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An Evaluation of Homocysteine in the Assessment of Vitamin B_{12} Status of Pasture-Fed Sheep

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A thesis submitted in partial fulfilment
of the requirements for the

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at

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By

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of M.Appl.Sc.

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by J.M.Furlong

Abstract

Homocysteine (Hcy) is a component of metabolic pathways that are associated with the exchange of methyl groups vital to the animal's survival. Vitamin B_{12} (cobalamin) has a key role in these methylation reactions and the measurement of Hcy in plasma could theoretically predict the clinical status of cobalamin (Cbl) deficiency in sheep, which is innately difficult to diagnose. Serum Hcy concentrations are evaluated in comparison with Cbl and another metabolic indicator of Cbl deficiency - methylmalonic acid (MMA), in situations of liveweight gain (LWG) responsiveness to cobalt (Co)/Cbl to determine its utility in pasture-fed sheep.

Improvement of HPLC methodology was carried out to resolve peak integrity, precision, run time and stability issues encountered previously in the assay of Hcy.

Eighty two pregnant ewes, half of which were supplemented with a Co bullet, were grazed together on a Southland property known to be Co deficient. Half of the lambs from each group were supplemented with 3mg microencapsulated Cbl (SMARTShot^{TM}) one month after lambing (day 0), providing four groups. Lambs were blood sampled and weighed, at monthly intervals, until approximately 210 days of age and were weaned at day 89.

A 40% difference in mean liveweight was recorded between the supplemented and unsupplemented lamb groups. Mean monthly total homocysteine (tHcy) concentration ranged from 1.5 to 4.5 μmol/l. On day 0 tHcy concentrations in lambs from supplemented

and unsupplemented ewe groups were 4.0 and 2.2 μmol/l; a difference which was significant ($p < 0.001$). Subsequently there were no differences between unsupplemented and supplemented lamb groups.

This lack of effect occurred despite plasma concentrations of MMA and Cbl at which response to supplementation could be expected and LWG responses to Cbl supplementation were observed. Unsupplemented lamb MMA and Cbl concentrations (>16 μmol/l and <170 pmol/l, respectively, compared to the deficient status thresholds from reference ranges of >13 μmol/l and <200 pmol/l respectively) were attained on day 57. No correlation between Hcy and MMA or Cbl concentrations was detected even in individual animals whose performance was severely compromised by deficiency. This suggested that the biological importance of the Hcy pathway means that it is highly conserved, even in extreme deficiency.

To pursue this further, data from a previous supplementation trial in which 34 individual unsupplemented animals had been removed due to welfare issues, due to severe ill-health, were examined. Mean tHcy concentrations ranging from 6 – 13 μmol/l were recorded, in association with Cbl concentrations below 200 pmol/l throughout and steady rise in MMA concentration to very high values (from 10 – 26 μmol/l). The severity of the deficiency at onset was characterised by rapid response to supplementation although tHcy showed no associated change as a consequence.

In conclusion, the measurement of tHcy as a metabolic indicator of Cbl deficiency in sheep on typical New Zealand pasture appears to have no value in detecting the incidence or assessment of the severity of the disease. The possibility that diet and other factors such as alternative pathways, including the betaine - methionine metabolic pathways, may regulate and conserve methylation pathways is discussed, but a conclusion that MMA is the first rate limiting pathway and the metabolic indicator of choice in predicting the clinical Cbl status in sheep, seems most plausible.

Keywords: Sheep, lambs, cobalt, homocysteine, methylmalonic acid, cobalamin, liveweight, plasma, HPLC.

