mean milk vitamin B₁₂ content (pmol/l) of Co bullet treated and control two-tooth ewes 1997/1998 ($\pm$ SEM)

	n	8/10/1997	n	31/10/1997	n	21/11/1997	n	15/12/1997	n	9/01/1998
SE	5	1394 $\pm$ 389	16	2203 $\pm$ 218	16	2017 $\pm$ 218	16	2357 $\pm$ 218	15	4078 $\pm$ 225
UE	4	819 $\pm$ 435	19	272 $\pm$ 200	16	416 $\pm$ 218	18	684 $\pm$ 205	18	1747 $\pm$ 205

n = Number of samples
For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.23: Mean milk vitamin B₁₂ content of Co bullet treated and control two-tooth ewes 1997/1998 ($\pm$ SEM)

Mean milk vitamin B₁₂ values for the 1998/1999 trial are given in Table 6.27 and Figure 6.24. There was a strong quadratic response to time ($p < 0.001$) and a highly significant effect due to treatment ($p < 0.001$), but no interaction of treatment over time. Although the OSE group had a much higher initial milk vitamin B₁₂ value

than the other three groups (4057 pmol/l versus an average of around 2200 pmol/l, respectively) there was no significant difference due to ewe age.

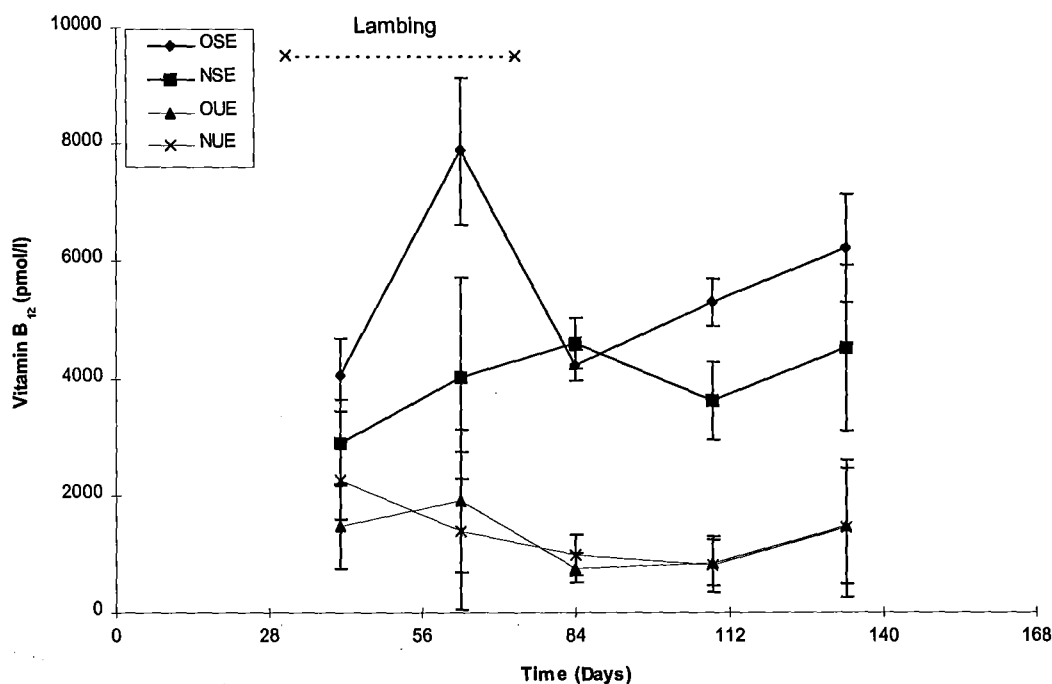
In the SE groups values rose initially to nearly 6000 pmol/l, then declined to around 4500 pmol/l before picking up again to around 5400 pmol/l at the end of the trial. In the UE groups values declined steadily to just below 1000 pmol/l before rising again to approximately 1600 pmol/l at the end of the trial. The difference between supplemented and unsupplemented ewes was highly significant ($p < 0.001$) at every sampling.

The only significant difference ($p < 0.001$) over time occurred in the OSE group in the first two samplings. Although there was no difference between the OSE and NSE groups, the OSE group had much higher milk vitamin B₁₂ values than in the previous year, whereas the NSE group's values were similar to the SE group from the previous year.

Table 6.27: Mean milk vitamin B₁₂ (pmol/l) of Co bullet treated and control two-tooth and four-tooth ewes 1998/1999 (± SEM)

Day	n	7/10/1998	n	29/10/1998	n	19/11/1998	n	14/12/1998	n	7/1/1999
		41		63		84		109		133
OSE	7	4057 ± 339	15	7729 ± 825	18	5125 ± 794	20	5287 ± 779	19	6273 ± 787
NSE	5	2900 ± 491	8	4014 ± 1232	7	4363 ± 1263	7	3756 ± 1263	8	4511 ± 1232
OUE	5	1489 ± 332	16	1771 ± 803	20	1040 ± 769	20	1121 ± 769	17	1806 ± 794
NUE	6	2260 ± 395	13	1371 ± 944	11	882 ± 979	13	775 ± 948	12	1399 ± 963

n = Number of samples
For abbreviations see 6.3.2



For abbreviations see 6.3.2

Figure 6.24: Mean milk vitamin B₁₂ of Co bullet treated and control two-tooth and four-tooth ewes 1998/1999 ($\pm$ SEM)

6.6 Discussion

6.6.1 Climate

Research from Australia (Gardiner, 1977) suggests that rainfall in winter determines the incidence of Co deficiency in spring. Deficiency seems to be much more likely when there has been a wet winter (Gardiner, 1977) or a wet spring with plenty of pasture growth after a dry winter (Lee, 1951). Therefore, comparisons of mean monthly rainfall and temperatures in the area of the farm have been made between 1996 and the following two years when the trials took place.

Rainfall in August and September 1996 was below average, followed by an above average wet October and plenty of pasture growth. This was the year when the farmer experienced problems with lamb growth and when high serum and urinary MMA in young lambs were recorded. The summer months were also wet. Cobalt had

been low as a result (hay made that season had a Co content of 0.05 µg/g DM). Lambs and sheep were in poor condition.

However, during the experimental period in 1997/1998 it had been particularly hot and dry, resulting in poor pasture growth and higher plant cobalt concentrations. In anticipation of a wetter season in the following year and to verify the results obtained in the previous trial, it was repeated in 1998/1999. But that year was another hot and dry one. Overall, rainfall in 1998 was ~ 20 % less than in the previous two years, and January 1998 in particular had only ~ 20 % of rain compared to January 1996 and 1997. Temperatures in 1998 were ~ 1°C higher than average, with February of that year having the highest recorded mean temperature (18.6°C) for the past 25 years.

Although the values for Co dropped to 0.09 mg Co/kg DM in the middle of the trial period in 1997/1998 and to 0.1 mg Co/kg DM in 1998/1999, they still remained above the threshold for deficiency set at 0.07 to 0.08 mg Co/kg DM for sheep and close to the threshold of 0.11 mg Co/kg DM for lambs (Andrews *et al.*, 1958). Therefore the animals' Co status was not as compromised as in the previous year and deficiency was only mild.

6.6.2 Target group for supplementation

In these trials the only significant difference in weight gain, indicative of Co/vitamin B₁₂ deficiency, occurred between the SE and UE groups in both years. The weight reduction that occurred in the 1997/1998 trial in the UE group but not in the SE group happened during early lactation, suggestive of depletion of vitamin B₁₂ from blood and tissues. A similar pattern of a drop in weight just after lambing was seen in the following year in all groups of ewes. This depletion was further supported by the below normal vitamin B₁₂ concentrations in plasma and liver and raised serum MMA concentrations around parturition and in early lactation in the UE group in 1997/1998. This rise in serum MMA around lambing time was also seen by Fisher and MacPherson (1991) in Co deficient ewes. Possibly the demands of late pregnancy on the energy and immune systems of the ewes were much increased during that time which is further illustrated by the fact that even the supplemented

ewes in both years showed the decrease of serum vitamin B₁₂ at lambing. The vitamin may have been channelled into the offspring at the expense of the parent's metabolic need. This is in contrast to Quirk and Norton (1987) who showed that failure to give priority to the metabolic needs of the offspring resulted in lambs excreting FIGLU whereas their dams did not.

Vitamin B₁₂ in serum and liver increased in all groups of lambs despite increases in MMA around the time of rumen development and no significant differences in LWG were seen between any of the groups. It may therefore be the ewe at lambing and during lactation that is at greatest risk of vitamin B₁₂ deficiency and not the lamb at weaning, as is commonly believed (Gardiner, 1977; Andrews, 1956 and 1972), though much may depend on the particular set of environmental conditions pertaining to these times.

6.6.3 Vitamin B₁₂ in times of shortage/excess

For the supplemented animals losses of vitamin B₁₂ from serum, i.e. reductions in plasma/serum vitamin B₁₂ concentrations, when they occurred, seem to be greater than for the unsupplemented sheep. This was illustrated at the third and last sampling for the SE group in 1997/1998, for the third sampling for the SE groups in 1998/1999 and for the last sampling for the lambs in 1997/1998. This suggests that the higher the vitamin B₁₂ status the more profligate the animal is, or *vice versa* that the vitamin is conserved when in short supply. Likewise, Smith and Marston (1970) found that faecal and urinary losses in vitamin B₁₂ deficient ewes were much lower than in those replete in vitamin B₁₂.

Similarly in 1998/1999, the NSE group had an increase of ~ 1000 pmol/l in serum vitamin B₁₂ about three weeks after receiving a Co bullet, whereas in the OSE group, having been supplemented in the previous year, a mean loss in serum vitamin B₁₂ was seen in the first two months. When the lambs in 1997/1998 received a Co bullet around weaning time it was the UE/UL group that had the steepest rise in plasma vitamin B₁₂. This is in agreement with Smith and Marston (1970) who found that the efficiency of conversion of Co into vitamin B₁₂ was higher in Co depleted animals (~ 15 %) than in those supplemented with Co (~ 3 %).

6.6.4 Effect of Co bullets

The bullet (2 g Co_3O_4), at a daily release rate of 0.6 to 1.2 % (personal communication with Schering-Plough), provided 12 to 24 mg/d (50 to 100 $\mu\text{mol/d}$) initially. Three moles of vitamin B_{12} can be synthesised from one mole of Co_3O_4 , therefore 150 to 300 μmol (200 to 600 mg) of the vitamin could potentially be produced by rumen bacteria if all the Co was converted to vitamin B_{12} . Since only 3 to 15 % of Co in the rumen is converted to vitamin B_{12} (Smith and Marston, 1970), 6 to 90 mg vitamin B_{12} could have been produced. However, Marston (1970) found that 1 mg Co/d, given *per os*, was enough to raise liver vitamin B_{12} concentrations to a maximum of around 1000 nmol/kg fresh tissue and that higher doses of Co added to the diet did not increase the amount absorbed. In fact, he estimated the amount of vitamin B_{12} from ruminal production to be $\sim 700 \mu\text{g/d}$ of which $\sim 34 \mu\text{g}$ were absorbed, viz $\sim 5 \%$.

The amount of Co the animals were getting from the bullet should, therefore, have been ample to saturate tissue concentrations. However, only the OSE group in 1998/1999 had reached liver vitamin B_{12} concentrations close to the maximum (1000 nmol/kg fresh tissue) reported by Marston (1970). When pasture Co increased in January 1998 it coincided with an increase in plasma and liver vitamin B_{12} in both the SE and UE groups. This could imply that the animals use pasture Co more readily than Co from supplementation, which is supported by the findings of Smith and Marston (1970). However, the rise in pasture Co in the following year increased serum and liver vitamin B_{12} in the untreated ewes only marginally. Since there seemed to be less Co deficiency in 1998/1999 than in the previous year, possibly the untreated ewes in the 1998/1999 trial were not as depleted as the ones in the year before (450 pmol/l versus 338 pmol/l serum vitamin B_{12} , respectively, at the start of the trial) and therefore there may have been less demand for the vitamin.

In the 1998/1999 trial the ewes that were treated with a Co bullet in the previous year had an initial advantage over the NSE group as far as serum and liver vitamin B_{12} were concerned. Their values continued to stay 25 to 50 % above those for the NSE group throughout the trial. It seemed that a) the bullets were still active after one year, showing a much greater efficacy than the one year stated by the

manufacurer and b) that the amount released by the bullets was not maximising vitamin B₁₂ production.

6.6.5 Vitamin B₁₂ and MMA transfer from ewe to lamb

The lambs from treated ewes had at least 30 % higher serum and liver vitamin B₁₂ concentrations than the ones from untreated ewes immediately after lambing and maintained that advantage at least until weaning even if they were unsupplemented themselves. In effect, treating the ewes lifted serum vitamin B₁₂ of the lambs from below 200 to above 300 pmol/l in both seasons and in liver from 86 to 140 nmol/kg fresh tissue in 1997/1998 and from 130 to 260 nmol/lg fresh tissue in 1998/1999. Obviously, the vitamin could be passed on through the placenta and the milk to the lamb, as observed by other researchers (Quirk and Norton, 1987; Grace, 1999a; Halpin and Caple, 1982).

Similar results were obtained by Quirk and Norton (1987) who found that daily doses of 0.03 and 0.06 mg Co/d, administered to the ewe, delayed the onset of deficiency symptoms (as indicated by raised urinary FIGLU and MMA) in lambs in a dose-related way, whereas Co bullets given to the dam avoided vitamin B₁₂ deficiency altogether in the offspring prior to weaning. On the other hand, they were unable to separate the lambs from the different treatments on the basis of serum vitamin B₁₂ which were uniformly low. The same observation regarding serum vitamin B₁₂ concentrations in lambs was made by Grace (1999a) and Halpin and Caple (1982). These authors therefore concluded that serum vitamin B₁₂ concentrations are not a reliable indicator of deficiency in PR lambs.

Grace *et al.* (1986) thought that milk provided most of the lamb's vitamin B₁₂ because they found that liver vitamin B₁₂ concentrations of foetal lambs were low. Yet in a later trial Grace (1999a) found that treating the ewe prior to mating increased liver vitamin B₁₂ concentrations in the lamb for at least the first month of life, compared to controls.

Not only vitamin B₁₂ but also MMA may be passed on from ewe to lamb. Fisher and MacPherson (1991) had suggested that MMA in lambs does not originate in the lambs themselves but rather is passed on from the dam to her offspring.

However, in these trials ewes with high MMA had lambs with low MMA and *vice versa*, indicating that MMA did not get passed on but stemmed from the lambs' own impaired metabolism in the processing of succinate to propionate. For instance, the two ewes which had MMA values of 26 and 39 $\mu\text{mol/l}$ suckled lambs which had MMA values of 8 and 5 $\mu\text{mol/l}$, respectively. The two lambs which had MMA values of ~ 80 $\mu\text{mol/l}$ were suckled by dams with MMA values of 13 and 6 $\mu\text{mol/l}$. Unfortunately, the ewes with even higher MMA values did not suckle lambs and therefore no comparisons could be made.

6.6.6 Lamb retention of injected vitamin B₁₂

The treated lambs had received 3000 μg (~ 2200 nmol) of vitamin B₁₂ between the first and second liver biopsy. This had increased liver concentrations by ~ 450 nmol/kg fresh tissue. The lambs weighed about 23 kg at the time of the second liver biopsy. Assuming that liver weight equals ~ 1.5 % of body weight (Wallace, 1948), 155 nmol would have been added to liver vitamin B₁₂ since the previous biopsy. This amounts to a retention of injected vitamin B₁₂ of ~ 7 %.

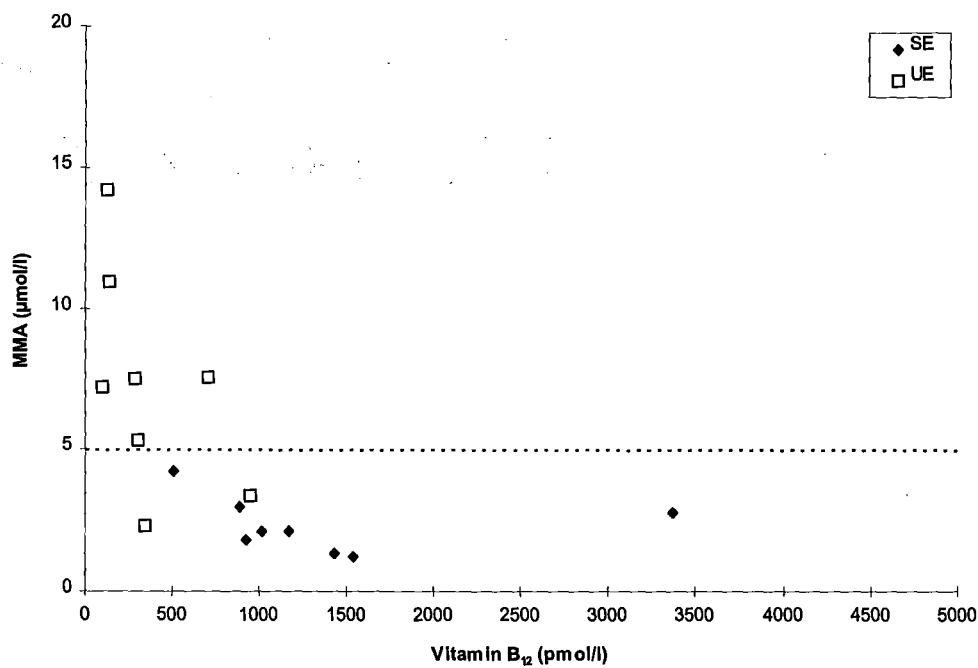
6.6.7 Threshold levels of MMA and vitamin B₁₂

MMA of 5 $\mu\text{mol/l}$ has been suggested as the upper level of normality for grass fed and 10 $\mu\text{mol/l}$ for concentrate fed sheep (O'Harte *et al.*, 1989a). The animals used in these trials were grazing, therefore comparisons were made initially between those unsupplemented ewes and lambs which had MMA concentrations below and above 5 $\mu\text{mol/l}$. Most untreated ewes in both years and untreated lambs in the 1997/1998 season had MMA above 5 $\mu\text{mol/l}$ at some stage.

Figures 6.25 to 6.28 give the mean vitamin B₁₂ concentrations for ewes and for lambs in both seasons in relation to MMA for each group at each sampling. The dotted line indicates the threshold of 5 $\mu\text{mol/l}$. An inverse relationship between vitamin B₁₂ and MMA was observed which was most obvious in the 1998/1999 ewes (Figure 6.28). When comparing serum vitamin B₁₂ and MMA concentrations in the lambs, the inverse trend was still observable, although not as pronounced as in the

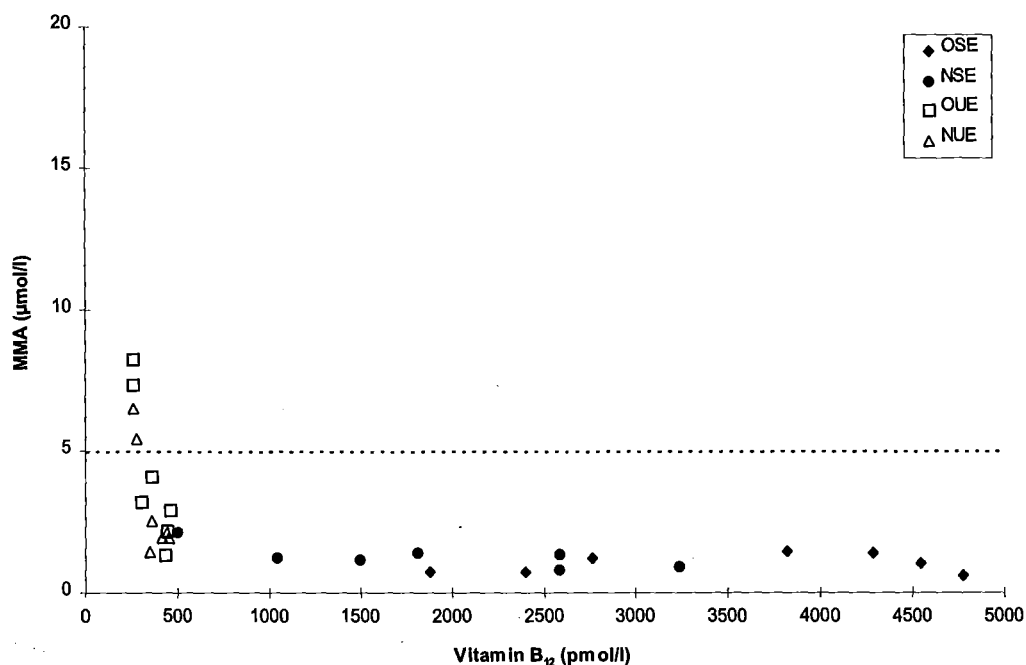
ewes (Figures 6.27 and 6.28). This was noticed even in the lambs of the second trial where MMA stayed below 5 $\mu\text{mol/l}$ at all times.

Although the unsupplemented ewes and lambs had vitamin B₁₂ values mostly below the threshold of normality of 500 pmol/l (New Zealand reference ranges from Labnet Invermay; Underwood and Suttle, 1999) and at times below 335 pmol/l (below which liveweight response to supplementation is considered likely by Labnet Invermay and which Underwood and Suttle, 1999, consider the lower level of a marginal vitamin B₁₂ status) there was not necessarily a correlation with MMA or liveweight changes.



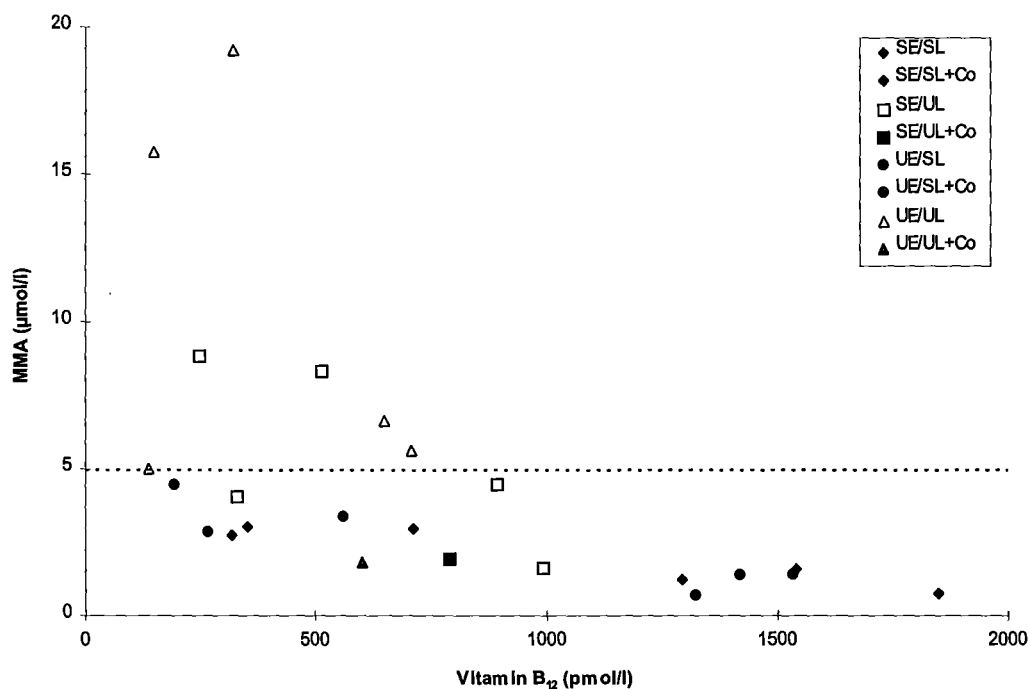
For abbreviations see 6.3.2

Figure 6.25: Relationship between plasma vitamin B₁₂ and MMA of Co bullet treated and control two-tooth ewes 1997/1998



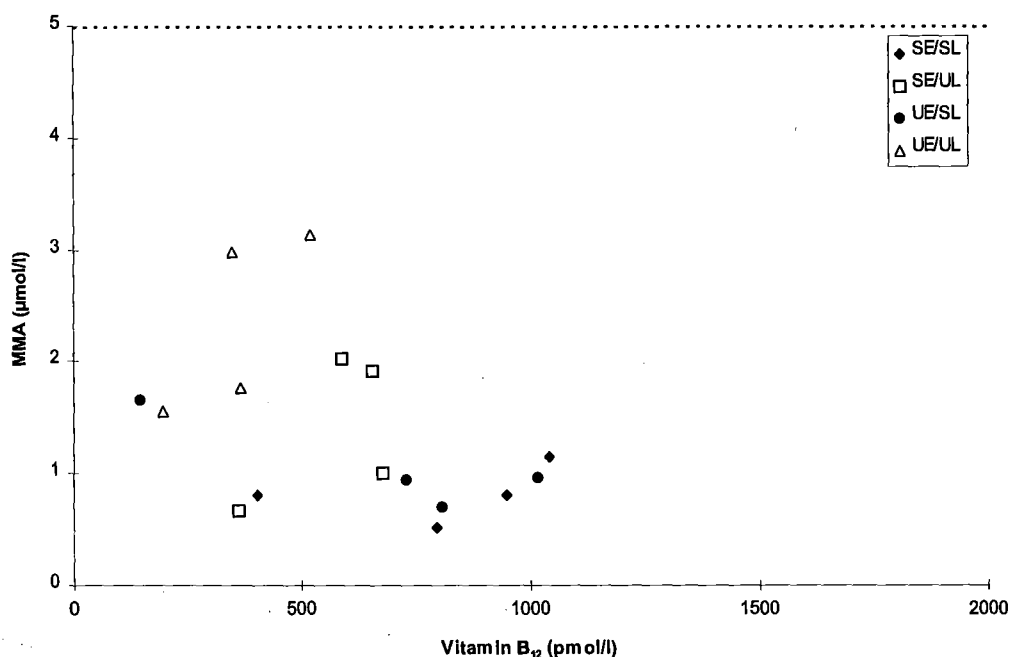
For abbreviations see 6.3.2

Figure 6.26: Relationship between serum vitamin B₁₂ and MMA of Co bullet treated and control two-tooth and four-tooth ewes 1998/1999



For abbreviations see 6.3.2

Figure 6.27: Relationship between plasma vitamin B₁₂ and MMA of vitamin B₁₂ injected and control lambs of Co bullet treated or control dams 1997/1998



For abbreviations see 6.3.2

Figure 6.28: Relationship between serum vitamin B₁₂ and MMA of vitamin B₁₂ injected and control lambs of Co bullet treated or control dams 1998/1999

In order to find a more fitting threshold for MMA in lambs and ewes the distribution of MMA among the 1997/1998 lambs was explored (Table 6.28). Although at least 90 % of those in the SL group had MMA concentrations below 5 μmol/l, only those in the UL group had MMA values above 10 μmol/l. However, since no difference in liveweight gain was observed in these groups the threshold above which vitamin B₁₂ deficiency is likely or imminent may lie closer to 20 μmol/l.

Table 6.28: Distribution of plasma MMA of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams in 1997/1998

MMA μmol/l	SE/SL %	SE/UL %	UE/SL %	UE/UL %
0-5.0	93	62	90	39
5.1-10.0	7	24	10	33
10.1-20.0	0	11	0	18
>20.1	0	2	0	10

For abbreviations see 6.3.2

Similarly, the 1997/1998 UE group was divided, for comparison, into animals whose MMA exceeded 10 $\mu\text{mol/l}$ at some stage of the trial (about two thirds of ewes), and into those whose MMA stayed below that threshold at every sampling (Table 6.29), in order to find out if this threshold was more appropriate as a marker of deficiency. There were no other markers (plasma and liver vitamin B₁₂, liveweight change or FEC) that showed any significant difference between those with MMA above or below 10 $\mu\text{mol/l}$, although the second liver sample just failed to reach significance ($p = 0.083$).

Table 6.29: Mean serum MMA values for control two-tooth ewes with MMA > 10 $\mu\text{mol/l}$ at some stage during the trial and those with MMA < 10 $\mu\text{mol/l}$ in 1997/1998 ($\pm$ SEM)

	MMA 1 $\mu\text{mol/l}$	MMA 2 $\mu\text{mol/l}$	MMA 3 $\mu\text{mol/l}$	MMA 4 $\mu\text{mol/l}$
MMA > 10	2.6 $\pm$ 0.26	6.0 $\pm$ 0.65	8.4 $\pm$ 0.51	18.4 $\pm$ 2.95
MMA < 10	1.9 $\pm$ 0.35	4.0 $\pm$ 0.86	5.0 $\pm$ 0.68	6.9 $\pm$ 3.93

	MMA 5 $\mu\text{mol/l}$	MMA 6 $\mu\text{mol/l}$	MMA 7 $\mu\text{mol/l}$	MMA 8 $\mu\text{mol/l}$
MMA > 10	13.7 $\pm$ 1.04	20.2 $\pm$ 9.03	9.6 $\pm$ 2.38	4.3 $\pm$ 0.77
MMA < 10	5.9 $\pm$ 1.38	4.6 $\pm$ 12.03	3.9 $\pm$ 3.17	1.8 $\pm$ 1.03

Lastly, animals who had MMA above 20 $\mu\text{mol/l}$ at some stage were singled out and their other traits investigated. Only three ewes and three lambs of the 1997/1998 trial (all unsupplemented) fell into this category. In the ewes, MMA ranged from 26 to 187 $\mu\text{mol/l}$, plasma vitamin B₁₂ from 60 to 350 pmol/l and liver vitamin B₁₂ from 70 to 85 nmol/kg fresh tissue. Their weight gain was in the upper half of the mean for that group. In the lambs, MMA ranged from 23 to 79 $\mu\text{mol/l}$, plasma vitamin B₁₂ from 90 to 450 pmol/l and liver vitamin B₁₂ from 70 to 160 nmol/kg fresh tissue. All three lambs had the smallest LWG in their group. Incidentally, they were not the offspring of the above ewes.

Further, lambs with low plasma and liver vitamin B₁₂ concentrations were identified. In 1997/1998, most lambs were born with liver vitamin B₁₂ concentrations below the limit of detection (70 nmol/kg fresh tissue). Of those, seven lambs also had plasma vitamin B₁₂ concentrations below the limit of detection (57 pmol/l). None of

these lambs had MMA > 10 $\mu\text{mol/l}$. In 1998/1999, the two lambs that had both serum and liver vitamin B₁₂ concentrations below the limit of detection had MMA well below 5 $\mu\text{mol/l}$. A rise in MMA for both ewes and lambs did not, therefore, necessarily appear to indicate low or depleted plasma or liver levels. The fact that LWG responses were not achieved suggests that the current range of serum and liver values for vitamin B₁₂ need revising and are certainly not valid for PR lambs.

6.6.8 Homocysteine

MMA is usually the first true indicator of the deficiency (after liver reserves become depleted), followed by hcy. Therefore only those plasma samples with MMA values above 5 $\mu\text{mol/l}$ were analysed for hcy, anticipating hcy values to be raised too. The hcy values in the present study rose to 24 $\mu\text{mol/l}$ which was slightly above what Kennedy *et al.* (1992a) considered normal, viz 20 $\mu\text{mol/l}$. However, the same authors (1994a), in a different trial, measured hcy values in Co sufficient lambs of ~ 38 $\mu\text{mol/l}$. Yet in neither of these trials did Kennedy *et al.* (1992a, 1994a) measure MMA.

Vellema *et al.* (1999) did measure both hcy and MMA in plasma in a trial with Co deficient and Co supplemented Texel twin lambs. Although they found a significant difference in both metabolites between Co sufficient and deficient lambs, and plasma MMA rose up to 80 $\mu\text{mol/l}$, highest hcy concentrations in Co deficient lambs were only ~ 15 $\mu\text{mol/l}$. Further, these workers (Vellema *et al.*, 1999) found that MMA and hcy increased at about the same time, making hcy as early an indicator for vitamin B₁₂ deficiency as MMA. This is in contrast to Kennedy *et al.* (1992a) who found that hcy did not increase significantly until ten weeks after plasma vitamin B₁₂ concentrations had decreased to below the threshold of normality, and twelve weeks after liveweight was significantly lower in lambs fed a Co deficient diet compared to Co replete control animals, thus making hcy a late indicator of deficiency.

When the results of the present trials were well below the level that Kennedy *et al.* (1994a) gave for vitamin B₁₂ replete ruminant lambs further analysis of samples

was abandoned. However, in the light of these latest findings by Vellema *et al.* (1999) analysis of hcy may well be warranted in future trials.

6.6.9 Milk composition and yield

6.6.9.1 Milk Protein

Milk proteins measured in this study matched well with the results of Peart *et al.* (1975) and Geenty (1979). Generally, protein values were slightly higher in the second year. When cobalt and plasma vitamin B₁₂ were at their lowest and MMA was at its highest, milk protein was at its lowest too. The most likely limiting amino acids for milk protein synthesis are methionine and lysine (Rusdi and van Houtert, 1997), and since methionine synthesis in the body depends on the presence of adequate amounts of me-cbl, this may be an indication that total protein could well be lowered in vitamin B₁₂ deficiency. This would be consistent with the findings of O'Halloran and Skerman (1961).

6.6.9.2 Milk Vitamin B₁₂

Vitamin B₁₂ was 1.5 to 5 fold higher in milk than in serum, indicating that vitamin B₁₂ must have been secreted into milk against a concentration gradient (Grace, 1999a). The higher values for sampling one would reflect that some samples still contained colostrum (which has been observed to have a much higher vitamin B₁₂ content than milk - O'Halloran and Skerman, 1961), and for sampling five that some ewes were drying up which could have made their milk more concentrated.

O'Halloran and Skerman (1961) compared the vitamin B₁₂ content of Co bullet treated ewes with controls. They found that colostrum of treated ewes had vitamin B₁₂ values around 75000 pmol/l, three times as high as that of control animals. After four weeks the vitamin B₁₂ content of milk had reduced to around 8500 pmol/l, ten times as high as that of the control ewes. Gregory (1954), who developed the assay method used by the above researchers, found that milk of Co replete animals had a vitamin B₁₂ concentration of ~ 7600 pmol/l, a comparable figure to the Co bullet treated animals of O'Halloran and Skerman (1961) after one month of lactation. At the end of October, after all the lambs were born, only the

OSE group in 1998/1999 had values comparable to these in the literature, viz ~ 7700 pmol/l. However, the SE group in the 1997/1998 trial had only ~ 2200 pmol/l and the NSE group ~ 4000 pmol/l, i.e. ~ 35 and ~ 50 % of the OSE groups' values. The UE groups had milk vitamin B₁₂ concentrations between 1300 and 1700 pmol/l in the 1998/1999 trial and only 270 pmol/l in the 1997/1998 trial.

6.6.9.3 *Milk Yield*

Estimated milk yield has been calculated from the lambs' growth rate. Table 6.30 and 6.31 show the results for the two years of the trial. From December onwards the results are likely to be somewhat overestimated since the lambs at that stage derive their energy increasingly from foraging rather than from milk alone.

Table 6.30: Estimated mean daily milk yield (litres/d) of Co bullet treated and control two-tooth ewes 1997/1998 (± SE)

	8/10/1997	31/10/1997	21/11/1997	15/12/1997	9/01/1998
SE	1.63 ± 0.078	1.45 ± 0.153	0.96 ± 0.095	0.90 ± 0.068	0.63 ± 0.092
UE	1.62 ± 0.074	1.55 ± 0.146	1.07 ± 0.090	0.98 ± 0.064	0.66 ± 0.090

For abbreviations see 6.3.2

Table 6.31: Estimated mean daily milk yield (litres/d) of Co bullet treated and control two-tooth and four-tooth ewes 1998/1999 (± SE)

	29/10/1998	19/11/1998	14/12/1998	7/1/1999
OSE	1.86 ± 0.186	1.39 ± 0.147	1.59 ± 0.132	0.80 ± 0.193
NSE	1.22 ± 0.302	1.54 ± 0.239	1.26 ± 0.214	1.38 ± 0.313
OUE	1.87 ± 0.186	1.08 ± 0.147	1.36 ± 0.132	0.67 ± 0.193
NUE	1.81 ± 0.228	0.90 ± 0.181	1.22 ± 0.162	1.03 ± 0.236

For abbreviations see 6.3.2

Milk yield (Table 6.32) has been measured by various researchers (Geenty, 1979; Peart *et al.*, 1975; Quirk and Norton, 1987; Jagusch *et al.*, 1972) in ewes on Co sufficient pastures as well as on low Co pastures with and without a Co bullet supplement. The estimated milk production in the two trials was within the range that these researchers have found (Figure 6.29) for the first six weeks. After that time comparisons are unrealistic because of the likely contribution of nutrients from pasture to LWG. Quirk and Norton (1987) found that supplementation with Co

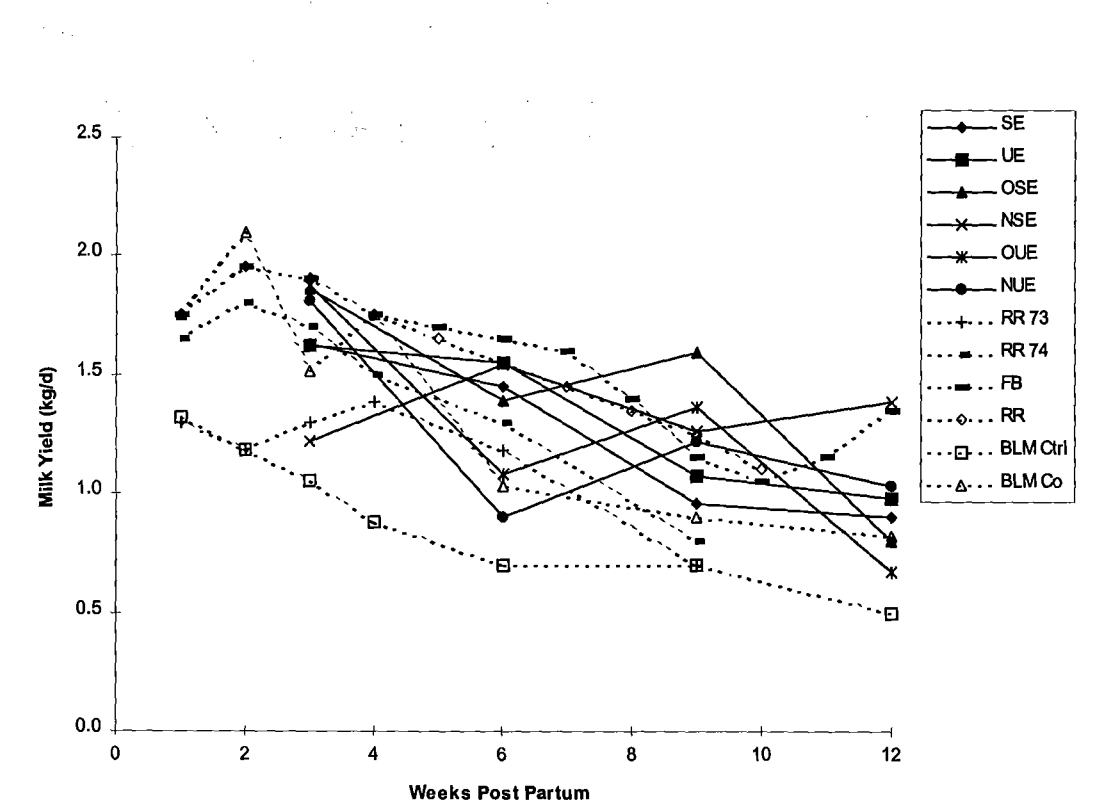
increased milk yield but this could not be substantiated in these trials, perhaps because the deficiency was too mild.

Table 6.32: Mean milk yield (litres/d) of ewes at various stages of lactation (as reported in the literature)

Researcher	Year	Breed/Trt*	Weeks of Lactation			
			3	6	9	12
Geenty	1979	RR 73	1.30	1.18	0.70	
Geenty	1979	RR 74	1.70	1.30	0.80	
Peart	1975	FB	1.90	1.65	1.15	1.35
Jagusch	1972	RR	1.90	1.55	1.25	
Quirk & Norton	1987	BLM Ctrl	1.05	0.70	0.70	0.50
		BLM Co	1.51	1.03	0.90	0.82

RR = Romney x Romney
FB = Finnish Landrace x Blackface
BLM = Border Leicester x Merino

Co = Co bullet
Ctrl = Control



RR = Romney x Romney
FB = Finnish Landrace x Blackface
BLM = Border Leicester x Merino
For other abbreviations see 6.3.2

Co = Co bullet
Ctrl = Control

Figure 6.29: Mean milk yield of various breeds of ewes (as reported in the literature) and data from this trial

From the vitamin B₁₂ content of milk and the milk yield an estimated mean vitamin B₁₂ intake of lambs from milk could be calculated (Table 6.33 and 6.34). Vitamin B₁₂ intake in December and January would not truly reflect what the lambs were receiving since some of their vitamin B₁₂ was then derived from Co intake and rumen vitamin B₁₂ production. The 1998/1999 lambs of OSE had a significantly higher ($p < 0.001$) vitamin B₁₂ intake than the other lambs at the start of the trial. Lambs from the SE groups received a significantly higher ($p < 0.001$) amount of vitamin B₁₂ than the UE lambs.

Table 6.33: Estimated mean daily vitamin B₁₂ intake (pmol/d) from milk of lambs suckling Co bullet treated or control dams 1997/1998 (± SE)

	31/10/1997	21/11/1997	15/12/1997	9/01/1998
SE	3632 ± 291	3299 ± 246	2317 ± 228	3791 ± 493
UE	439 ± 67	658 ± 130	695 ± 195	1738 ± 179

For abbreviations see 6.3.2

Table 6.34: Estimated mean daily vitamin B₁₂ intake (pmol/d) from milk of lambs suckling Co bullet treated or control dams 1998/1999 (± SE)

	29/10/1998	19/11/1998	14/12/1998	7/1/1999
OSE	16460 ± 6693	5879 ± 760	8342 ± 1515	7123 ± 2407
OUE	2411 ± 585	793 ± 177	1187 ± 259	1337 ± 324
NSE	5377 ± 839	7596 ± 985	4516 ± 660	6065 ± 1091
NUE	2293 ± 584	1230 ± 252	1076 ± 208	1639 ± 289

For abbreviations see 6.3.2

From the daily intake of the vitamin, the amount and percentage of vitamin B₁₂ retained in the liver for untreated and treated lambs could be estimated (Tables 6.35 and 6.36). Treated lambs retained around 8 % of the vitamin in their livers, whereas the UE/UL group retained 50 to 70 %. This suggests the less the animal has available the greater is the absorption and retention of the vitamin, as has been postulated by Smith and Marston (1970a).

Table 6.35: Mean vitamin B₁₂ retention in the livers of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1997/1998

	Liver increase	Milk + trt B ₁₂	Liver retention	Left for metabolism/other tissues/excretion
	pmol/d	pmol/d	%	pmol/d
SE/SL	2875	35416	8.1	32541
SE/UL	961	3029	31.7	2068

UE/SL	2559	32726	7.8	30167
UE/UL	630	862	73.1	232

For abbreviations see 6.3.2

Table 6.36: Mean vitamin B₁₂ retention in the livers of vitamin B₁₂ injected and control lambs from Co bullet treated or control dams 1998/1999

	Liver increase	Milk + trt B ₁₂	Liver retention	Left for metabolism/other tissues/excretion
	pmol/d	pmol/d	%	pmol/d
SE/SL	2860	38086	7.5	35226
SE/UL	1281	10019	12.8	8737
UE/SL	2612	33210	7.9	30598
UE/UL	822	1598	51.4	776

For abbreviations see 6.3.2

6.6.10 Faecal egg count

Co/vitamin B₁₂ supplementation had a beneficial effect on lowering the parasitic egg burden in sheep. This was particularly obvious in the 1997/1998 season. MacPherson *et al.* (1987) and Paterson and MacPherson (1990b) have found greater severity of *Ostertagia* infection in Co depleted calves, with impairment of neutrophil function, followed by reduction in liveweight gain. Similar results have been obtained for sheep by Judson *et al.* (1985).

Anderson and Andrews (1955) suspected that parasites contributed to the unthriftiness of Co deficient lambs. Significant parasitic burdens of *Ostertagia* in vitamin B₁₂ deficient lambs have been found by other workers too (Clark *et al.*, 1978; Clark, 1982; Ferguson *et al.*, 1989). However, Downey (1965 and 1966a) found that Co deficient lambs had less parasitic burden in the initial stages of infestation than Co supplemented lambs when the parasite was either *Trichostrongylus axei* or *Haemonchus contortus*, but when infected with *Ostertagia circumcincta* Co deficient lambs showed higher egg burdens than the supplemented ones (Downey, 1966b).