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ABBREVIATIONS

μg	Microgram
μl	Microlitre
μM	Micromolar
ABD-F	7-Fluorobenz-2-oxa-1,3-diazole-4-sulfonamide
AdoCbl	Adenosylcobalamin
ATP	Adenosine triphosphate
BCFA	Branch chain fatty acids
BHMT	Betaine-homocysteine methyltransferase
Cbl	Cobalamin
CNCbl	Cyanocobalamin
Co	Cobalt
CO_2	Carbon dioxide
d	Day
DM	Dry matter
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra –acetic acid
g	Gram
GLDH	Glutamate dehydrogenase
GSH	Glutathione
HC's	Haptocorrins
HCl	Hydrochloric acid
Hcy	Homocysteine
holoHC	Holohaptocorrrin
HPLC	High performance liquid chromatography
ISTD	Internal standard
kg	Kilogram
l	Litre
LWG	Liveweight gain
M	Molar
MeCbl	Methylcobalamin
mg	Milligram

min	Minute
ml	Millilitre
MMA	Methylmalonic acid
MTHF	5, 10 – methylenetetrahydrofolate
N	Nitrogen
Na	Sodium
NH_3	Ammonia
OHCbl	Hydroxycobalamin
OWLD	Ovine white liver disease
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
ppm	Parts per million
RNA	Ribonucleic acid
Rpm	Revolutions per minute
SAH	*S* - adenosylhomocysteine
SAM	*S* - adenosylmethionine
SE	Supplemented ewe
SESL	Supplemented ewe/supplemented lamb
SEUL	Supplemented ewe/unsupplemented lamb
SL	Supplemented lamb
TC, TCII	Transcobalamin II
TCA	Tricarboxylic acid
TCI, TCIII	Transcobalamin I, Transcobalamin III (haptocorrins)
tHcy	Total homocysteine
THF	Tetrahydrofolate
UE	Unsupplemented ewe
UESL	Unsupplemented ewe/supplemented lamb
UEUL	Unsupplemented ewe/unsupplemented lamb
UL	Unsupplemented lamb
VFI	Voluntary food intake

CHAPTER ONE

INTRODUCTION

Homocysteine is a metabolically important amino acid generated by the enzymic de-methylation of methionine and sits at an essential crossroad which regulates the fate of sulphur containing compounds. Its remethylation requires an adequate supply of vitamin B_{12}, also known as cobalamin (Cbl), for this methylation process to proceed. The sulphur containing amino acid methionine is often the first limiting amino acid in diets for ruminants and also a significant donor of methyl groups, which may be of particular importance in the growing lamb. It has been thought that homocysteine (Hcy) could be used as a nutritional biomarker of Cbl deficiency in sheep to aid accurate diagnosis especially in situations of marginal deficiency. Elevated concentrations can be caused by decreased methylation of Hcy to form methionine and therefore if measured could highlight the metabolic status of the animal.

Cobalamin has a molecular weight of 1355 and the formula $C_{63}H_{88}O_{14}N_{14}PCo$ and is of microbial origin. Cobalt (Co) is required in the diet as a constituent of Cbl. As Co is not stored in the body in significant quantities, ruminants must consume Co frequently for adequate Cbl supply. A Co deficiency is, therefore, considered a Cbl deficiency in ruminants, as rumen microbes are unable to synthesise sufficient Cbl causing ensuing effects on propionate metabolism and methylation processes.

Once the Cbl stores in the liver and other tissues are depleted deficiency occurs which can impact on production through loss in liveweight and can in severe cases eventuate in the death of the animal. A fatty liver disease in sheep known as ovine white liver disease (OWLD) may also occur in severe deficiency with an accumulation of triglycerides and free fatty acids in the liver. Sheep are more susceptible to Co deficiency than cattle and responses to changes in dietary Co supply are rapid with increased appetite and rapid weight gain achieved within days of increased Co intake. The remission of anaemia, however, occurs more slowly. See Plate 1.1

Plate 1.1. Photograph depicting the consequences of Cbl deficiency on the health and welfare status of the animal severely compromised as a result of the disease. Buère *et al.*, (1993).

Two distinct forms of Cbl with contrasting co-enzyme functions are produced from its metabolism, namely methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl). Methylcobalamin is utilised in the resynthesis of methionine by methylation of Hcy allowing the co-enzyme methionine synthase to supply methyl groups. Adenosylcobalamin influences energy metabolism as cofactor to the co-enzyme methylmalonyl-CoA mutase to form succinate from propionate allowing the production of glucose.