Gardiner (1966), who infected sheep with *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Ostertagia* spp., found lower serum vitamin B₁₂ concentrations in sheep with *T. colubriformis* infection. Since Co and feed intake had remained constant he concluded that the decline of serum vitamin B₁₂ was due to intestinal damage caused by *T. colubriformis* which led to a lack of absorptive capacity. He also noticed an increase of serum vitamin B₁₂ levels when the sheep

were *in extremis* due to the infection, probably because of liver cell necrosis and subsequent release of stored vitamin into the circulation. Andrews (1972) stated that internal parasites reduce the amount of vitamin B₁₂ that is stored in the liver. He implied that a high FEC could therefore contribute to Co deficiency on marginal pastures. High plasma and low liver vitamin B₁₂ concentrations have also been found in humans with liver disease (Baker *et al.*, 1998).

As increased levels of immune markers have been found in animals affected by *Ostertagia* spp. infection vitamin B₁₂ may confer some immunity, possibly via the me-cbl pathways since methionine plays a vital role in the immune response of an animal. Elevated levels of other immune markers, such as IgE, Interleukin-1 and -6, and tumour necrosis factor (Sykes *et al.*, 1992), have also been found with increased FEC. It seems a vicious circle is created whereby the infection depletes the animal of vitamin B₁₂ and therefore reduces immunity which in turn leads to a higher FEC.

In fact, the highest FEC was noted in the 1997/1998 UE group just after lambing, when vitamin B₁₂ resources were stretched. Familton *et al.* (1995) also noticed this post-parturient rise in FEC in undrenched ewes which leads to increased pasture contamination. This, in turn, contributes to the establishment of primary infection in lambs. A greater worm burden was indeed seen in the UL groups from the time the lambs became ruminant. Judson *et al.* (1985) made similar observations in Co deficient weaner lambs.

Familton *et al.* (1995) also observed that FEC decreased at weaning and stayed low from then on. This was in contrast to these findings where FEC rose again in the UE group at weaning time in 1997/1998. In the following year, ewe FEC rose just before lambing in the two-tooths and just after lambing in the four-tooths, and declined from then on until the end of the trial. The lower FEC in the OE groups in the second trial may be due to their more mature immune system, thus having developed more resistance. This is supported by the findings of Sykes *et al.* (1992) and Familton and McAnulty (1996).

Noteworthy is too that *Ostertagia* spp. infection leads to an increase in abomasal pH (Familton and McAnulty, 1997; Sykes *et al.*, 1992). This would not only impede the digestion of protein, but also the release of IF which is needed to bind and absorb vitamin B₁₂. Affected animals therefore may be disadvantaged

doubly as far as their available vitamin B₁₂ is concerned: they already have a compromised vitamin B₁₂ status, leading to the higher infection, and the higher the infection, the less they absorb.

CHAPTER 7

Comparison of tissue vitamin B₁₂ concentrations with methylmalonic acid in the period after weaning

7.1 Introduction

For lambs, weaning is the most vulnerable time as far as vitamin B₁₂ deficiency is concerned (Andrews, 1956 and 1972). Until that time, lambs still receive some simple sugars (lactose) as well as vitamin B₁₂ from milk. After weaning, however, lambs depend almost entirely on gluconoeogenesis from volatile fatty acids, chiefly propionate. Hence there is increased demand on the propionate - succinate metabolic pathway which requires vitamin B₁₂ as a cofactor. The lambs then also rely entirely on their own vitamin B₁₂ production from ruminal bacteria and Co from herbage.

In this chapter the metabolic changes in vitamin B₁₂ status and changes in metabolic pathways affected by vitamin B₁₂ are investigated in lambs in the period after weaning. In 1998, three farms in Southland where Co deficiency had supposedly occurred in previous years were chosen by local veterinarians. In 1999, Farm 1 was followed up with another, similar trial.

7.2 Aim

These trials intended to compare changes in serum and liver vitamin B₁₂ concentrations with serum MMA and weight gain to determine the critical time and tissue levels of vitamin B₁₂ and a threshold concentration for MMA in lambs at and after weaning at which weight gain response to supplementation would occur.

7.3 Experimental design

Three farms participated in the 1998 trial, with 50 lambs from each. Farm 1 was located 46°10' latitude south and 168°10' longitude east. The soil was

Drummond (melanic) soil (recorded as being low to marginal in Co - Andrews, 1972) and the lambs were Coopworths. Farm 2 was located 46°07' latitude south and 167°40' longitude east. The soil was Monowai complex Glenelg (glacial outwash) soil. The lambs were Romney x Texel. Farm 3 was located 46°15' latitude south and 169°46' longitude east. The soil type was YG Earth and the lambs were Romney. The pasture on all three farms was traditional perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.).

The trial started at weaning and ran for four to five months. The animals were sampled from 26/11/1997 to 6/4/1998 on Farm 1, from 7/1/1998 to 16/4/1998 on Farm 2 and from 11/12/1997 to 16/3/1998 on Farm 3. Table 7.1 gives an outline of the treatment and the samples taken each time. Of the 50 lambs used from each farm ten animals were treated with a Co bullet (PermaCo, 2 g Co) at the start of the trial. They were allocated to group A. Each month, another ten lambs from the remaining untreated group received a Co bullet (groups B, C, D). All animals were weighed monthly. Group E (the last ten animals) was only to be used if the difference in liveweight between treated and untreated groups was more than 1 kg (shown in italics in Table 7.1). Blood samples were taken only from those animals that were about to be treated (Ctrl) and from those that had been treated. Liver biopsies were taken only from the ten lambs that were about to be treated, and from the same randomly chosen five lambs from group A after they had been treated.

Table 7.1: Outline of experimental design of 1998

Week	0		4		8		12		16	
	n	S	n	S	n	S	n	S	n	S
Group A (treated)	10	w,b,l		w,b,l*		w,b,l*		w,b,l*		w,b,l*
Untreated lambs	40	w		w		w		w		w
Group B (treated)			10	w,b,l		w,b		w,b		w,b
Untreated lambs			30	w		w		w		w
Group C (treated)					10	w,b,l		w,b		w,b
Untreated lambs					20	w		w		w
Group D (treated)							10	w,b,l		w,b
Untreated lambs							10	w		w
Group E (treated)									10	w,b,l
Untreated lambs									0	
Treated	10		20		30		40		50	
Untreated	40		30		20		10		0	

n = number of lambs S = samples taken * = 5 lambs only
w = weight b = blood l = liver

A similar trial was conducted the following year. Farm 1 from the previous trial was chosen to investigate that season’s weaner lambs for their vitamin B₁₂ status. Figure 7.1 outlines the trial:

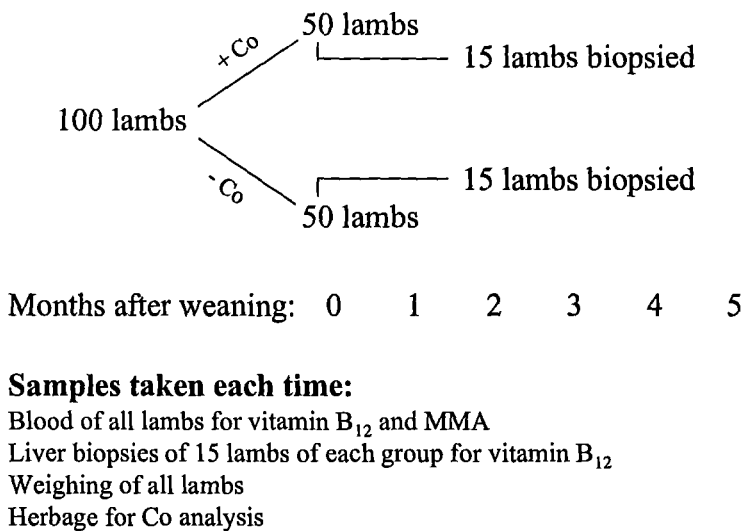


Figure 7.1: Outline of experimental design in 1999

At weaning, 100 Coopworth lambs were hierarchically divided by weight into two groups of 50 each. One group (Trt) was treated with two Co bullets (to

reduce the possibility of regurgitation or coating), the untreated group (Ctrl) served as control. At the start of the trial (before treatment) and at monthly intervals for six months, all lambs were weighed, had blood samples taken, and the same 15 lambs from each group (matched initially by weight and taken from a range of weights) were subjected to a liver biopsy. However, because of a misunderstanding, only the liver biopsied lambs in the treated group had blood samples taken on the first occasion. Herbage samples were taken for Co analysis. This trial ran from 25/11/1998 to 20/4/1999.

In both trials, the lambs were subjected to normal farming practice, such as drenching.

7.4 Materials and methods

7.4.1 Sampling

Sample collection was done monthly on the farms by local veterinarians and followed the protocol given in Chapter 6.4. Blood and liver samples were sent to our laboratory. Pasture samples were sent directly to the laboratory at AgResearch Soil Fertility Service (Hamilton, New Zealand). Co was measured on washed and unwashed samples.

7.4.2 Statistical analysis

All the data were analysed using Genstat. In the 1998 trial the mean values of each group for serum vitamin B₁₂, MMA and liver vitamin B₁₂ before supplementation were compared to those values obtained after the lambs had been treated. Therefore, the first sampling of every group was used as a baseline of untreated lambs (UL) over time. The values for this 'group' of unsupplemented animals was then compared with the mean values for each group after supplementation. Data was also analysed over time, and a group x time interaction was calculated. Orthogonal polynomial analysis of the data was performed to test differences in treatments with repeated observations in time on the basis of differences in the coefficients for components of the shape of the curve relating

parameters measured to time. The weights of the animals for each group were compared using ANOVA.

In the 1999 trial ANOVA was performed on all the traits. Means and overall SEM of the treated and untreated groups were calculated as well as the interaction between treatment x time of the various groups (Kenward, 1987). Degrees of freedom have been adjusted using the Greenhouse-Geisser epsilon (Greenhouse and Geisser, 1959) since not all measurements were independent of each other. The shapes of the curves were determined by orthogonal polynomial analysis to identify their components.

For both years, SED and overall SEM have been determined and, together with Student's t-tests, the LSDs were found for various p-values ($p < 0.05$, < 0.01 and < 0.001).

7.5 Results

7.5.1 1998 trials

7.5.1.1 Serum vitamin B₁₂

On Farm 1 (Figure 7.2) analysis of variance revealed a significant difference ($p < 0.01$) in the linear components of the group x time interaction. This was due to the increase in vitamin B₁₂ concentrations in the UL 'group' from sampling two onwards while the treated groups showed a decline.

The UL 'group' showed a decline from from 710 pmol/l to 194 pmol/l ($p < 0.05$) in the first month after weaning, followed by a trend for a steady but non-significant increase to 457 pmol/l at the end of the trial about five months after weaning. Supplementation simply maintained or restored concentrations in all groups to values seen at weaning with the exception of group C which had a significantly higher ($p < 0.001$) serum vitamin B₁₂ level at three months (1386 pmol/l) compared to the untreated lambs. Yet this rise was short-lived and dropped significantly ($p < 0.01$) at four months to 803 pmol/l.

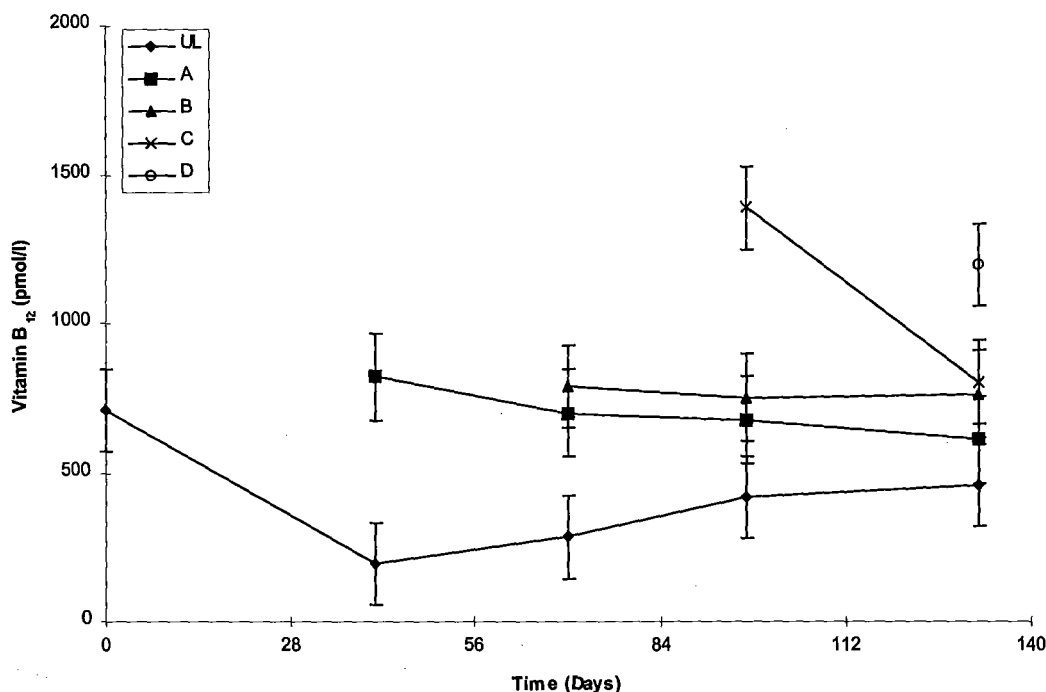


Figure 7.2: Serum vitamin B12 concentrations of Co bullet treated and untreated lambs on Farm 1 ($\pm$ SEM)

On Farm 2 (Figure 7.3) the group $\times$ time interaction was non-significant. There was, however, a highly significant difference ($p < 0.001$) between the groups and a significant difference ($p < 0.05$) due to time because of the drop that occurred earlier in the UL 'group' than in the others.

Serum vitamin B₁₂ was 952 pmol/l at weaning. The decline to 347 pmol/l in the UL 'group' was highly significant ($p < 0.01$) one month after weaning and values then did not change significantly until the end of the trial. Supplementation maintained the level of vitamin B₁₂ observed at weaning but supplemented groups (B and C) followed the trend of the UL 'group' in showing a decline of serum vitamin B₁₂ to 590 and 852 pmol/l, delayed by one or two months, respectively.

The decline in serum vitamin B₁₂ in this trial could be approximated by fitting the following exponential decay curve: $y = a + b^{-c(\text{days} + m1 + m2)}$ where $m1$ is the displacement due to the second curve, and $m2$ that of the third curve. The values obtained for the various parameters were: $a = -154 \pm 491$, $b = 1012 \pm 537$, $c = 0.0121 \pm 0.0079$, $m1 = -65.6 \pm 12.7$ and $m2 = -94.1 \pm 13.7$. The r^2 value was 0.40. Half life could be estimated at 58 ± 38 days.

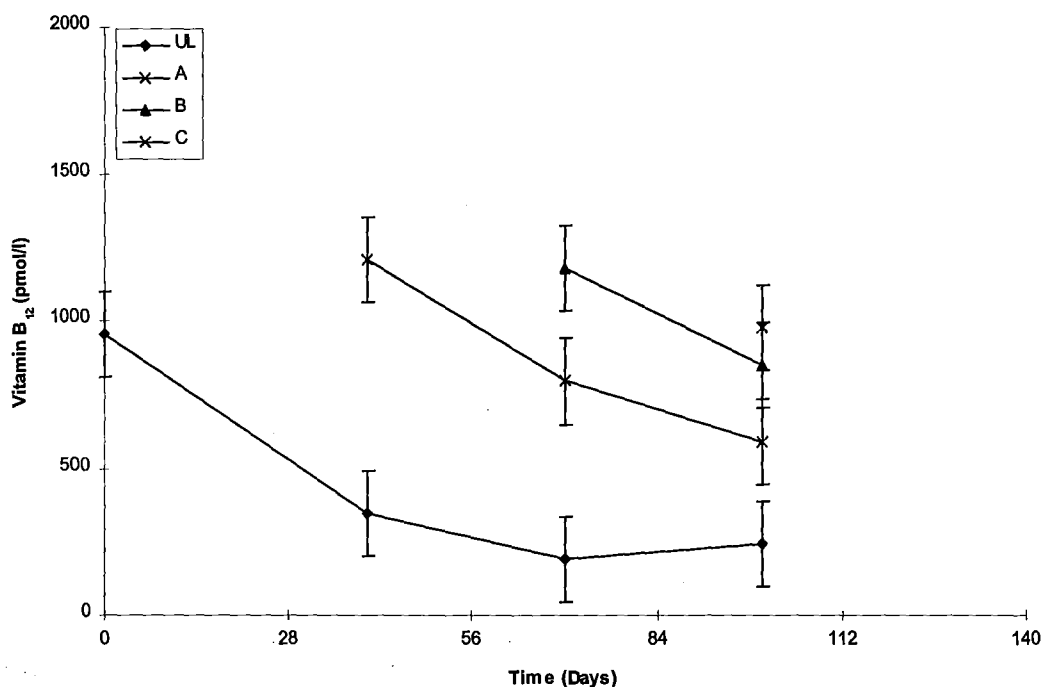


Figure 7.3: Serum vitamin B₁₂ concentrations of Co bullet treated and untreated lambs on Farm 2 ($\pm$ SEM)

On Farm 3 (Figure 7.4) the change in serum vitamin B₁₂ over time and the difference between the groups was highly significant ($p < 0.001$ for both). There was no group $\times$ time interaction, indicating that the change in concentration for the groups was essentially the same. The curves had significant quadratic components ($p < 0.001$), reflecting an initial decline and consequent increase in concentration with time.

The UL 'group' started with a mean serum vitamin B₁₂ of 480 pmol/l. In untreated lambs serum vitamin B₁₂ concentrations fell non-significantly to 234 pmol/l one month after weaning before rising again significantly ($p < 0.05$) to 729 pmol/l at the end of the trial. Supplementation maintained the concentrations seen at weaning and increased values ($p < 0.05$) to 639 pmol/l at one month after weaning. Serum vitamin B₁₂ levels subsequently declined in group A to 514 pmol/l before rising to 822 pmol/l at the end of the trial. There was no significant difference in the serum values between groups A and UL in the last two samplings.

Group B had a mean serum vitamin B₁₂ value of 950 pmol/l one month after supplementation, highly significantly different ($p < 0.001$) from the UL 'group' at

that time and higher than values in group A ($p < 0.05$). There was a non-significant increase to 1267 pmol/l in group B two months after treatment. Group C was treated two months after weaning and had a mean serum vitamin B₁₂ of 1853 pmol/l one month later. This was significantly higher ($p < 0.001$) than at any time in the UL ‘group’ or in any of the other supplemented groups.

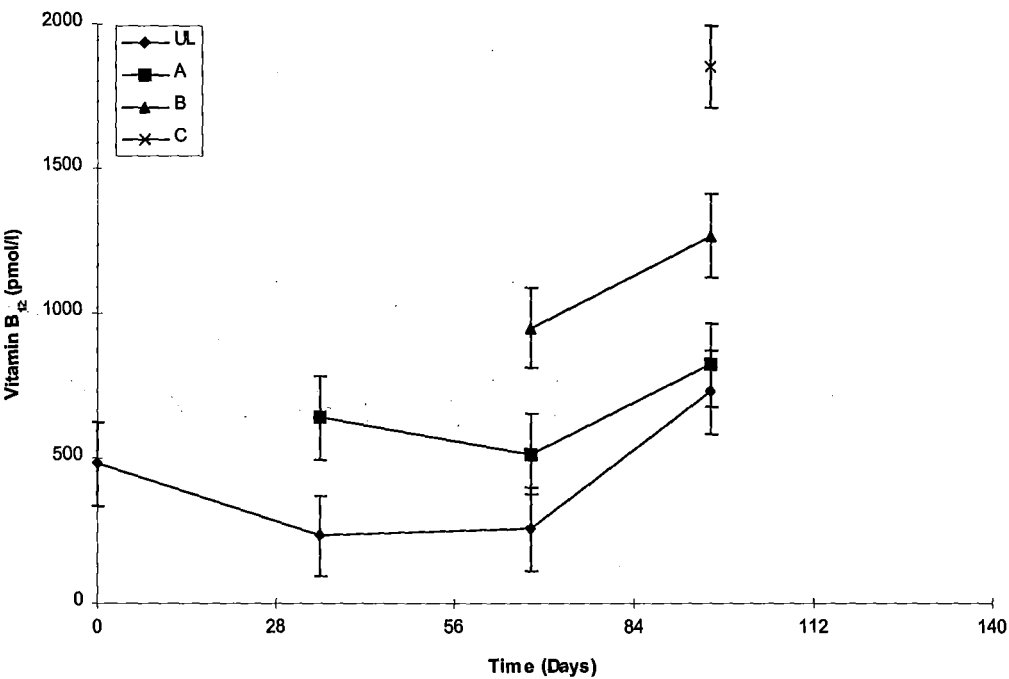


Figure 7.4: Serum vitamin B₁₂ concentrations of Co bullet treated and untreated lambs on Farm 3 ($\pm$ SEM)

7.5.1.2 Liver vitamin B₁₂

The mean liver vitamin B₁₂ values for Farm 1 are given in Figure 7.5. There was a highly significant difference between the two groups and a highly significant change over time ($p < 0.001$ for both). However, there was no group x time interaction, indicating that the curves for the two groups were parallel.

Liver vitamin B₁₂ in the UL ‘group’ fell from 210 to 95 nmol/kg fresh tissue one month after weaning ($p < 0.001$) before increasing to 174 nmol/kg fresh tissue three months after weaning ($p < 0.01$). Group A followed the trend of the UL ‘group’

although their liver vitamin B₁₂ values were significantly ($p < 0.05$) higher at every sampling than those of the UL 'group'.

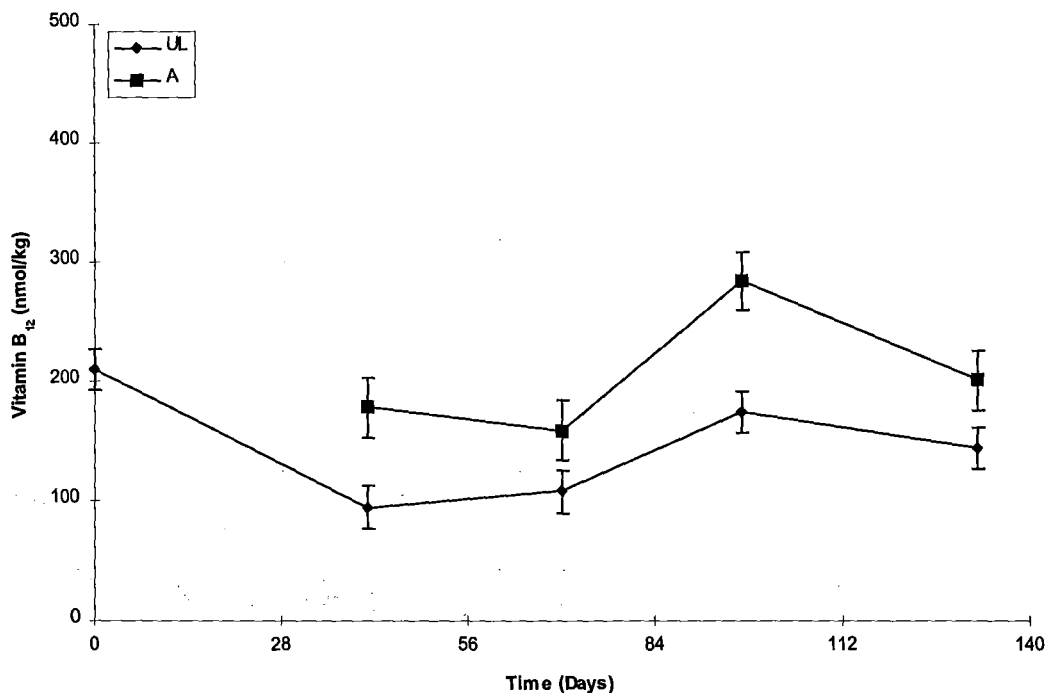


Figure 7.5: Liver vitamin B₁₂ concentrations of Co bullet treated and untreated lambs on Farm 1 ($\pm$ SEM)

Figure 7.6 displays the mean liver vitamin B₁₂ values for Farm 2. The overall difference between the groups was highly significant ($p < 0.01$) and so was the change over time ($p < 0.001$). There was no group x time interaction; the curves ran parallel. The difference between the curves was mainly due to linear components ($p < 0.001$).

Untreated animals had liver values of 318 nmol/kg fresh tissue at weaning which dropped to 152 nmol/kg fresh tissue at the end of the trial ($p < 0.05$). Treatment increased liver stores initially to 406 nmol/kg fresh tissue in group A. Values then decreased at a similar rate to the UL 'group' to 260 nmol/kg fresh tissue. However, this decrease was not statistically significant.

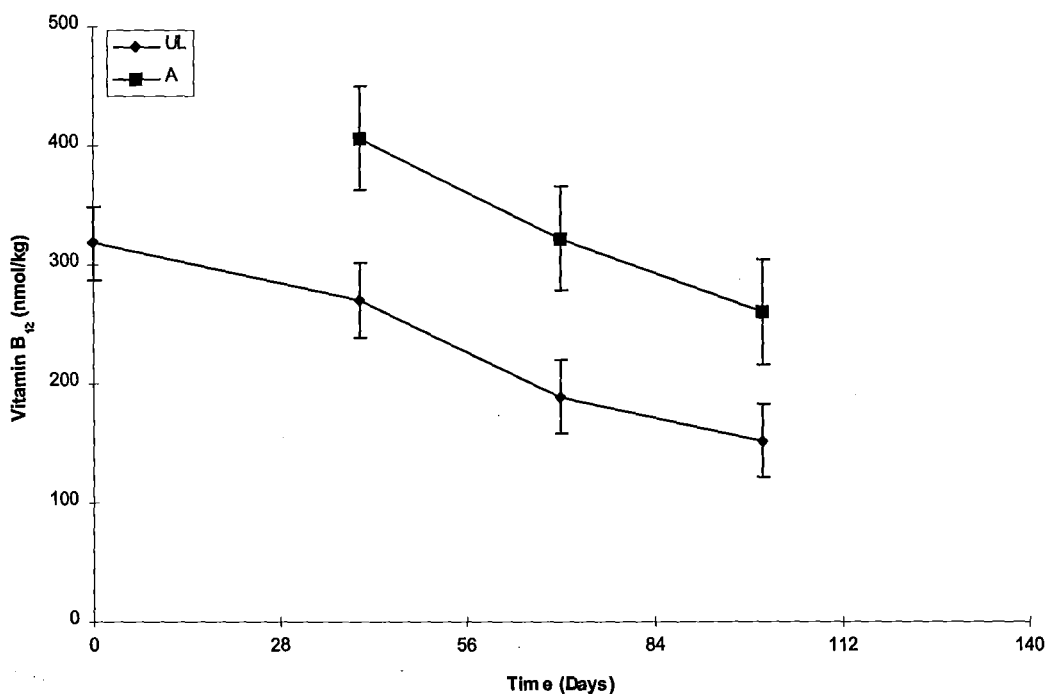


Figure 7.6: Liver vitamin B₁₂ concentrations of Co bullet treated and untreated lambs on Farm 2 ($\pm$ SEM)

Mean liver vitamin B₁₂ values for Farm 3 are given in Figure 7.7. Essentially there was no difference between the two groups, only a highly significant ($p < 0.001$) change over time, due to the initial drop of vitamin B₁₂ concentration in the UL 'group'. There was no group x time interaction. The shape of the curves was basically linear ($p < 0.001$).

The lambs started with a mean liver vitamin B₁₂ concentration of 301 nmol/kg fresh tissue at weaning. In the UL 'group' this dropped significantly ($p < 0.001$) to 154 nmol/kg fresh tissue one month later. Liver values fluctuated non-significantly and finished with 125 nmol/kg fresh tissue at the end of the trial. For group A liver values dropped non-significantly after supplementation from 248 to 220 nmol/kg fresh tissue at the end of the trial. The difference between the UL 'group' and group A was non-significant.

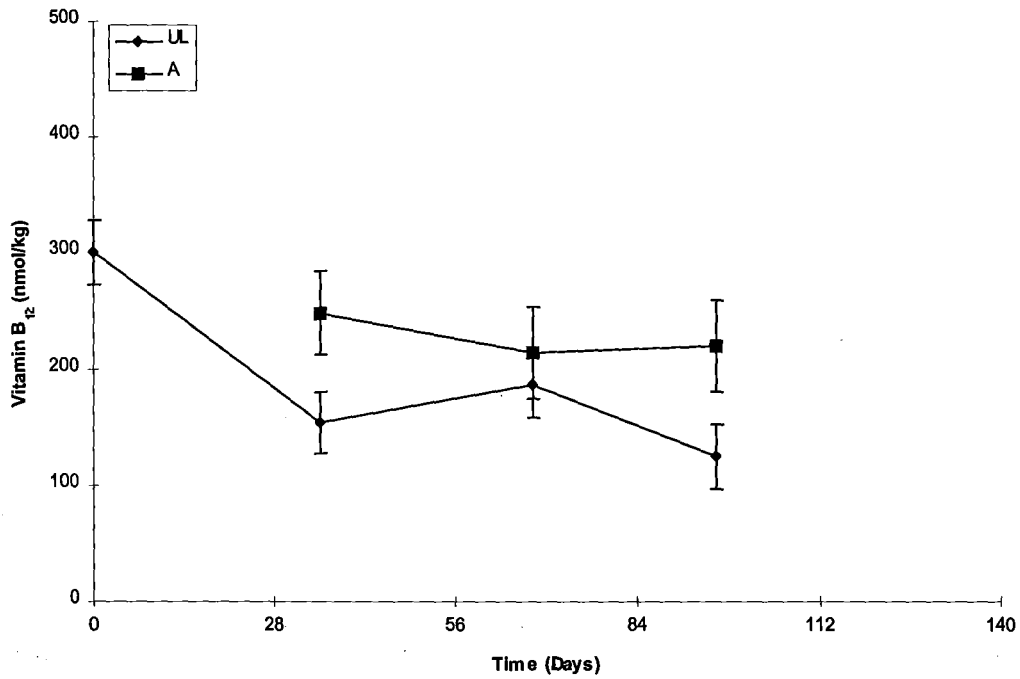


Figure 7.7: Liver vitamin B₁₂ concentrations of Co bullet treated and untreated lambs on Farm 3 ($\pm$ SEM)

7.5.1.3 *Methylmalonic acid*

MMA values for Farm 1 are shown in Figure 7.8. There was a highly significant ($p < 0.01$) group $\times$ time interaction in the quadratic component due to the UL 'group' showing significantly bigger changes in value over time.

The lambs started with MMA of 2.3 $\mu\text{mol/l}$ at weaning. The values for the UL 'group' rose significantly ($p < 0.001$) for the next two months to 6.8 $\mu\text{mol/l}$ before dropping back ($p < 0.01$) to 3.5 $\mu\text{mol/l}$ at the end of the trial. MMA for the supplemented groups stayed between 1.6 and 2.5 $\mu\text{mol/l}$ throughout the trial.

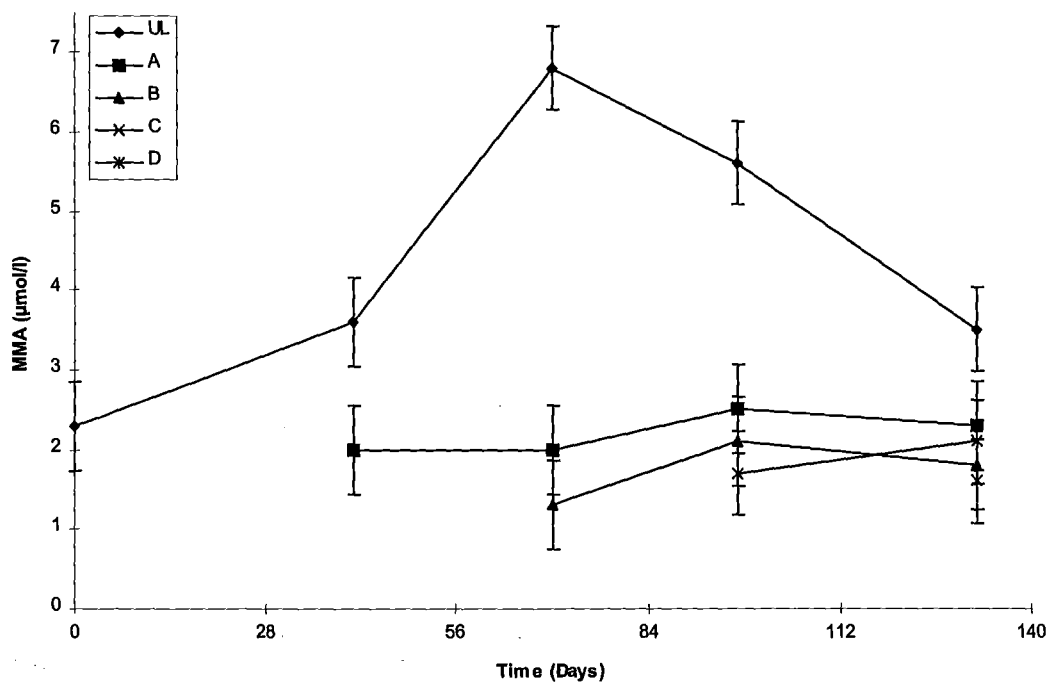


Figure 7.8: Serum MMA concentrations of Co bullet treated and untreated lambs on Farm 1 ($\pm$ SEM)

MMA values on Farm 2 are given in Figure 7.9. The difference between the UL 'group' and the treated groups as well as the change of MMA over time had highly significant ($p < 0.001$) quadratic components.

MMA values in the UL 'group' fell significantly ($p < 0.001$) from 4.7 to 1.7 $\mu\text{mol/l}$ one month after weaning before increasing again ($p < 0.05$) to 3.4 $\mu\text{mol/l}$ at the end of the trial. The supplemented groups had MMA values of 1.2 to 1.6 $\mu\text{mol/l}$ one month after treatment which increased non-significantly to around 2.5 $\mu\text{mol/l}$ at the end of the trial.

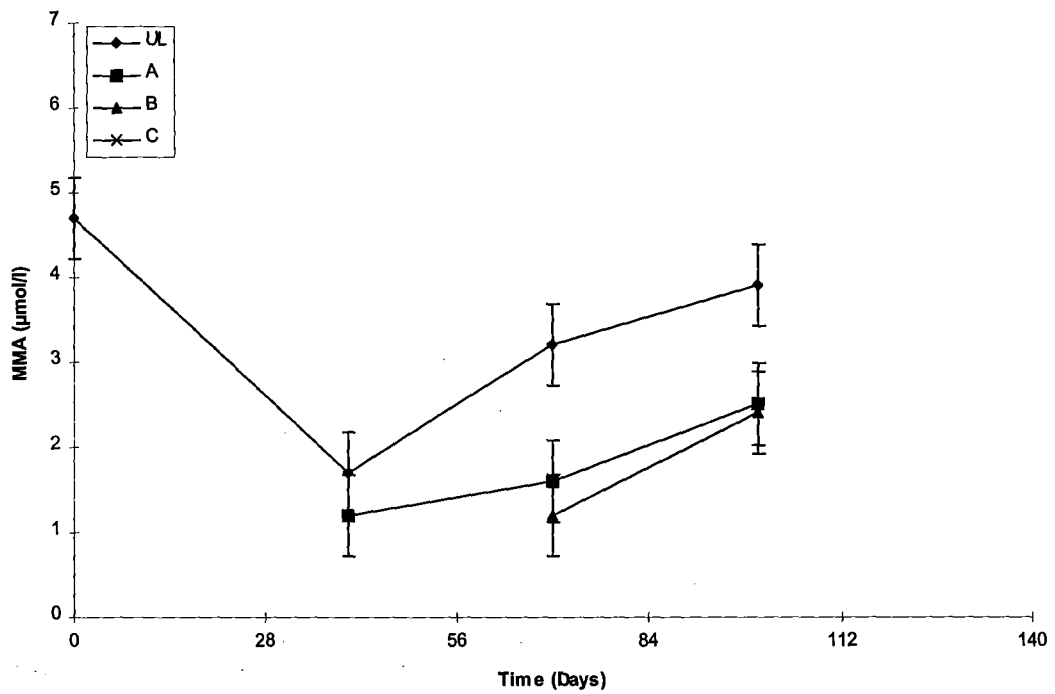


Figure 7.9: Serum MMA concentrations of Co bullet treated and untreated lambs on Farm 2 ($\pm$ SEM)

For Farm 3 MMA values are given in Figure 7.10. There was a highly significant difference ($p < 0.001$) between the treated and untreated groups as well as the change of MMA over time, as seen in the difference ($p < 0.001$) in the quadratic components. There was no significant group $\times$ time interaction.

Values in the UL 'group' increased significantly ($p < 0.01$) from 1.8 to 6.3 $\mu\text{mol/l}$ two months after weaning and dropped back to 3.1 $\mu\text{mol/l}$ ($p < 0.01$) at the last sampling. The treated groups had MMA values between 1.2 and 2.1 $\mu\text{mol/l}$ throughout the trial.

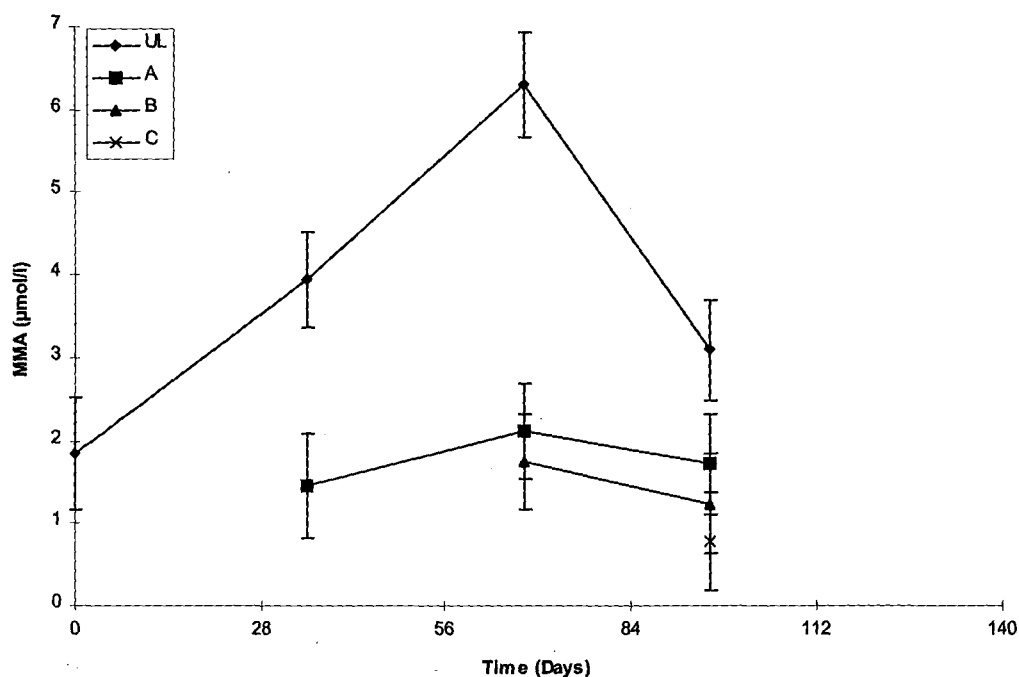


Figure 7.10: Serum MMA concentrations of Co bullet treated and untreated lambs on Farm 3 ($\pm$ SEM)

7.5.1.4 *Live Weight*

Figure 7.11 shows the live weight of the lambs on the three farms. On Farm 1 the weight difference between groups A and E at the fourth sampling was greater than 1 kg, therefore the trial continued for one more month with the inclusion of group E. Farmers on Farms 2 and 3 wanted to sell some of their lambs so sampling stopped after four months. There was no significant difference in weight between groups A to E on any of the farms. The only significant change in weight occurred over time as the animals were growing ($p < 0.001$).

Table 7.2 shows the mean LWG for the various groups as well as the mean LWG for the treated and untreated lambs. When LWG was compared in the groups there was no significant difference between any of the groups although there was a trend for the treated lambs to have a slightly greater LWG than the untreated lambs. The lambs grew more rapidly in the first four to five weeks. Growth rate was then reduced slightly during the next two months. The lambs on Farm 1 experienced another growth spurt during the last month of the trial.

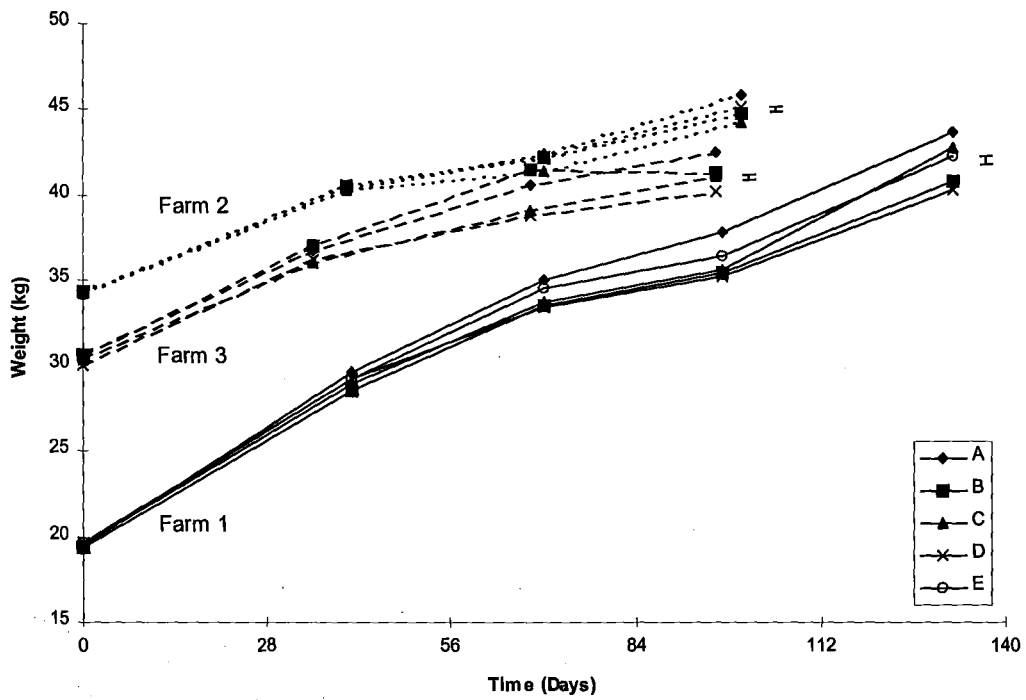


Figure 7.11: Liveweight (kg) of Co bullet treated and untreated lambs on the three farms ($\pm$ SEM given at end of graphs)

Table 7.2: Daily weight gain (kg) of Co bullet treated and untreated lambs on the three farms

Farm 1	Days	0 to 41	42 to 70	71 to 97	98 to 132	SEM
	Group A	0.24	0.19	0.10	0.17	0.020
	Group B	0.22	0.18	0.06	0.15	0.020
	Group C	0.23	0.17	0.07	0.21	0.020
	Group D	0.23	0.15	0.07	0.15	0.020
	Group E	0.23	0.19	0.07	0.17	0.020
	Treated Untreated	0.23	0.19 0.17	0.08 0.07	0.18 0.16	
Farm 2	Days	0 to 40	41 to 70	71 to 100		SEM
	Group A	0.15	0.08	0.12		0.033
	Group B	0.16	0.06	0.09		0.033
	Group C	0.16	0.04	0.10		0.033
	Group D	0.16	0.09	0.09		0.033
	Group E	0.16	0.06	0.10		0.033
	Treated Untreated	0.16	0.06 0.08	0.11 0.10		
Farm 3	Days	0 to 35	36 to 68	69 to 96		SEM
	Group A	0.18	0.14	0.05		0.013
	Group B	0.19	0.16	0.00		0.013
	Group C	0.16	0.11	0.06		0.014
	Group D	0.18	0.09	0.04		0.014
	Treated Untreated	0.18	0.14 0.12	0.03 0.05		

7.5.2 1999 trial

7.5.2.1 Pasture cobalt

Table 7.3 gives the results of the Co analysis in 1999. Except for the first sample Co levels stayed below 0.07 µg/g DM throughout the trial in washed samples.