It has been shown that a severe elevation in plasma total homocysteine (tHcy) concentration suggesting a decreased capacity to methylate Hcy, occurs in Cbl deficient states in sheep fed a barley-based diet (Kennedy *et al.*, 1992) or in cattle fed a maize-silage based diet (Stangl *et al.*, 2000). As the measurement of plasma concentrations of methylmalonic acid (MMA) are now seen as an early diagnostic marker of ovine Cbl clinical and subclinical deficiency, as compared to Cbl alone, in sheep fed on a barley-based diet (O'Harte *et al.*, 1989, Kennedy *et al.*, 1991), or in sheep in grazing systems (McMurray *et al.*, 1985, Gruner *et al.*, 2004, 2004a, 2004b) comparison between tHcy and

MMA as metabolic indicators of deficiency at the tissue level requires evaluation as indices of Co/Cbl deficiency in sheep at pasture.

The following studies describe the results of field experiments conducted on grazing livestock used to characterize total homocysteine concentration in lambs as a consequence of severe Cbl deficiency.

- Main experiment – To evaluate plasma tHcy concentration in relation to that of MMA, Cbl and liveweight gain performance as a predictor of the level of deficiency and metabolic pathway proclivity. (Trial A)
- The suitability of tHcy as an indicator of Cbl status is substantiated with data from a previously conducted trial comparing mean group and individual animal data from animals severely affected by Cbl deficiency. (Trial B)

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Ruminants require cobalt in their diet in order to facilitate the synthesis of vitamin B_{12}. Micro-organisms in the rumen incorporate dietary cobalt (Co) into a corrin ring structure to furnish vitamin B_{12} (Cbl) supply. Cobalt is, therefore, an essential nutrient for sheep and cattle where a deficiency primarily leads to Cbl deficiency in the host animal. Non-ruminant animals cannot incorporate Co into its physiologically active form and therefore require Cbl either directly in food or indirectly by ingesting faeces, which has been enriched by the synthetic activity of microbes in the large intestine (Underwood & Suttle, 1999). Concentrations of Cbl in the blood of ruminants generally reflect dietary Co intake with liver tissue reserves to a lesser extent. Liver Cbl concentrations can range from < 48 to > 1000 nmol/kg fresh tissue with blood levels ranging from < 80 to > 900 pmol/l depending on Co intake (Grace, 1994).

2.2 Background

2.2.1 Cobalt supply

Cobalamin contains 4.4% Co (Underwood & Suttle, 1999) and Co supply is dependant either on pasture content or supply in a concentrate. Depending on intake and nature of the diet about 13% dietary Co is converted to Cbl, which in sheep represents about 700 μg of Cbl per day (Grace, 1994). Cobalt concentrations can vary widely within forage species and with soil conditions. Seasonal variation can also affect the adequacy of Co supply with rapid pasture growth lowering Co levels during a wet spring season. Soil contamination can greatly increase herbage concentration in a dry season allowing for a higher degree of soil ingestion. Generally, the Co content of pastures tends to increase in late autumn and winter and to decrease in spring and summer (Andrews, 1956). McNaught, (1948) suggested, in early New Zealand studies, that pasture levels of 0.07 –

0.08 ppm Co in the dry diet was considered the minimum requirement for sheep and cattle. Later studies suggested the minimum level of "pasture associated" Co required by growing lambs to be 0.11 ppm DM with lambs being the most sensitive to Co deficiency, followed by mature sheep, calves, and mature cattle (Andrews, 1956). Grace, (1994) concludes that the Co requirement of sheep on pasture is 0.1mg Co/kg DM. Growth responses to Co or Cbl supplementation is anticipated when herbage Co levels fall below 0.08 to 0.1 mg/kg DM (Underwood & Suttle, 1999; Andrews *et al.,* 1958; Andrews, 1956 & 1972).

2.2.2 Cobalamin metabolism

Cobalamin is a highly water-soluble vitamin, the site of storage being the liver, where it is poorly stored, but various amounts are also found in other tissues including the kidney, where it is stored in the free form, and bound to transport proteins in the circulation. It is considered that the kidney plays an important role in the regulation of Cbl metabolism with Cbl deficiency and Cbl loading producing greater changes in Cbl concentration in the kidney than in the liver (Scott *et al.,* 1984). Little is known about the route of excretion in ruminants but it is thought to be via urinary excretion and biliary secretion to the faeces.