Table 7.3: Pasture Co (µg/g DM) in 1999

	3/12/1998	16/12/1998	21/01/1999	23/02/1999	23/03/1999	19/04/1999
Washed	1.98	0.06	0.04	0.06	0.07	0.07
Unwashed	1.48	0.05	0.06	0.04	0.06	0.19

7.5.2.2 *Serum vitamin B₁₂*

The mean serum vitamin B₁₂ values for the 1999 trial are given in Figure 7.12. There was a highly significant treatment x time effect ($p < 0.001$) due to an initial increase from ~ 500 pmol/l to ~ 2500 pmol/l in the Co bullet treated lambs. Serum vitamin B₁₂ concentrations then fluctuated between 350 and 700 pmol/l in the Ctrl group and between 1278 and 2000 pmol/l in the Trt group.

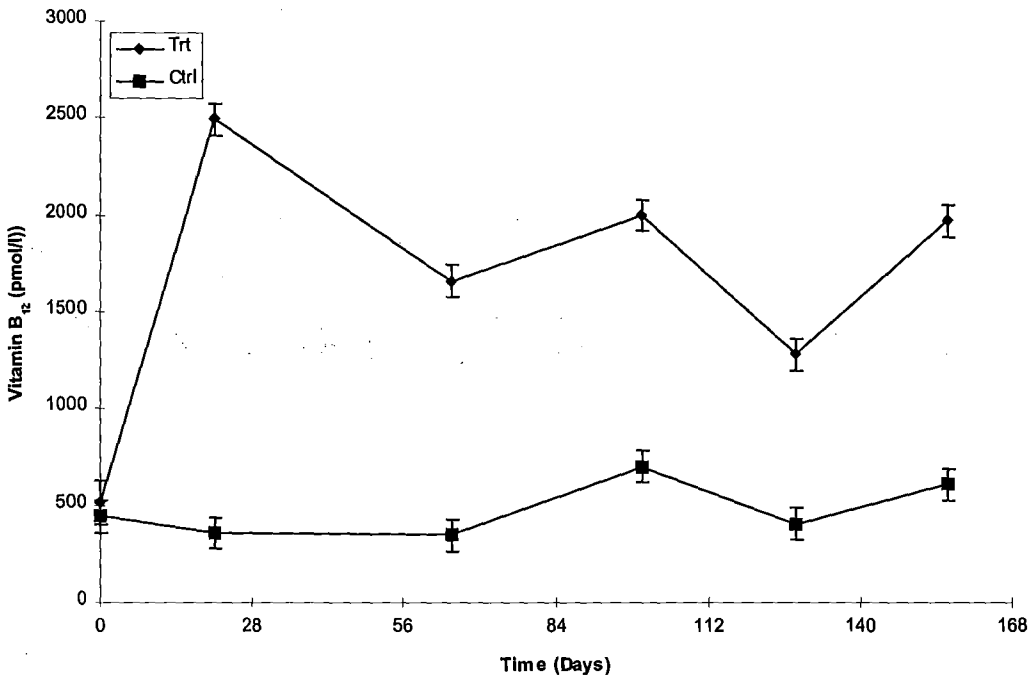


Figure 7.12: Serum vitamin B₁₂ concentrations of Co bullet treated and control lambs 1999 ($\pm$ SEM)

7.5.2.3 *Liver vitamin B₁₂*

The mean liver vitamin B₁₂ concentrations for the two groups are given in Figure 7.13. There was a highly significant treatment x time interaction with differences attributable to linear components ($p < 0.001$).

The Ctrl group started with a significantly higher ($p < 0.05$) value than the Trt group (529 versus 427 nmol/kg fresh tissue). Concentrations in both groups decreased after weaning to around 350 nmol/kg fresh tissue (a highly significant drop for the Ctrl group). Values in the Ctrl group further decreased to 225 before

increasing to 288 nmol/kg fresh tissue ($p < 0.05$). Concentrations in the Trt group remained constant for the following two samplings and then increased to around 450 nmol/kg fresh tissue ($p < 0.05$).

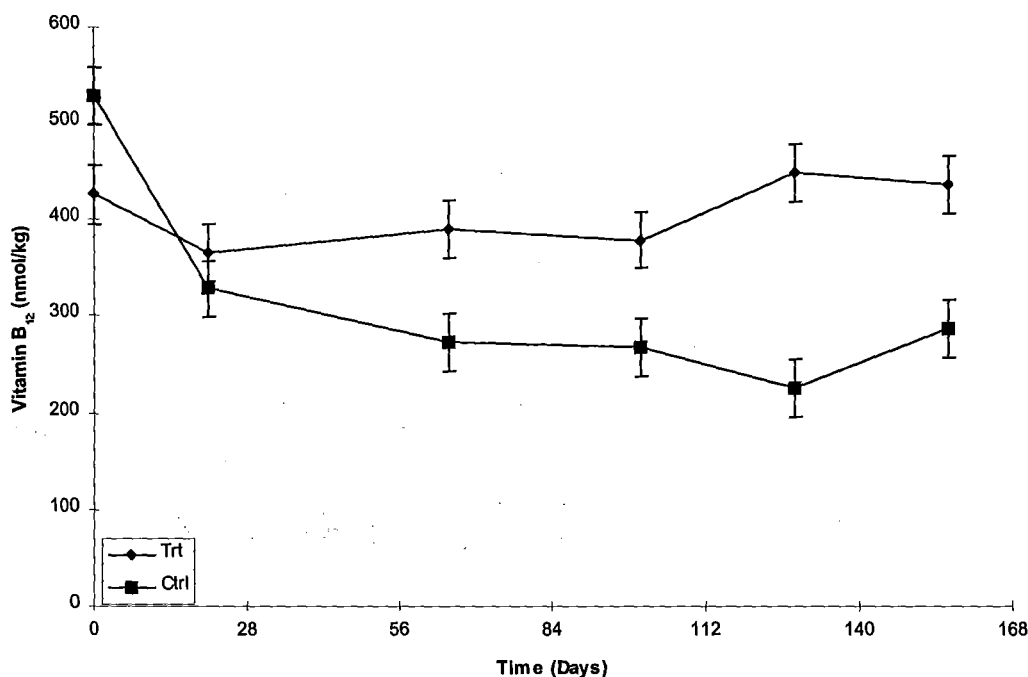


Figure 7.13: Liver vitamin B₁₂ concentrations of Co bullet treated and control lambs 1999 ($\pm$ SEM)

7.5.2.4 Methylmalonic acid

Values for MMA are given in Figure 7.14. There was a highly significant treatment $\times$ time interaction with quadratic and cubic components ($p < 0.001$ for both) due to the initial rise of MMA in the Ctrl group, followed by a decrease.

The Ctrl group had a non-significantly higher value than the Trt group at the start of the trial (2.9 and 2.0 $\mu\text{mol/l}$, respectively). MMA then increased in the Ctrl group to 4.5 $\mu\text{mol/l}$ before dropping back to 3.2 $\mu\text{mol/l}$. Both changes were highly significant ($p < 0.001$). MMA then decreased further to 2.5 $\mu\text{mol/l}$ at the end of the trial. In the Trt group MMA dropped significantly ($p < 0.001$) after supplementation to 1.0 $\mu\text{mol/l}$ and stayed around that value until the end of the trial.

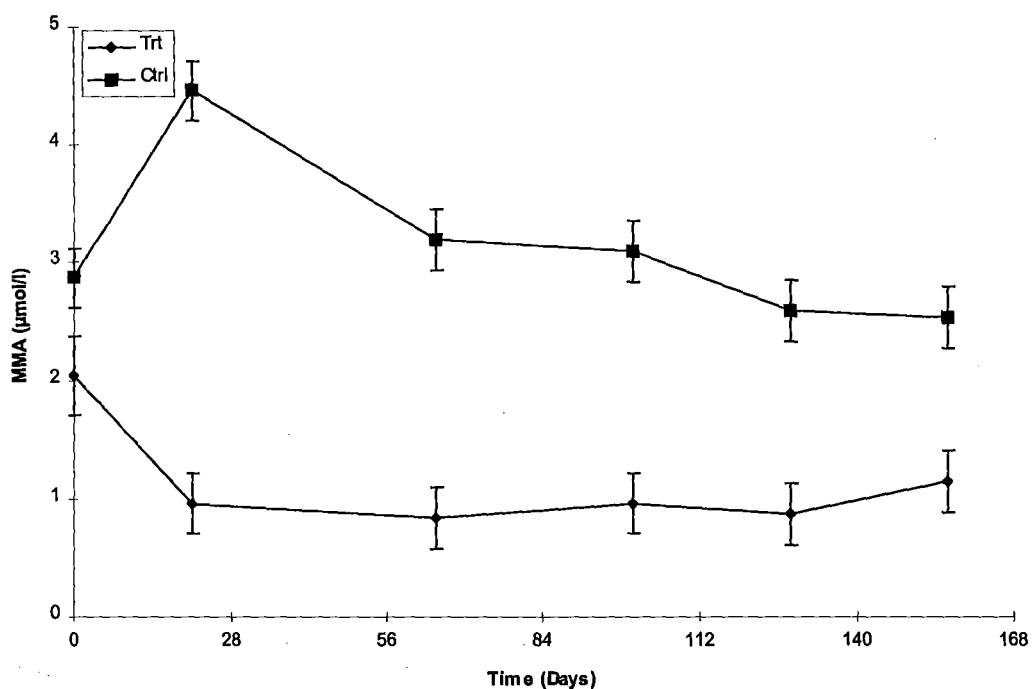


Figure 7.14: Serum MMA concentrations of Co bullet treated and control lambs 1999 ($\pm$ SEM)

7.5.2.5 *Weight*

The mean weight of Trt and Ctrl groups has been given in Figure 7.15. There was no difference between the two groups at any stage. However, the weight increase over time was highly significant ($p < 0.001$) at every sampling as the lambs were growing. The curve was essentially linear with small quadratic and cubic components ($p < 0.001$ for all three components).

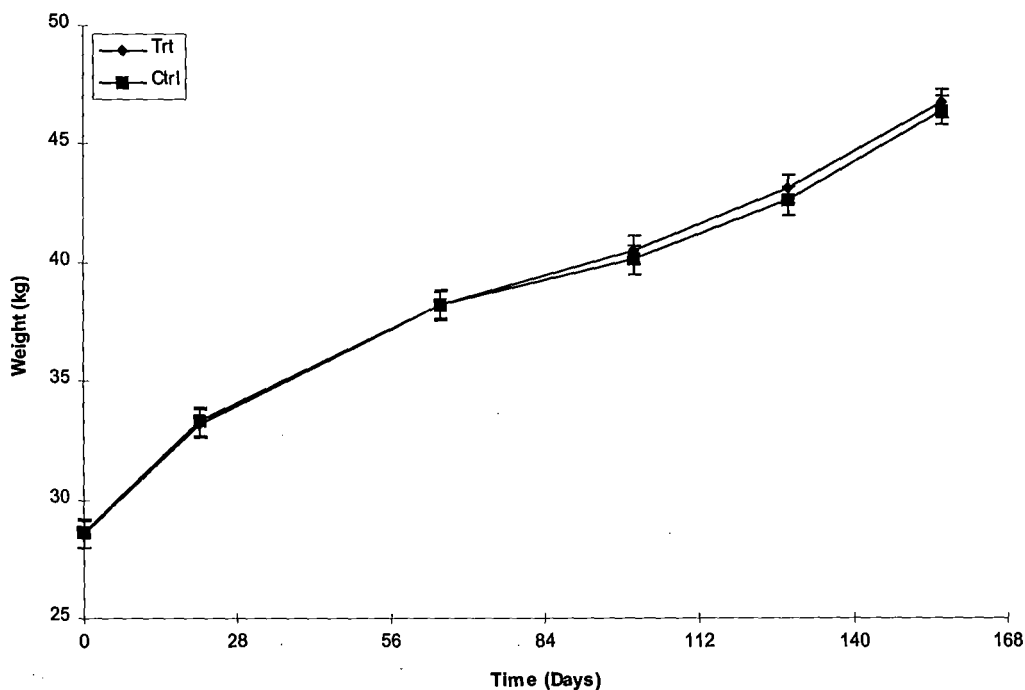


Figure 7.15: Weight of Co bullet treated and control lambs 1999 ($\pm$ SEM)

The mean for the LWG of the Ctrl and Trt groups is shown in Table 7.4. There was a highly significant difference ($p < 0.001$) in the growth rates achieved in the different time periods. The lambs gained weight more slowly the longer they were weaned, although they had another growth spurt around five months after weaning.

Table 7.4: Liveweight gain (in kg) of Co bullet treated and control lambs in 1999 (SEM = $\pm$ 0.008)

Days	0 - 21	22 - 65	66 - 100	101 - 128	129 - 156
Trt	0.18	0.15	0.06	0.10	0.13
Ctrl	0.18	0.14	0.05	0.09	0.13

7.6 Discussion

7.6.1 Pasture Co

Pasture Co was only analysed in 1999. It was much higher in the first sample than in the rest. It seems unlikely that Co content of the plants or the soil would have dropped so radically in such a short time. It is therefore possible that this sample was contaminated. This value was consequently excluded from consideration. The higher Co value in the last unwashed sample was most likely due to soil contamination since iron was also markedly elevated in that sample.

7.6.2 Threshold levels for vitamin B₁₂ deficiency

The farms involved in these trials had been picked by veterinarians as having problems regarding vitamin B₁₂ status. In 1998 a decrease in serum vitamin B₁₂ levels was indeed seen straight after weaning on all three farms in the untreated lambs whereas in 1999 levels stayed even in the Ctrl group. Except for Farm 3 where serum vitamin B₁₂ increased again at the end of the trial for the untreated lambs values in both years stayed below 500 pmol/l, the threshold in the New Zealand reference range above which levels are considered to be adequate.

On all farms one month after weaning serum levels were around or below 335 pmol/l in the untreated lambs, where an economic response to supplementation of 10 g LWG or greater is considered possible (Clark *et al.*, 1985 and 1989; New Zealand reference ranges). Underwood and Suttle (1999) also regarded a marginal zone for deficiency in weaned lambs from 336 to 500 pmol/l. Yet Clark *et al.* (1985) conceived a 'zone of doubt' for serum vitamin B₁₂ concentrations between 200 and 330 pmol/l. Later, Clark (1998) suggested a possible reference range for two to twelve month old lambs where serum vitamin B₁₂ concentrations below 340 pmol/l were considered to be low. According to these reference ranges the lambs in these trials were vitamin B₁₂ deficient.

Reference values for liver vitamin B₁₂ concentrations below which deficiency is likely have been given as 282 nmol/kg fresh tissue by Clark (1998) and Clark *et al.* (1989). Liver values in 1998 were between 100 and 200 nmol/kg in the UL 'groups' on all farms for most of the trial and tended to decrease with time. Further, in 1999

pasture Co values were below the threshold of 0.07 to 0.08 $\mu\text{g/g}$ DM for sheep and 0.11 $\mu\text{g/g}$ DM for lambs (Andrews *et al.*, 1958) throughout the 1999 trial. Deficiency in the lambs was therefore likely.

On three occasions (Figures 7.8 and 7.10) MMA was slightly elevated above the 5 $\mu\text{mol/l}$ threshold for grass-fed animals put forward by O'Harte *et al.* (1989a). Further, there was a general trend of higher MMA values with lower serum vitamin B₁₂, as illustrated in Figure 7.16. The mean serum vitamin B₁₂ values and their corresponding MMA values from all the farms at each sampling are plotted.

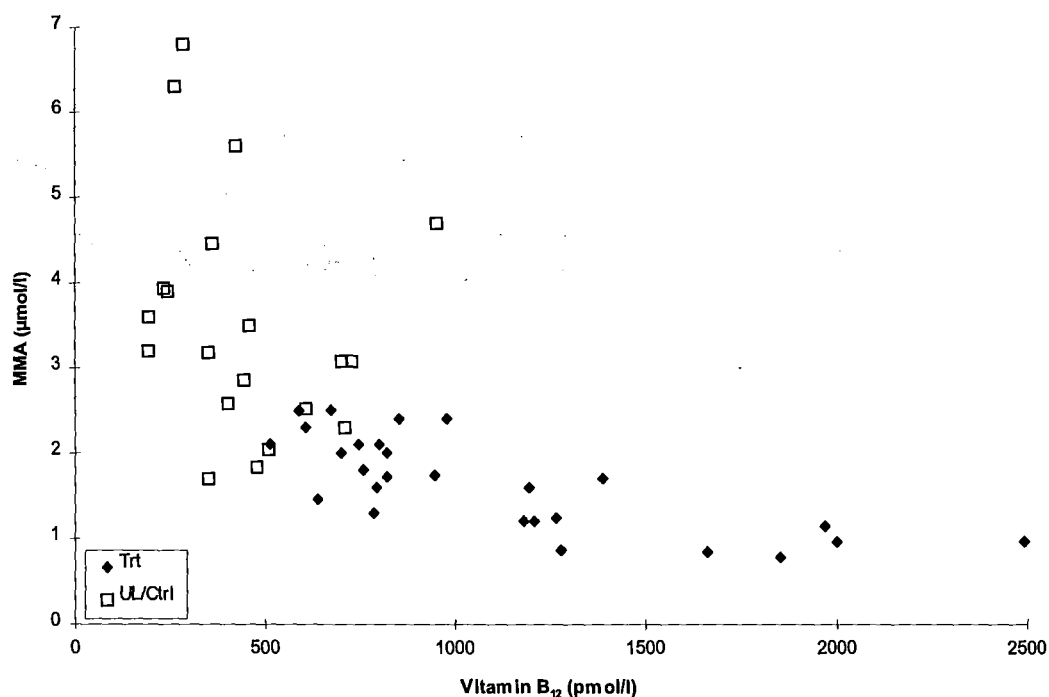


Figure 7.16: Serum vitamin B₁₂ versus MMA of Co bullet treated and control lambs

Yet LWG was the same in treated and untreated animals for all the farms in these trials. Comparison of MMA with serum vitamin B₁₂ over the length of the trials showed no consistent pattern of increasing MMA with decreasing serum or liver vitamin B₁₂, as had been seen very clearly in deficient animals (Kennedy *et al.*, 1991b). On Farm 2, for instance, serum and liver vitamin B₁₂ and MMA concentrations all declined initially. Both liver vitamin B₁₂ and MMA concentrations fell at the last sampling on Farm 3 and at the first sampling in 1999 while serum

vitamin B₁₂ concentrations rose. Even when different traits (MMA, serum and liver vitamin B₁₂ and LWG) in individual lambs were compared no pattern indicating deficiency could be found.

Therefore thresholds for serum and liver vitamin B₁₂ and MMA may need to be revised as vitamin B₁₂ deficiency was not seen in these trials. McMurray *et al.* (1985) gave 220 pmol/l and Judson *et al.* (1987) 200 pmol/l as the threshold for serum vitamin B₁₂ in sheep in Northern Ireland and Australia, respectively. Judson *et al.* (1987) further suggested 100 nmol/kg fresh tissue as the threshold for liver vitamin B₁₂. Obviously these values are more in agreement with the results from these trials and may reflect more realistically a marginal or deficient status in weaned lambs.

7.6.3 Efficacy of Co bullets

In 1998, when lambs received one Co bullet each, there was elevation of serum vitamin B₁₂ to a maximum of 1200 pmol/l one month after treatment. Although supplementation with Co halted the drop in liver vitamin B₁₂ that occurred in the untreated lambs after weaning it did not consistently and continuously elevate it. Evidence suggests that the administration of Co bullets is not a sure way of supplementation (Judson *et al.*, 1995). Other factors, as outlined in 7.6.4, may play a role.

In 1998, animals were rather variable in their individual responses to supplementation. Between samplings decreases of > 1500 pmol/l and increases of > 3000 pmol/l were seen, especially early after weaning. This is also obvious from the rather large SEM in 1998. The bullets in 1998 may have become somewhat coated and were therefore not releasing the normal amount of Co. In some animals in 1998 the bullet may have been regurgitated. However, best individual responses were obtained from two months after weaning onward which would support the notion that the bullets were coated rather than regurgitated.

In 1999, when two Co bullets were administered to each lamb, treatment raised serum levels to around 2500 pmol/l and the rise in serum vitamin B₁₂ was more consistent in all the lambs, as seen in the much smaller SEM compared to the

previous year. Therefore it may be advisable to use either two bullets or a bullet and a grub screw to avoid coating (Judson *et al.*, 1992), at least in weaner lambs, to ensure efficacy. Alternately, long-acting vitamin B₁₂ injections may prove to be the treatment of choice in this case and trials (Grace, 1999c; Grace and Lewis, 1999; Judson *et al.*, 2000; Grace and West, 2000; Grace and Sinclair, 1999; Grace, 1999d; McSporran, personal communications) are under way.

There was a tendency for all the groups in each trial (except for the last sampling on Farm 3) to maintain or decrease from previous serum vitamin B₁₂ levels after an initial rise. In Farm 2 an exponential decay curve could be fitted, indicating that vitamin B₁₂ blood stores were depleted at a set rate. However, none of the other farms showed this exponential decay pattern. It may therefore be a spurious result.

7.6.4 Influence of seasonal and environmental factors

Vitamin B₁₂ and MMA concentrations in the treated lambs tended to follow the trend in the untreated ones, albeit with slightly higher vitamin B₁₂ and slightly lower MMA values, as seen for example in the rise in liver vitamin B₁₂ on Farm 1 at sampling four or the rise in serum vitamin B₁₂ levels on Farm 3 at the end of the trial. It was therefore more likely that these changes were determined by metabolic or environmental factors.

Climatic conditions may have been such that grass growth was stunted (hot and dry), or the animals may have been transferred to a paddock with very short pasture so that soil ingestion was high (Judson *et al.*, 1988). The lambs could have also been transferred to a pasture with different plant composition, such as a higher proportion of white clover, since clovers contain higher amounts of Co than grasses. The fructan content of the pasture, which places a higher demand on the propionate/succinate pathway, could have been lower (due to a lesser proportion of perennial ryegrass in pasture composition - Ulvund and Pestalozzi, 1990), thus freeing some of the vitamin from its metabolic task. The manganese content of a different paddock could have been lower, thus plant Co would have been more readily available.

7.6.5 Weight inconsistencies

There was a difference between the farms in mean weight at weaning. Generally, the earlier the trial started in 1998 the lighter the lambs. Yet in 1999 weaning took place at the same time as in the previous year on Farm 1 and the lambs weighed on average 9 kg more at that time than in 1998. On the other hand, the lambs on Farm 1 grew faster after weaning in the first year than in the second year. Although these lambs had similar serum vitamin B₁₂ concentrations in both years the liver vitamin B₁₂ concentrations were markedly higher in the second year. Possibly the better vitamin B₁₂ status in the second year could have contributed to this difference in weight.

Although the lambs in both years started with a LWG generally considered desirable (~ 220 g/d - Grace, 1999d) this reduced to only 40 to 90 g/d after four to six weeks after weaning in treated and untreated animals. Obviously other factors, such as shortage of feed supply or possibly other deficiencies, played a role in this poor growth performance since all the animals were affected by it.

CHAPTER 8

Development of a method for the separation of corrinoids in ovine tissues by HPLC

8.1 Introduction

Vitamin B₁₂ is present in mammalian tissues in its coenzyme forms, ado-cbl and me-cbl, as well as OH-cbl, small amounts of CN-cbl and traces of sulfito-cbl (Gimsing and Beck, 1986; Jacobsen *et al.*, 1986). Further analogues have been identified which lack vitamin B₁₂ activity and can be potentially harmful to the organism. Since many of the common assays for vitamin B₁₂ cannot distinguish between these different forms, no information can be gained about their relative amounts and the extent to which such variation could play a part in the problem of diagnosis of Co supplement responsive conditions.

8.2 Aims

- 1) To develop an HPLC assay for the separation of corrinoids in liver.
- 2) To provide information on the variation of corrinoids depending on the age of the sheep and vitamin B₁₂ status.
- 3) To make a preliminary examination of the feasibility of measuring corrinoids in other biological samples.

8.3 Method development for the separation of corrinoids by HPLC

8.3.1 Method

Separation and identification of the different corrinoids was achieved by HPLC, using a modified version of the method described by Djalali *et al.* (1990). Methods employed by other researchers (Gimsing and Beck, 1986; Green *et al.*,

1974; Fie *et al.*, 1994) have been adapted to maximise the speed and efficiency with which samples can be analysed, whilst maintaining extraction efficiency and sample purity.

8.3.1.1 *Principles of extraction*

a) The samples were incubated with cadmium acetate (CdAc) in order to maximise the release of corrinoids, especially OH-cbl, from binding proteins and from non-specific binding sites on histidine residues of other proteins. (Comparisons have been carried out in our laboratory between extractions with and without this stage, and an approximate 50 % increase in overall extraction was observed.)

b) To free the corrinoids from their protein binders in plasma the proteins needed to be denatured. This was achieved by boiling plasma in an acidic buffer, the method used by Labnet Invermay. It is a simplification of a method originally described by Djalali *et al.* (1990). These workers used a hot ethanolic extraction of plasma. This introduced an extra freeze drying step, as the ethanol had to be removed before the sample could be put through a Sep-Pak column. This extra freeze-drying step can be avoided. Comparisons have been performed in our laboratory and the two extraction methods produce similar results.

c) Since varying amounts of salt in the sample will change the mobility of the corrinoids (Gimsing and Nexø, 1983) desalting was done on Sep-Pak columns with tertiary butanol used as the eluent so that the samples could be easily frozen before freeze drying.

8.3.1.2 *Source of reagents and materials*

OH-cbl, CN-cbl, me-cbl, ado-cbl, cbi (cobinamide dicyanide - a vitamin B₁₂ analogue), borax, bovine serum albumin and cadmium acetate were purchased from Sigma (Auckland, New Zealand). Sep-Pak cartridges were obtained from Waters Association, Milford, MA, USA, and HPLC columns from Phenomenex, Auckland, New Zealand.

8.3.1.3 *Preparation of samples*

All procedures were carried out under dim red illumination (photographic safe light), unless otherwise stated, as the coenzymes, once freed from their binders, are light sensitive and convert to OH-cbl within minutes (Nexø and Olesen, 1982; own unpublished results). The initial part of the method varied according to the biological material to be extracted. The extractions were all carried out in 28 mm i.d. plastic centrifuge tubes.

Liver was weighed (~ 0.5 g, minimum 0.02 g) and homogenised in isotonic saline (5 ml, 0.9 % NaCl). **SI fluid** was centrifuged and the supernatant pipetted off (5 ml). **Plasma and milk** were used without modification (5 ml).

Faeces (2 g) was first treated with 6 ml of deproteinising buffer, containing aprotinin (KIU 5000 U/l), phenylmethanesulphonyl fluoride (0.02 mM, M_r 174.2), sodium azide (3 mM, M_r 65.0) and Triton X100 (0.05 % v/v) in sodium phosphate buffer (0.1 M) adjusted to pH 7.4, and incubated under rotative agitation for 24 h at 4°C (cold store). This was followed by centrifugation at 19000 g for 60 min at 4°C. The supernatant was used either immediately or stored at -80°C and used within 20 days of collection.

To the solution to be extracted (5 ml of either liver extract, plasma, milk, SI fluid or faeces extract), one volume (5 ml) of CdAc x 2 H₂O (0.4 M, M_r = 266.52) was added and the mixture allowed to stand at 4 to 8°C overnight (cold store) to release OH-cbl from non-specific binding to histidine residues. Two volumes of extracting buffer (10 ml 0.4 M sodium acetate, M_r 86.05, adjusted to pH 4.0 with 0.4 M HCl, M_r 36.46) were added, the top of the centrifuge tubes capped with foil and the mixture placed in a boiling water bath for 20 min.

Sep-Pak C18 cartridges were fitted with a syringe style solvent reservoir containing a polyethylene frit (20 µm) and prepared by wetting with acetonitrile (2 ml), followed by water (5 ml). This was done on a 'vacustat' manifold, which will hold up to 20 cartridges and allows the application of a vacuum.

After cooling in ice the extraction mixture was centrifuged at 17,400 g for 10 min at 4°C. The supernatant liquid was then decanted into a reservoir attached to a Sep-Pak cartridge. The solution was slowly drawn through the cartridges with the assistance of a vacuum pump. The cartridges were then washed with water (15 ml)

and afterwards removed from the vacuum manifold. The corrinoids were eluted by slowly passing tertiary butanol (20 % v/v, 6 ml) through the cartridge with a syringe. The eluent was collected in a Quickfit boiling tube (~ 25 mm i.d.).

The boiling tubes were placed in a foil-wrapped polystyrene cool box and frozen (overnight or with the assistance of liquid nitrogen) before being freeze-dried overnight to remove all solvent residues. The remaining solid was redissolved in water (200 µl) and filtered through a syringe filter (4 mm, 0.45 µm) into a foil-wrapped tinted glass HPLC vial with insert.

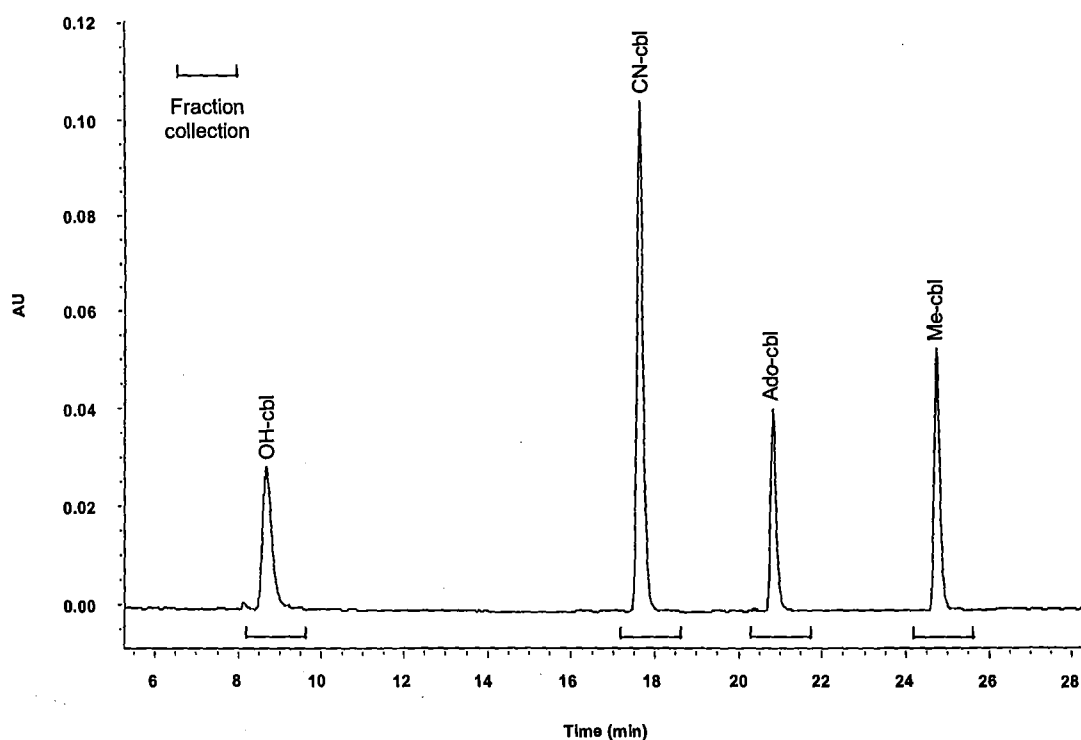
8.3.1.4 HPLC separation

Separation was achieved using a C18 column (Prodigy model) fitted with a C18 guard column, using the gradient elution program outlined in Table 8.1. Curve 6: linear gradient; curve 1: return to initial condition. Solvent A for the HPLC consisted of phosphoric acid (0.085 M, M_r 98), adjusted to pH 3.1 with ammonium hydroxide. Solvent B was acetonitrile.

Table 8.1: Gradient elution program for corrinoid analysis on HPLC

time	flow ml/min	% A	% B	curve
0	0.5	90	10	
40	0.5	50	50	6
48	0.5	30	70	6
53	1	30	70	6
65	1	90	10	1

Detection was achieved using UV 365 nm. Initially, a mixture of standard solutions (3 µM) of OH-cbl, CN-cbl, ado-cbl and me-cbl (100 µl) were injected in order to obtain the retention times of each of the corrinoids (Figure 8.1). They eluted in the following order: OH-cbl, CN-cbl, ado-cbl, and me-cbl.



AU: Absorption Units

Figure 8.1: Retention times of 3 μ M standard solutions of corrinoids on HPLC

Each sample (100 μ l) was then injected and fractions collected from 0.5 min before the start of the cobalamin peak to 1 min after the cobalamin peak (measured from standards). Any material in between the cobalamin peaks was collected and pooled. This fraction was postulated to contain the vitamin B₁₂ analogues. Since CN-cbl was overlaid by an analogue peak CN-cbl could not be collected separately. Therefore CN-cbl was collected in the same fraction as the analogues. As CN-cbl is present in biological tissues in only negligible amounts this would not have substantially altered the distribution of corrinoids. Previous experiments in which fractions were collected and measured every 30 sec showed that no measurable B₁₂ eluted before OH-cbl or after me-cbl. Measurement of the exact retention time of each cobalamin is a crucial part of the analysis as it was assumed that the retention time of the standards was the same as that of the samples where it was not possible to distinguish the individual corrinoids on the chromatogram.

Vitamin B₂ (riboflavin) coeluted with ado-cbl and was seen as a very large peak on the chromatogram of liver samples (not seen as clearly with plasma) with a very similar retention time to ado-cbl (~ 22 min). This was a useful indicator peak

because if this was observed to shift from sample to sample, then it could be assumed that the corrinoids were shifting also. If the peak due to vitamin B₂ (noticeable by its yellow colour) did not elute into that fraction allocated to ado-cbl then the standards needed to be run again and the retention times checked. This could happen if the column had not equilibrated sufficiently.

When the vitamin B₂ peak was not clearly visible, as in the case for plasma, then other peaks or the general peak pattern could be used to indicate the reproducibility of the retention times. By superimposing chromatograms of many plasma runs it was possible to see if the peaks due to the major components of the plasma were shifting. If they did shift then the column needed to be equilibrated longer and/or the sample re-run.

8.3.1.5 *Fraction collection*

An aliquot (200 µl) of bovine serum albumin (0.02 % v/v, BSA) in a borax buffer (0.082 M, sodium tetraborate decahydrate, M_r 381.36) adjusted to pH 9.8 was added to each collection tube. This prevented binding of the vitamin B₁₂ to the vessel and also maintained the sample at slightly alkaline pH, where it is more stable than in the HPLC eluent. Fractions were collected automatically using the Gradifrac fraction collector, programmed using 'break points' to collect fractions of the appropriate time length. The fraction collector needed to be started manually in synchrony with the injection on the HPLC. When fitted with the large tube holder (28 mm) large centrifuge tubes or large conical tubes could be used to collect fractions. The fractions were freeze dried directly in these tubes.

Fractions were redissolved in 0.5 to 10 ml water so that the final concentration fell within the measurement range of plasma vitamin B₁₂ (57 - 1500 pmol/l) and then sent for RIDA vitamin B₁₂ analysis at Labnet Invermay. It was better to make the fractions more concentrated rather than too weak as they could easily be diluted. Based on the results, proportions of each cobalamin, and their analogues, were estimated.

8.3.2 Quantification

The proportion of each cobalamin extracted using this process was estimated as follows:

Plasma: The vitamin B₁₂ concentration of a sample was measured before and after extraction to establish a mean background level in the sample and its extracts. Further plasma samples from the same batch (n=2) were then divided into four aliquots and each was spiked with 4000 pmol/l of one of the corrinoids (ado-cbl, me-cbl, OH-cbl and cbi) and measured before and after extraction. The samples were mixed thoroughly and left to stand for several hours to ensure binding of the added cbl to apo-binders in plasma. They were then prepared as described above.

The concentration of vitamin B₁₂ in the sample before extraction (1190 pmol/l) was then subtracted from the concentrations of the spiked samples before extraction to obtain a value that would reflect the added amount of vitamin B₁₂. It became obvious from the small difference in vitamin B₁₂ concentration between the samples before and after addition of cbi that Labnet Invermay's assay method was not able to measure this analogue. Labnet Invermay uses chick serum as a binder which does not allow the measurement of all the analogues present.

It was assumed that in the light all forms of cbl converted to OH-cbl. Therefore the proportion of OH-cbl in the sample after extraction, compared to before the extraction, was used to establish the percentage recovered after extraction. This amounted to 29 % of 1190 pmol/l or 345 pmol/l. This amount (345 pmol/l) was then subtracted from the vitamin B₁₂ concentration of all the spiked samples after extraction. After considering the presence of background vitamin B₁₂ before and after extraction, the results for the recovery of each corrinoid were: OH-cbl 29 %, ado-cbl 50 % and me-cbl 64 %.

These results are very similar to those obtained by Djalali *et al.* (1990) for human plasma, viz OH-cbl 38 %, ado-cbl 45 %, me-cbl 60 %. These researchers also estimated an extraction recovery for analogues, 69 %, which we were not able to do. Therefore, the value of 69 % for analogues was adopted in our calculations.

Liver: It was assumed that the recovery from liver would be the same as from plasma. Since the added vitamin B₁₂ needed to be protein-bound in order to

give a realistic value for the extraction, this could not be guaranteed with a solid material like liver.

SI fluid: The procedure followed the one outlined for plasma. Five aliquots of a SI fluid sample were measured before and after extraction to establish a mean background level in the sample and its extracts. Further samples (n=4) were then spiked with the different corrinoids at the level needed to significantly increase their vitamin B₁₂ content, and then measured before and after extraction. After considering the presence of background vitamin B₁₂ before and after extraction, the results ($\pm$ SE) were: OH-cbl 9.0 ± 0.7 %, ado-cbl 78.9 ± 4.3 %, me-cbl 85.6 ± 6.7 %. For the recovery of analogues, the value of 69 %, set for human plasma by Djalali *et al.* (1990), was used.

These extraction efficiencies were taken into account as a factor when calculating the proportion of each cobalamin present in the original sample.

8.3.3 Precision

Reproducibility studies were performed on the distribution of corrinoids in liver (n=5), SI fluid (n=6) and plasma (n=3) samples (Table 8.2). The samples (n) were taken from the same liver or batch of fluid, respectively. Liver was homogenised and the fluids (SI and plasma) thoroughly mixed before the aliquots were taken. The low SE values show that reproducibility was excellent and that the method could therefore be employed with confidence to the separation of corrinoids in liver, SI fluid and plasma.

Table 8.2: Reproducibility studies (% distribution) of the separation of corrinoids by HPLC in liver, SI fluid and plasma ($\pm$ SE)

	n	ado-cbl	me-cbl	OH-cbl	analogues
Liver	5	64.8 ± 1.1	1.5 ± 0.4	7.0 ± 1.5	26.6 ± 1.7
SI Fluid	6	26.4 ± 0.9	6.0 ± 0.8	14.9 ± 2.7	52.7 ± 2.4
Plasma	3	31.8 ± 1.0	7.9 ± 0.7	47.3 ± 2.2	13.1 ± 0.6

Retention times of the cbl standards, measured on eleven occasions over a three month period, did not vary significantly (Table 8.3). It is therefore reasonable

to assume that the retention times of corrinoids in biological samples also stayed constant.

Table 8.3: Mean retention times (min) of cobalamin standards ($\pm$ SE) separated by HPLC

Retention times	n	OH-cbl	ado-cbl	me-cbl
	11	10.2 $\pm$ 0.12	22.4 $\pm$ 0.09	26.4 $\pm$ 0.09

8.4 Analysis of liver tissue from sheep on different farms and at different ages on varying Co/vitamin B₁₂ supplementation regimens

Estimations of the contribution of the forms of corrinoids to total vitamin B₁₂ in liver tissue were then made. As this chapter deals mainly with the development of the separation method and with testing it on various biological tissues the samples were not chosen according to a balanced design for statistical analysis. Rather, the amount of liver tissue available determined the number of samples in each category, hence the unequal numbers. Basically, these liver samples were used to get an overview of the distribution of corrinoids and to see if there was evidence of significant variation in the distribution in different situations.

A summary of the samples (n=68) that were analysed from the various farms is given in Table 8.4. Considering that the last liver sample of the lambs in Chapter 6 was analysed it was counted in the ruminant category since the lambs were about three months old then with a functional rumen and close to being weaned. Corrinoids were compared between farms, between ruminant lambs (L) and ewes (E), and between supplemented (Co, OH-cbl) and unsupplemented (Ctrl) animals. Samples from two farms from a preliminary trial (Ha and Jo) were also included.

Ado-cbl made up the largest fraction of corrinoids in the liver, around 35 to 75 %. Analogues constituted the second largest fraction with 15 to 35 %. The OH-cbl fraction was between 6 and 38 % and me-cbl was the smallest fraction with values between 1 and 10 %.

No obvious major differences were found between lambs and ewes, and ruminant and PR lambs. Figure 8.2 shows the distribution in graphical form. In this

figure all the data from Table 8.4 have been included as well as the non-propionate treated PR lambs from Chapter 4 which brought the total number of samples up to 72. Vitamin B₁₂ in the form of ado-cbl tended to be higher in the supplemented groups (n=11) than in the Ctrl groups (n=61) (56.0 and 42.1 %, respectively), and with only two exceptions the proportion of analogues tended to be lower in supplemented than in unsupplemented groups (29.0 and 20.5 %, respectively).

Table 8.4: Amounts of estimated vitamin B₁₂ (nmol/kg) in each corrrinoid fraction (percentages in parenthesis) in liver tissue of supplemented and unsupplemented ewes and ruminant lambs from various farms sampled during the study

Chapter	Farm/Year	E/L	Trt	n	ado-cbl	me-cbl	OH-cbl	analogues	Total B ₁₂
6	1997/1998	E	Ctrl	3	124 (47.9)	7 (3.0)	67 (29.4)	48 (19.8)	246
6	1997/1998	E	Co	2	485 (62.1)	17 (2.0)	163 (19.8)	131 (16.1)	796
6	1997/1998	L	Ctrl	2	74 (44.5)	3 (2.2)	37 (21.6)	54 (31.7)	168
561	1997/1998	L	B ₁₂	1	325 (58.0)	23 (4.1)	88 (15.7)	125 (22.3)	561
6	1998/1999	E	Ctrl	34	113 (39.5)	10 (3.5)	76 (27.3)	89 (30.3)	288
7	Farm 1	L	Ctrl	1	120 (63.1)	18 (9.4)	12 (6.4)	40 (21.1)	190
7	Farm 1	L	Co	1	56 (58.9)	7 (7.1)	10 (10.0)	23 (24.0)	96
7	Farm 2	L	Ctrl	2	190 (62.7)	10 (5.7)	32 (9.7)	71 (21.9)	303
7	Farm 2	L	Co	1	231 (74.6)	8 (2.6)	22 (7.2)	48 (15.6)	309
7	Farm 3	L	Ctrl	10	76 (37.3)	5 (3.5)	70 (31.7)	52 (27.5)	203
7	Farm 3	L	Co	3	100 (51.7)	5 (3.0)	49 (21.5)	52 (23.8)	206
*	Ha	L	Ctrl	3	179 (35.2)	8 (1.7)	142 (28.4)	171 (34.7)	500
*	Ha	L	Co	1	373 (42.3)	12 (1.3)	334 (37.9)	162 (18.4)	881
*	Jo	L	Ctrl	3	214 (52.9)	13 (3.2)	87 (23.6)	79 (20.3)	393
*	Jo	L	Co	1	254 (41.6)	27 (4.6)	165 (28.0)	152 (25.8)	598

* Animals from farms used in a pre-trial in 1997

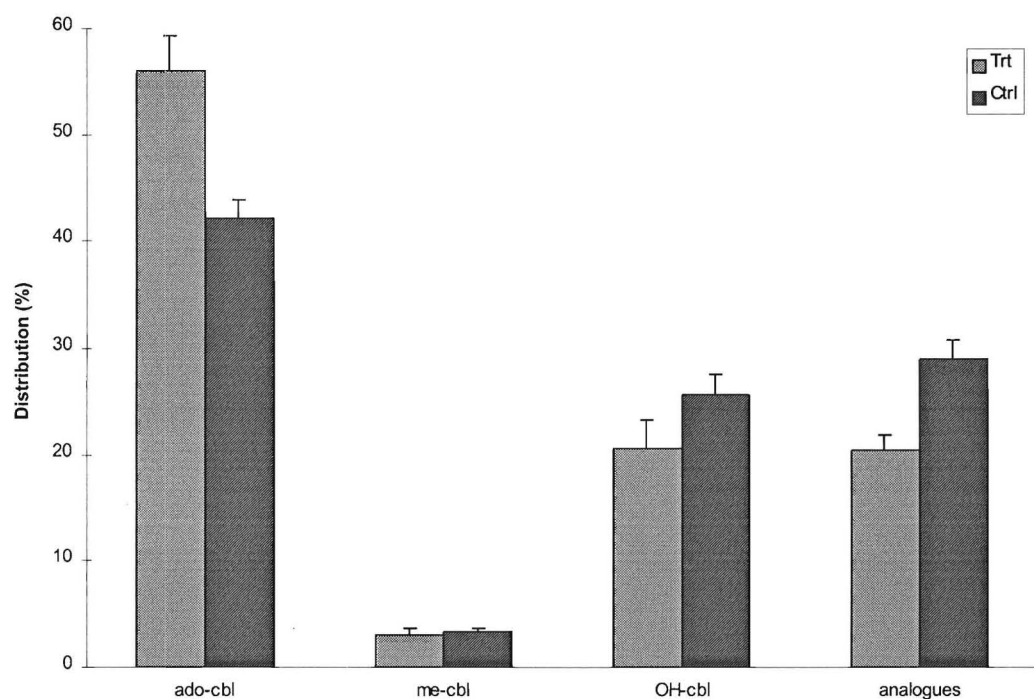


Figure 8.2: Distribution of corrinoids (%) in liver of lambs and ewes treated with vitamin B₁₂ or Co bullets, and control animals ($\pm$ SE)

The corrinoid distribution has been examined according to total liver vitamin B₁₂ concentration and the results are given in Figure 8.3. In this analysis there was a tendency for the proportions of ado-cbl to slightly increase and of me-cbl and analogues to decrease with increasing liver vitamin B₁₂ concentrations.

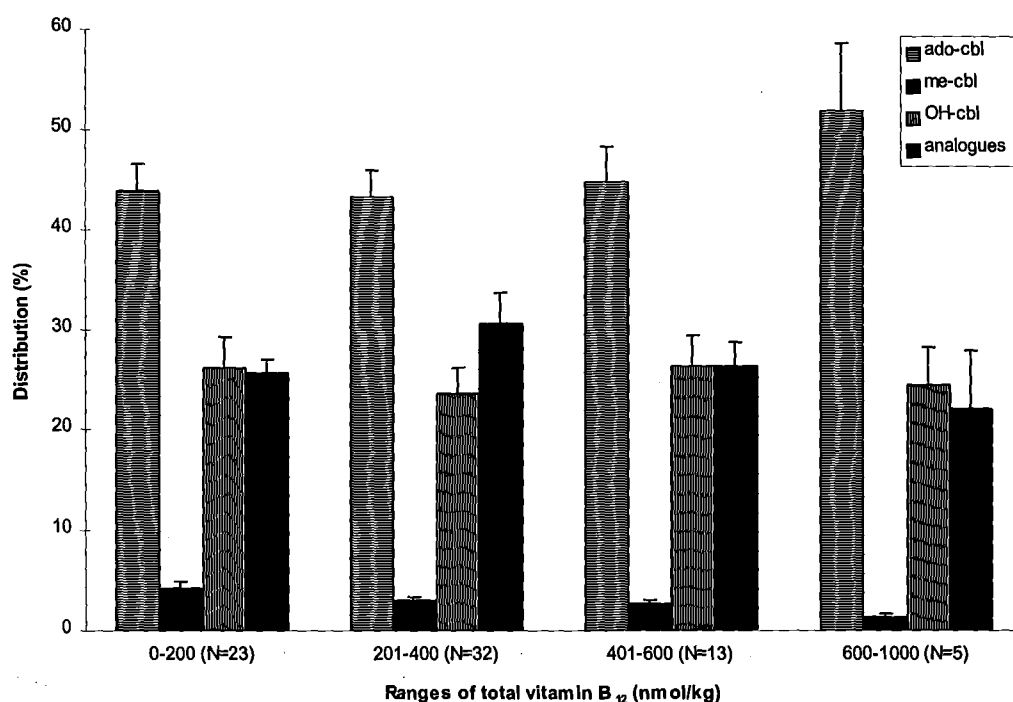


Figure 8.3: Distribution of corrinoids (%) in liver of lambs and ewes in different total vitamin B₁₂ ranges ($\pm$ SE)

Comparisons of corrinoids in liver samples of lambs were also made. Liver samples from one lamb in each of the four different treatment groups in Chapter 4, taken before the start and at the end of the trial, were analysed for corrinoids and the results presented in Table 8.5. Unfortunately, the second sample of the lamb in group +B₁₂/+Pr was lost during extraction. There was no other pair in this group available for analysis because of the small amount of liver tissue that could be taken from those lambs, especially during the first biopsy.

When comparing the vitamin B₁₂ treated and untreated lambs there was a suggestion that there may be a large difference in the OH-cbl component. Unfortunately, this was based on the data from one lamb. However, the OH component in the vitamin B₁₂ treated lamb (23.1 %) was many times greater than the values observed in the other lambs (between 1.3 and 6.6 %). This phenomenon was not observed in the ruminant vitamin B₁₂ treated lambs in Chapter 6. However, sample numbers were so small that no definitive conclusions could be drawn.

Table 8.5: Distribution of corrinoids in livers of lambs before (1) and after (2) treatment with propionate and vitamin B₁₂ injections

Fraction	Treatment	Distribution (1) (%)	Vit. B ₁₂ (1) (nmol/kg)	Distribution (2) (%)	Vit. B ₁₂ (2) (nmol/kg)
ado-cbl	+B ₁₂ /-Pr	73.5	176	61.1	587
	+B ₁₂ /+Pr	77.3	178		
	-B ₁₂ /-Pr	30.4	103	65.0	150
	-B ₁₂ /+Pr	64.2	77	67.5	182
me-cbl	+B ₁₂ /-Pr	2.6	6	0.3	3
	+B ₁₂ /+Pr	4.4	10		
	-B ₁₂ /-Pr	0.7	2	0.8	2
	-B ₁₂ /+Pr	0.9	1	2.3	6
OH-cbl	+B ₁₂ /-Pr	4.8	12	23.1	222
	+B ₁₂ /+Pr	4.8	11		
	-B ₁₂ /-Pr	3.3	11	3.1	7
	-B ₁₂ /+Pr	6.6	8	1.3	3
analogues	+B ₁₂ /-Pr	19.0	46	15.5	148
	+B ₁₂ /+Pr	13.5	31		
	-B ₁₂ /-Pr	65.6	223	31.1	72
	-B ₁₂ /+Pr	28.3	34	28.9	78

8.5 Separation of corrinoids in biological samples other than liver

In order to validate the method for biological samples other than liver and to see whether it may be possible to separate corrinoids in other tissues as well as to provide the basis for more quantitative metabolism studies the analysis was carried out on blood, SI fluid and faeces. The latter was of particular interest as it was hoped that metabolism studies could be established or that faecal samples may provide a method of detection of a responsive condition in the field. These investigations are very preliminary, as time constraints did not allow follow-up at this stage.

8.5.1 Blood

Six blood samples from Co replete ruminant sheep were assayed. Me-cbl was only detected in one sample, being below the detection limit in the other five in the plasma sample size used. The values for me-cbl were therefore omitted. The greatest

proportion of corrinoids was due to OH-cbl, followed by ado-cbl and finally analogues (Table 8.6 and Figure 8.4).

Table 8.6: Distribution and vitamin B₁₂ of corrinoids in ovine plasma (± SE)

	n	ado-cbl	me-cbl	OH-cbl	analogues
Distribution (%)	6	27.7 ± 4.4	0	52.0 ± 6.4	17.6 ± 2.6
B ₁₂ (pmol/l)	6	113 ± 35.6	0	197 ± 45.6	65 ± 16.0

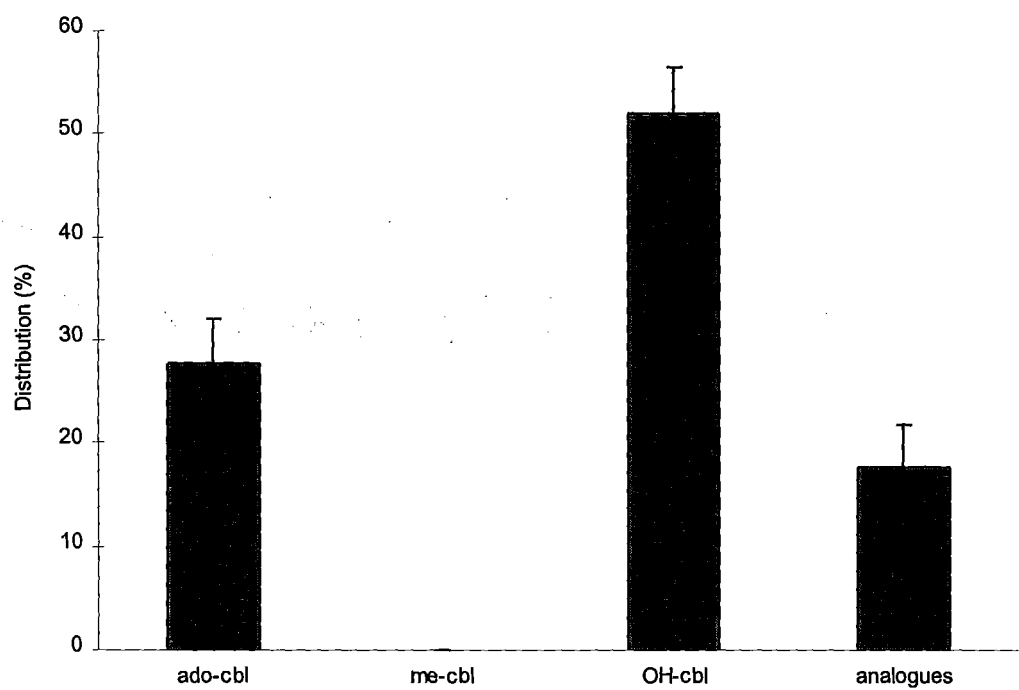


Figure 8.4: Distribution of corrinoids in ovine plasma (± SE)

8.5.2 Small intestinal fluid

Five samples of SI fluid were taken from a canulated sheep and analysed for their corrinoid content. The results are given in Table 8.7 and Figure 8.5. The greatest proportion of corrinoids was due to analogues, followed by ado-cbl, OH-cbl and me-cbl.

Table 8.7: Distribution and vitamin B₁₂ of corrinoids in ovine small intestinal fluid (± SE)

	n	ado-cbl	me-cbl	OH-cbl	analogues
Distribution (%)	6	24.6 ± 0.9	6.0 ± 0.8	14.9 ± 2.7	52.7 ± 2.4
Vitamin B ₁₂ (pmol/l)	6	21.9 ± 0.8	4.9 ± 0.7	12.3 ± 2.4	43.8 ± 2.2

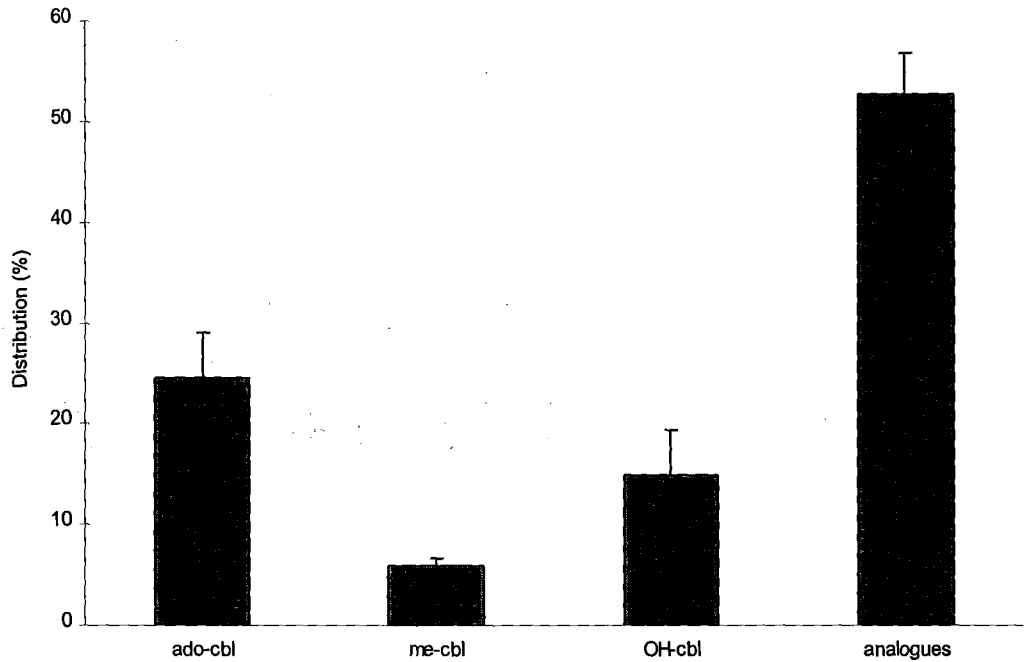


Figure 8.5: Distribution of corrinoids in ovine small intestinal fluid (± SE)

8.5.3 Faeces

Two sheep were placed in metabolic cages for four days and their faeces collected every 24 h. Both sheep received 2000 µg OH-cbl by injection (time 0) immediately after the second batch of 24 h faeces had been collected. Faeces continued to be collected for two more 24 h cycles. Each collection was thoroughly mixed and two samples from each sheep were taken for analysis.

The results are summarised in Table 8.8 and illustrated in Figure 8.6. The SEM was calculated on the four samples obtained each time - two from each sheep. Neither the distribution nor the vitamin B₁₂ content of faeces changed after OH-cbl loading.

Table 8.8: Distribution (%) and vitamin B₁₂ content (nmol/kg fresh matter) of corrinoids in faeces of two sheep before and after OH-cbl loading ($\pm$ SEM)

Days		-1	0	1	2
Distribution	ado-cbl	8.9 $\pm$ 1.2	7.4 $\pm$ 1.0	6.0 $\pm$ 1.1	6.6 $\pm$ 1.1
	me-cbl	5.9 $\pm$ 1.1	5.4 $\pm$ 1.0	5.8 $\pm$ 1.0	3.7 $\pm$ 1.0
	OH-cbl	20.0 $\pm$ 4.2	17.9 $\pm$ 3.6	23.1 $\pm$ 3.6	22.0 $\pm$ 3.6
	analogues	57.0 $\pm$ 10.7	48.7 $\pm$ 9.3	58.9 $\pm$ 9.3	59.9 $\pm$ 9.3
Vitamin B ₁₂	ado-cbl	79 $\pm$ 12.0	62 $\pm$ 10.4	50 $\pm$ 10.4	59 $\pm$ 10.4
	me-cbl	52 $\pm$ 9.7	45 $\pm$ 8.4	48 $\pm$ 8.4	33 $\pm$ 8.4
	OH-cbl	178 $\pm$ 32.9	151 $\pm$ 28.5	192 $\pm$ 28.5	186 $\pm$ 28.5
	analogues	580 $\pm$ 42.7	583 $\pm$ 36.9	548 $\pm$ 36.9	597 $\pm$ 36.9

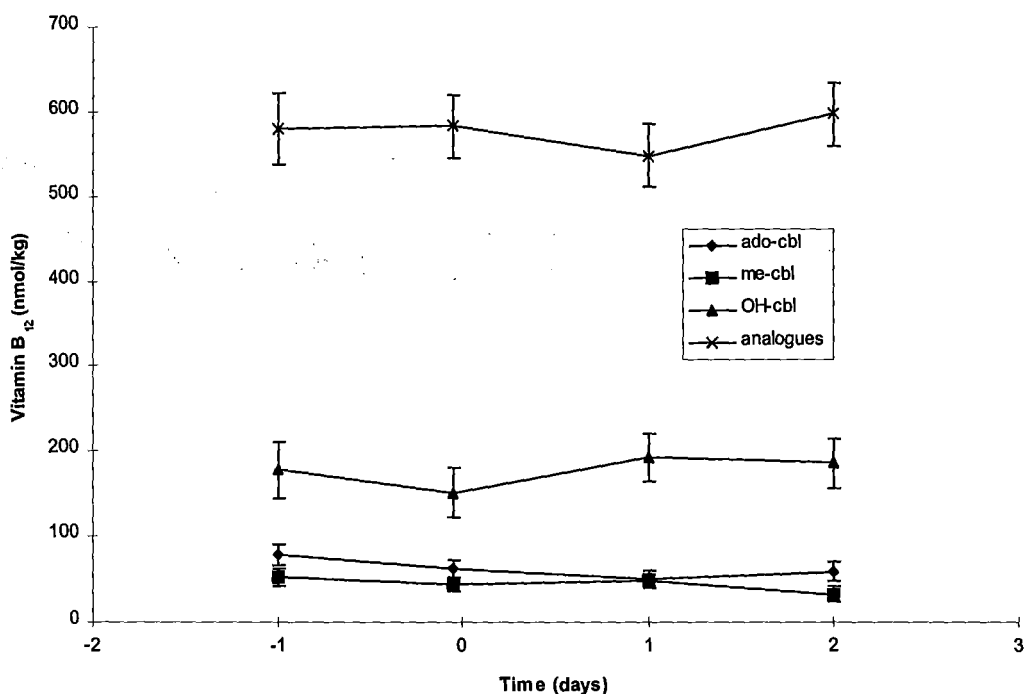


Figure 8.6: Vitamin B₁₂ content of the different corrinoids in faeces before and after OH-cbl loading ($\pm$ SEM)

Urine samples were also taken in like manner, i.e. collected over 24 h for each day, in order to estimate the loss of injected vitamin B₁₂. However, attempts to measure the vitamin in urine failed as inconsistent results were obtained.

8.5.4 Other tissues

Bile and rumen fluid have also been investigated but were found to be unsuitable for extraction using this procedure. Bile contained compounds which decomposed the Sep-Pak column, and rumen fluid did not extract to give consistent or sensible results.

Full fat homogenised and pasteurised cowsmilk was used to investigate the corrinoids in milk. The result of one sample gave ado-cbl: 39.4 %, me-cbl: 3.7 %, OH-cbl: 25.0 % and analogues: 31.9 % (with recovery coefficients as for plasma).

8.6 Discussion

8.6.1 Presence of analogues

Labnet Invermay, where all our samples were analysed for vitamin B₁₂ content, use a chicken serum method developed by Green *et al.* (1974). The authors state that their results were higher than the ones obtained with *E. gracilis* and *L. leichmannii*, both of which measure analogues to some extent. This suggests that analogues contribute to the vitamin B₁₂ results from Labnet Invermay. This was confirmed here by corrinoid separation and subsequent RIDA where analogues were recorded for all biological tissues. However, as was seen with cbi not all analogues are picked up by Labnet Invermay's method. Therefore the amount of analogues measured would have been an underestimation of the total amount of analogues present in the sample.

However, the fact demonstrated here that a significant proportion of the Labnet Invermay assay comprises analogues (15 to 30 %) has implications for the interpretation of vitamin B₁₂ values obtained for biological samples such as liver, plasma and milk. In theory, the 'true' vitamin B₁₂ status of an animal may be considerably lower than assumed from the results of the assay. This is an academic point if the reference ranges are established by the same assay and the proportion of analogues is constant. It may well be that, in part at least, the higher threshold levels in use as the reference range in New Zealand may reflect uncertainties in the analysis

and variation in the proportion of contribution of analogues. Whether there is more variation in these under New Zealand pastoral conditions than elsewhere which would thus explain the higher reference range is a question worth pursuing. My preliminary studies have not suggested large variation but it could be critical at truly marginal vitamin B₁₂ deficiency situations.

8.6.2 Corrinoids in liver

A summary of the corrinoids in sheep and human tissue is given in Table 8.9. In sheep liver the highest proportion of corrinoids was due to ado-cbl. Considering that ruminants cannot absorb much glucose from their diet and hence rely on gluconeogenesis, this result may not be surprising. However, PR lambs, which do not yet require ado-cbl for gluconeogenesis as they can meet their glucose needs from the lactose in milk, also had high proportions of ado-cbl. On the other hand, they may have a greater need for protein synthesis to facilitate their rapid growth in body weight and wool. Therefore one might have expected a lower proportion of ado-cbl in favour of me-cbl to produce methionine which is needed for growth and immunity (Fisher and MacPherson, 1991; Black and Reis, 1979). Yet no differences in liver corrinoid distribution have been found between PR lambs and mature sheep. In fact, there was no difference either when comparing the corrinoid components in sheep and human liver. This suggests liver storage of coenzymes is determined by factors other than the supposed activity of the metabolic pathway.

However, ado-cbl in liver made up a significantly lower proportion of total vitamin B₁₂ in unsupplemented animals than in supplemented ones. These differences in the distribution as well as vitamin B₁₂ content of ado-cbl may have been due to it being the most active metabolic pool and possibly the form in which surplus vitamin B₁₂ is excreted. This may also point towards the primary metabolic defect in vitamin B₁₂ deficiency originating from impairment in the propionate-succinate pathway, as has been suggested by some researchers (Marston *et al.*, 1961 and 1972; Smith and Marston, 1971), rather than from a deficiency of me-cbl as Kennedy *et al.* (1991b) thought. On the other hand, these reflections will need to be taken with care since no deficiency state was seen in the animals and it is therefore

not possible to associate any particular corrinoid concentration with any particular state of deficiency.

There was certainly no evidence that when MMA was elevated that the proportion of ado-cbl was reduced, although its actual concentration was. However, the data collected were too few to warrant any definite predictions. But, because of the lack of response to treatment in the field trials it was decided not to carry on with sample analysis, especially since the corrinoid separation is labour intensive and expensive.

The high amount of OH-cbl in the liver of one lamb treated with OH-cbl injections (compared to the untreated lambs - Table 8.5) could have been a remnant of the form in which vitamin B₁₂ was injected, viz OH-cbl, which would indicate absorption of the administered corrinoid form into liver. This is in contrast to Gimsing *et al.* (1982) who found that although the dominant form of cbl in blood was the one that was administered therapeutically (CN-cbl or OH-cbl) this was converted to the coenzymes once it was taken up by the liver.

The finding of nearly 30 % of analogues in sheep liver is in contrast to Marston (1970) who postulated that vitamin B₁₂ activity in liver was almost entirely due to vitamin B₁₂ itself and that only negligible amounts of analogues were absorbed or otherwise they were destroyed very rapidly.

8.6.3 Corrinoids in other biological samples

In humans, me-cbl contributes the highest proportion of corrinoids in blood (Table 8.10). Djalali *et al.* (1990) measured corrinoids in human plasma with HPLC/RIDA and found similar amounts of ado-cbl and me-cbl (39 and 33 %, respectively) and only small amounts of analogues (9 %) in plasma. Other researchers (van Kapel *et al.*, 1983; Matthews, 1979; Nexø and Olesen, 1982) found a much higher percentage of me-cbl (47 to 65 %), measured with HPLC/RIDA and TLC/bioautography, respectively (Table 8.10). Van Kapel *et al.* (1983) found a much larger proportion of analogues (20 to 30 %) than Djalali *et al.* (1990), an amount similar to the results of this research (18 %). The variation between authors and

methods is large and therefore perhaps only semi-quantitative statements can be made.

Gimsing and Beck (1989), however, deny the existence of analogues in (human) blood and consider any such occurrence to be the result of cbl degradation during the assay procedure (RIDA). Yet the fractions collected and assayed from sheep blood in the current trial indicate that analogues do exist in plasma, at least in sheep.

HC, as the main carrier for endogenous vitamin B₁₂ in human blood, has been found to be the preferred carrier of me-cbl, whereas TC II is considered to be the preferred carrier of ado-cbl (Nexø *et al.*, 1979). In sheep, TC II seems to be the main carrier for endogenous vitamin B₁₂ (Schultz and Judson, 1985), perhaps because of its association with ado-cbl which constitutes the highest fraction of corrinoids in sheep blood. This may then explain the difference in binders between humans and sheep: the main plasma binder could be the one that carries the coenzyme that is present or required most by the particular species. This area warrants further investigation.

Table 8.9: Mean distribution (%) of corrinoids (± SE) in biological tissues of sheep (results from the current study) and humans (literature)

Source	Tissue	ado-cbl	me-cbl	OH-cbl	CN-cbl	analogues	
Sheep	Plasma	27.7 ± 4.4	0	52.0 ± 6.4		17.6 ± 2.6	
	Liver	45.3 ± 1.7	3.3 ± 0.3	24.1 ± 1.6		27.6 ± 1.5	
	SI fluid	26.4 ± 0.9	6.0 ± 0.8	14.9 ± 2.7		52.7 ± 2.4	
	Faeces	20.8 ± 1.7	7.1 ± 0.5	5.1 ± 0.5		56.0 ± 4.3	
Human	Djalali <i>et al.</i> (1990)	Plasma	38.6 ± 16.7	33.1 ± 13.8	18.3 ± 7.6	1.4 ± 1.0	8.7 ± 5.2
	v. Kapel <i>et al.</i> (1983)	Plasma	8.5 ± 2.9	46.9 ± 4.5	40.4 ± 7.1	4.2 ± 3.0	20 - 30
	Nexø & Olesen (1982)	Plasma	30	60	< 10		
	Matthews (1979)	Plasma	23	65	10	2	
	Matthews (1979)	Liver	61	1	38	0	
	Matthews (1979)	Bile	50	4	39	7	
	Djalali <i>et al.</i> (1990)	Faeces					84
	Adjalla <i>et al.</i> (1993)	Faeces					70 - 90

* mean values for 2 sheep during 4 days

In SI fluid and faeces, the proportions of ado-cbl were about half, and of me-cbl and analogues twice as high, compared to liver and many times higher than in plasma. SI fluid and faeces seem very similar in their cbl distribution, yet the vitamin

B₁₂ content of SI fluid was 100 times higher than in faeces (83000 versus 800 - 950 pmol/l), suggestive of a large proportion of vitamin B₁₂ being absorbed. SI contains all the corrinoids from rumen digestion on their way to either absorption or elimination and therefore a much higher amount than in faeces was to be expected. (This comparison should be interpreted with caution, though, as SI fluid was taken from one sheep only.)

Gawthorne (1969) estimated the activities of corrinoids in sheep rumen contents as two thirds 'true' vitamin B₁₂ and one third analogues. Therefore a higher proportion of analogues than measured in SI fluid and faeces would be expected. However, since Labnet Invermay only measures some of the analogues, their actual amount would be underestimated in the assay. Vitamin B₁₂ in human faeces has been observed to contain between 70 and 90 % of analogues (Adjalla *et al.*, 1993; Djalali *et al.*, 1990).

It has been postulated that faeces was the main route by which excess corrinoids were eliminated from the system (Smith and Loosli, 1957; Smith and Marston, 1970a). However, in the loading trial no differences in faecal vitamin B₁₂ concentrations were found before and after supplementation. It may well be that any initial excess was eliminated via urine and that elimination via bile and faeces occurred at a later stage. This is supported by Gräsbeck *et al.* (1958) who found that in human volunteers most of the radiolabeled injected vitamin B₁₂ was eliminated via urine in the first two to three days. After that, the urinary elimination nearly stopped but it continued in bile for at least one more month. Faecal collection in the loading trial stopped after two days post-injection; it is possible that a rise in faecal corrinoids could have been observed had the trial continued.

8.6.4 Comparisons of corrinoids between different species and biological tissues

In our study, liver contained half the proportion of OH-cbl and twice that of ado-cbl than blood. This could imply a conversion of OH-cbl to the coenzyme forms in the liver (Linnell, 1975). According to Seetharam and Alpers (1994) the TC II-

bound cbl (in blood) is converted to the coenzymes within the cells after entry of the TC II-cbl complex.

Matthews (1979) found that the proportion of me-cbl had decreased drastically from blood to liver, whereas ado-cbl had increased. In humans, at least, me-cbl tends to be the predominant coenzyme form in blood, whereas ado-cbl seems to dominate in the liver. In contrast, there was far less variation in corrinoids between the sheep tissues analysed. Me-cbl is low in all of them, but ado-cbl is still the main form of cbl in the liver. Low values for plasma me-cbl have also been found in other species, e.g. the baboon, rat, guinea pig, cat and dog (Matthews, 1979).

In human neonates, on the other hand, the proportion of me-cbl in blood is much higher than in adults (Matthews, 1979; Linnell, 1975). It is possible that a similar pattern exists for ruminants. However, measuring corrinoids in PR lambs' blood was not attempted in this study. It would have been a valuable adjunct, though, particularly in the propionate trial described in Chapter 4. If differences existed between the +Pr and -PR lambs in the proportion of me-cbl (and ado-cbl) it could mean that the conversion of one coenzyme form to another may be driven by demand.

OH-cbl was present in human (Matthews, 1979) and sheep liver in relatively high proportions (38 and 25 %, respectively). In addition, sheep liver also contained significant proportions of analogues. Whether they do or do not exist in human liver is not known since no data was found to confirm or deny their existence.

Further, the presence of analogues as well as OH-cbl showed that not all cbl is stored in the liver in its coenzyme form. Kolhouse and Allen (1977b) found only 65 % of endogenous cbl in liver bound to the intracellular binding proteins methylmalonyl-CoA and methionine synthase, and hence in the form of ado- or me-cbl. In the present study, around half of the corrinoids in sheep liver were in coenzyme form. However, considering the variation in data from our results as well as those obtained by various authors for plasma corrinoids these values may be an approximation only.

The separation of corrinoids certainly warrants further research as only an attempt has been made here to scan the field. Trials set up for the measurement of

corrinoids in vitamin B₁₂ deficient and replete sheep may reveal differences in coenzyme concentrations and distributions that were not seen in the limited samples analysed. It may even be possible to draw conclusions from a comparison of corrinoids in plasma with what is bound to TC II and HC.

CHAPTER 9

General discussion

The reason this work was undertaken and supported by industry was from concern about the apparent increase in diagnosis of vitamin B₁₂ deficiency on farms in New Zealand. Yet in the farm trials no response to supplementation was detected, other than a greater LWG between supplemented and unsupplemented ewes in Chapter 6, despite serum and liver vitamin B₁₂ concentrations often being below the current reference ranges. Unsupplemented ewes on Co deficient pastures had mean serum vitamin B₁₂ concentrations as low as 100 pmol/l and their offspring between 100 and 200 pmol/l. Even most lambs from vitamin B₁₂ replete ewes had serum vitamin B₁₂ concentrations around or below 335 pmol/l, the threshold set for diagnostic purposes in New Zealand below which deficiency is likely. None of the PR lambs had serum vitamin B₁₂ concentrations above 500 pmol/l (the minimum level to be considered normal). (Incidentally, the same marginal range from 335 to 500 pmol/l was also recently suggested by Underwood and Suttle, 1999). This work therefore suggests that these reference ranges are probably too high and need to be revised.

The justification for the high reference values in New Zealand is unclear, since in contrast to the New Zealand serum reference ranges and those of Underwood and Suttle (1999), Judson *et al.* (1987) from Australia suggested deficiency below 200 pmol/l and a normal state above 400 pmol/l, and McMurray *et al.* (1985) from Northern Ireland used 220 pmol/l as the threshold for deficiency.

Liver vitamin B₁₂ concentrations have been considered to be a more reliable indicator of Co deficiency on the basis that they represent a 'store' as opposed to current intake. However, the above researchers differ too in their assessment of liver status. Underwood and Suttle (1999) suggested a marginal range of 280 to 340 nmol/kg fresh tissue for all groups of ruminants whereas Judson *et al.* (1987) proposed adequacy at levels above 200 nmol/kg fresh tissue for PR lambs as well as ruminants and likely deficiency only at levels below 100 nmol/kg fresh tissue for ruminant sheep. These latter values are in contrast to the New Zealand diagnostic

reference range (marginal range between 280 and 375 nmol/kg fresh tissue). In the present research, mean liver vitamin B₁₂ concentrations of around 100 nmol/kg fresh tissue in untreated ewes and around or below the limit of detection (70 nmol/kg fresh tissue) in untreated PR lambs were found without any response to supplementation. This would suggest that adjustments to the laboratory's thresholds of deficiency may need to be carried out. In fact, Clark (1998) did suggest a lower marginal range for mature sheep of 100 to 200 nmol/kg fresh tissue. However, this suggestion for downward revision has occurred since this work had begun and is not yet reflected in current laboratory reference ranges.

Due to the unreliability of serum vitamin B₁₂ assays, in that they vary widely between laboratories (Millar and Penrose, 1980, Schultz, 1987), in that they only measure the vitamin 'in transit' and only, at best, reflect current rumen synthesis (Underwood and Suttle, 1999), and the problems involved in obtaining a liver biopsy sample, accurate diagnosis of Co deficiency has been difficult. Field trials have used liveweight gain and/or change of lambs and sheep in response to Co/vitamin B₁₂ supplementation. Other markers of deficiency have been sought that could indicate more easily and with more certainty if individual animals or the flock as a whole was deficient or not.

Although MMA assays have been adopted in clinical and research laboratories in conjunction with serum vitamin B₁₂ concentrations to determine deficiency states, establishment of a threshold above which serum MMA concentrations indicate likely response to supplementation have not yet been satisfactorily established for pastoral conditions. For example, O'Harte *et al.* (1989) suggested 5 µmol/l for grass-fed and 10 µmol/l for concentrate-fed sheep as the critical threshold for MMA. Ulvund (1990a) suggested a broader range, 5 to 15 µmol/l, as a gauge for the onset of deficiency. Underwood and Suttle (1999) question the merit of measuring MMA in marginally deficient lactating ewes and suckling lambs.

The present work has not really resolved this issue since responses to supplementation were not achieved. However, it is possible to suggest from these studies that the MMA threshold for forage fed animals is likely to be in excess of 20 µmol/l. Despite the low serum and liver vitamin B₁₂ concentrations found in lambs

and unsupplemented sheep in the trials described in Chapters 6 and 7, mean MMA values in the region of ~ 15 $\mu\text{mol/l}$ in ewes and ~ 20 $\mu\text{mol/l}$ in lambs (Table 9.1) and response of both vitamin B₁₂ and MMA to treatment, no differences in LWG in lambs and estimated milk production between treated and untreated ewes could be detected. The only response observed was an 11 % higher LWG in the supplemented ewes in the 1997/1998 trial (Chapter 6). Only in one lamb in which MMA reached 80 $\mu\text{mol/l}$ was there evidence of a lower LWG in comparison to the other lambs in the flock.

When vitamin B₁₂ in the ewes in Chapter 6 in the 1997/1998 trial was increased from 100 to 1000 pmol/l in serum and from 100 to 400 nmol/kg fresh tissue in liver, and serum MMA was reduced from 15 to 2 $\mu\text{mol/l}$ as a result of Co bullet application a positive LWG response was obtained. This LWG difference was achieved during a small window when metabolic demand was high. However, lambs had higher MMA than ewes with no response to supplementation. The critical value for vitamin B₁₂ deficiency may therefore lie close to the values obtained for the unsupplemented ewes in Chapter 6 in 1997/1998 and 1998/1999, i.e. between 100 and 200 pmol/l for serum and 100 and 200 nmol/kg fresh tissue for liver vitamin B₁₂ and possibly between 15 and 20 $\mu\text{mol/l}$ for MMA.

Table 9.1: Lowest mean serum and liver vitamin B₁₂ and highest mean MMA concentrations in unsupplemented animals from trials described in Chapters 6 and 7

Animals (Chapter)	Serum vitamin B ₁₂ (pmol/l)	Liver vitamin B ₁₂ (nmol/kg)	MMA ($\mu\text{mol/l}$)
Ewes 1997 (6)	100	100	15
Ewes 1998 (6)	260	220	7.5
Lambs 1997 (6)	135	70	20
Lambs 1998 (6)	130	130	3
Lambs Farms 1-3 (7)	200-230	100-150	5-7
Lambs 1999 (7)	350	225	5

More recent work from our group which has followed these studies in the 1999/2000 season (a season more conducive to vitamin B₁₂ deficiency) (unpublished data) has indicated that a mean MMA of above 20 $\mu\text{mol/l}$ could be a more realistic threshold above which deficiency is likely. However, more trials are needed to ascertain and confirm that 20 $\mu\text{mol/l}$ is indeed the threshold for MMA under New

Zealand conditions above which response to supplementation will occur with certainty, in order to give veterinarians and farmers clearer guidelines as to when supplementation is merited.

The trial described in Chapter 6 showed that Co bullets given to ewes during pregnancy not only significantly increased ewe vitamin B₁₂ status but also that of their offspring, indicating significant transfer across the placenta and also in milk. Similar observations were made by other researchers (Halpin and Caple, 1982; Grace *et al.*, 1986). Serum vitamin B₁₂ concentrations were 100 to 340 pmol/l higher in untreated lambs from treated ewes than in untreated lambs from untreated ewes. Liver vitamin B₁₂ concentrations in lambs were increased from 71 to 119 nmol/kg fresh tissue in 1997/1998 and from 127 to 262 nmol/kg fresh tissue in 1998/1999 when supplementing the dams. This advantage was maintained, if not increased, until weaning. In many situations this would be sufficient to maintain adequate vitamin B₁₂ status in lambs until weaning and may be a justified use of Co bullets, especially in view of the evidence for a LWG response in the ewe.

Further, the bullets were still maintaining increased serum and liver vitamin B₁₂ concentrations in the host animal twelve months later. The previously supplemented ewes in Chapter 6, 1998/1999, maintained a higher vitamin B₁₂ status in serum and liver, compared to the newly supplemented ewes, throughout the trial. The Co bullet was obviously still effective after 18 months and adding to the release of Co from the bullet administered in the second year. This was considerably longer than the findings of Judson *et al.* (1992) that intraruminal pellets only lasted for about one year. However, these researchers (Judson *et al.*, 1992 and 1995) reported other trials where the effect of Co bullets lasted between 14 weeks and five years.

Although the bullets were still active in the ewe in the second year and milk vitamin B₁₂ concentrations were higher in the OSE than the NSE in 1998/1999, this did not result in further elevation in the serum and liver vitamin B₁₂ concentrations in the offspring. There may not be a quantitative relationship between vitamin B₁₂ concentrations in milk and vitamin B₁₂ concentrations in serum and liver of lambs above a certain threshold and therefore vitamin B₁₂ intake in the lambs. At low Co status, however, serum and liver vitamin B₁₂ concentrations in lambs were influenced by milk vitamin B₁₂ concentrations in both trials.

In these trials, Co bullets readily increased serum and liver vitamin B₁₂ concentrations in ruminant animals to a level well above the minimum threshold of what is considered normal (500 pmol/l for serum, 375 nmol/kg fresh tissue for liver - New Zealand reference ranges). Highest serum concentrations reached nearly 5000 pmol/l in ewes and about half that in weaner lambs. In liver, highest concentrations were 900 and 600 nmol/kg fresh tissue, respectively.

Yet despite this increase following the administration of a Co bullet and ample release of Co from bullets (as shown by the calculations in Chapter 6), maximum blood vitamin B₁₂ levels had not been reached. In the 1998 trial with weaner lambs (Chapter 7) highest serum vitamin B₁₂ concentrations were only 1850 pmol/l and highest liver vitamin B₁₂ concentrations were only 400 nmol/kg fresh tissue. Besides, in none of the animals in Chapters 6 or 7 that were supplemented with Co bullets did serum or liver vitamin B₁₂ levels show a plateauing off that would be expected when maximum concentrations have been reached. Fluctuations in serum and liver vitamin B₁₂ concentrations were observed in supplemented animals in both trials described in Chapter 7. The release rate of the bullet could be much less than stated by the manufacturer or, more likely, rumen fermentation may be influenced by the type and quality of the feed and type and amount of rumen micro-organisms which in turn may affect the amount of vitamin B₁₂ produced and absorbed. Climatic and seasonal fluctuations, as described by Lee (1951), could also influence the Co status of the animal. Further work in this area is warranted.

The attempt to calculate availability of vitamin B₁₂ represented a novel approach based on the approach by Suttle (1974) to estimate copper availability. The estimate obtained - 10 % - was considerably higher than the estimate of availability of vitamin B₁₂ from microbial production by Marston (1970) of 3 to 5 %. However, even this may be questioned in view of the findings from the field trials described in Chapter 6. In this latter trial the suckled lambs in 1998/1999 could be calculated to have had vitamin B₁₂ intakes of only 1200 to 2300 pmol/d, based on milk vitamin B₁₂ concentration and estimated milk production. Serum vitamin B₁₂ concentrations increased during the suckling period from 194 to 362 pmol/l. In the 1997/1998 trial the lambs were calculated to receive only 450 to 1200 pmol/d and still maintained serum vitamin B₁₂ concentrations, thus suggesting a daily requirement of < 1200

pmol to maintain serum concentrations. (For these calculations only the first two months of lactation have been considered as there would have been little vitamin B₁₂ available to the lamb from sources other than milk, such as rumen vitamin B₁₂ production.) On the other hand, the non-supplemented indoor lambs in Chapters 4 and 5 were only able to maintain plasma vitamin B₁₂ despite intakes of 1800 to 4000 pmol/d vitamin B₁₂ from the milk replacer. The findings in the suckled lambs are, however, in accordance with Grace *et al.* (1986) who calculated that between 2600 and 3700 pmol/d is required by the suckled lamb for normal growth.

Yet in the lambs in Chapter 5 administration of about 24,000 pmol/d was required to stimulate increase in serum vitamin B₁₂ concentrations. This suggests that vitamin B₁₂ in milk must be about 20 times more absorbable than the OH-cbl used in Chapter 5. There seem to be four possibilities. Firstly, vitamin B₁₂ in milk may be in a form or complexed in such a way that it is more readily available than that in commercial vitamin B₁₂ supplements. Secondly, the very young lamb may well have a developed rumen, significant herbage intake and microbial vitamin B₁₂ production during very early life, and there is evidence for rumen development by four weeks of age (Joyce and Rattray, 1970). Thirdly, and arising from the latter, propionate production from such fermentation may have stimulated vitamin B₁₂ uptake in serum as observed in the propionate supplemented lambs in Chapter 4. Fourthly, the lamb may be profligate in the use of the available vitamin B₁₂ - the more it has the more it can afford to waste (Smith and Marston, 1970).

There are, however, other reports which suggest an availability of vitamin B₁₂ from milk of around 100 % (O'Halloran and Skerman, 1961). The present studies do show the importance of milk supply for the vitamin B₁₂ status of lambs in the pre-weaning period, and clearly suggest that the status of the ewe will have an impact through her milk.

So far only a few studies have been done with pre-ruminant lambs. The research is hampered by being labour-intensive and limited in the time before the lambs become ruminant. However, in order to understand the intricate metabolism that leads to vitamin B₁₂ deficiency in these animals more work is needed. Assaying plasma binding proteins is one aspect that may yield further insight into the ability of PR lambs to retain the vitamin. This may add another perspective to the information

already gained from MMA, serum and liver vitamin B₁₂ concentrations, and LWG in order to ascertain with more certainty when a deficiency is likely to occur.

CHAPTER 10

Conclusions

This research set out to investigate the metabolic pathways in which vitamin B₁₂ is involved, to ascertain the vitamin B₁₂ requirements of PR lambs, to find the crucial time when supplementation may be needed and to establish more accurate reference values for serum and liver vitamin B₁₂ and MMA in sheep and lambs. Since the experimental periods on farms turned out to be 'drought' seasons where Co deficiency was not observed these goals have only been partially achieved.

The following conclusions were drawn from this research:

Vitamin B₁₂ reference ranges

- > The marginal ranges for serum and liver vitamin B₁₂ concentrations probably lie between 100 and 200 pmol/l and 100 to 200 nmol/kg fresh tissue, respectively.
- > Vitamin B₁₂ deficient sheep on pasture probably have MMA concentrations of at least 10 to 15, if not up to 20, µmol/l.

Co supply from bullets

- > One Co bullet per ewe can last up to 18 months.
- > This could also ensure adequacy of vitamin B₁₂ in the offspring in the first season.

Vitamin B₁₂ storage in pre-ruminant lambs

- > Liver concentrations in lambs and ewes supplemented with supra-physiological doses of vitamin B₁₂/Co can reach up to 900 nmol/kg fresh tissue.
- > Liver retention of supra-physiological doses was calculated as being between 7 and 10 % during one to three months of supplementation.

Vitamin B₁₂ absorption and requirements of PR lambs

- > The total amount of the vitamin B₁₂ administered by IM occurred in plasma.
- > The vitamin B₁₂ contained in milk may be absorbed ~ 100 %.
- > PR lambs may require between 1200 and 4000 pmol (~ 1.6 to 5.4 µg) per day, depending on age.

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