Cobalamin cannot traverse cellular plasma membranes; it therefore forms complexes with three mammalian tissue fluid proteins to aid transportation. These include gastric intrinsic factor, transcobalamin II (TC, TCII) present in plasma, and haptocorrins (TCI, TCIII) found in most secretions. Intrinsic factor secreted from the stomach is essential for the intestinal uptake of Cbl, whereas TCII facilitates the cellular uptake of Cbl from plasma and various tissue fluids into a wide spectrum of cells. Lack of synthesis of either intrinsic factor or TCII leads to haematological, gastrointestinal, and/or neurological disorders. Haptocorrins (HC's) are considered a Cbl storage protein forming the complex holohaptocorrin (holoHC), which accounts for much of the total Cbl bound in serum.

There are two distinct forms of Cbl – methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl) along with two other less important ones i.e. cyanocobalamin (CNCbl) and hydroxycobalamin (OHCbl). Cobalamin acts as a cofactor for two enzymes, methylmalonyl-CoA mutase and methionine synthase. A deficiency in

Cbl can cause an increase in the plasma concentrations of methylmalonic acid (MMA) and homocysteine (Hcy). The primary metabolic defect in Cbl deficiency is a block in the utilisation of propionic acid. This is a volatile free fatty acid and is a major source of energy in the ruminant. Underwood & Suttle, (1999) state that AdoCbl influences energy metabolism by facilitating the formation of glucose by assisting methylmalonyl-coenzymeA mutase to form succinate from propionate, chiefly in the liver. An increase in serum propionic acid and MMA in deficient animals is thought to be responsible for anorexia (Clark & Miller, 1983).

Methylcobalamin assists a number of methyltransferase enzymes by acting as a donor of methyl groups. It is important in enabling methionine synthase to supply methyl groups to a wide range of molecules, including formate, noradrenaline, myelin, and phospatidyl ethanolamine and in the synthesis of methionine. Methionine facilitates the transport of folic acid (folate) into the liver cells and is also necessary for optimum growth and wool production. A restricted dietary intake of methionine from anorexia induced by Cbl deficiency could affect the way sheep govern the interaction between the two metabolic pathways (McMurray *et al.,* 1985). It therefore raises the question as to which metabolic pathway is critical to sheep in the maintenance of growth and wool production when Cbl supplies are limited.

2.2.3 Diagnosis of Cobalamin deficiency

The signs and symptoms of Cbl deficiency are non-specific i.e. a marked depression in appetite, poor growth rates, anaemia, diarrhoea, ill thriven appearance and a watery lachrymation. Biochemical changes, as a consequence of ovine Cbl deficiency in the metabolism of propionic acid, phospholipids, propionate and succinate in the rumen are well characterised, although complex (Kennedy *et al.,* 1995). In severe cases it is also responsible for a fatty degeneration of the liver known as ovine white liver disease (OWLD). Kennedy *et al.,* (1994, 1997) experimentally produced this disease in lambs fed on a diet only lacking in Co, where the liver showed a highly significant increase in the concentration of triglycerides and free fatty acids. If an animal remains undiagnosed then deficiency of Cbl can ultimately result in death of the animal. Traditionally serum Cbl levels have been taken and diagnosis and treatment made on this assessment but

investigation into the metabolic pathways of Cbl depletion i.e. MMA and Hcy accumulation could more accurately diagnose supplementation requirements. Indeed MMA has gained widespread acceptance as a diagnostic marker of Co deficiency. Debate as to the true value of Hcy measurements, however, still remains.

2.3 Homocysteine

2.3.1 Metabolism

Homocysteine is a thiol-containing amino acid produced by the intracellular demethylation of methionine. It is exported into plasma where it circulates, mostly in its oxidised form, bound to plasma proteins. Smaller amounts of reduced homocysteine and disulphide homocysteine (HCY-SS-HCY) are also present. Total homocysteine (tHcy) represents the sum of all Hcy species found in plasma or serum (free plus protein bound). Reference to tHcy concentrations may be expressed as Hcy or tHcy and are presented here as written in the original reference.

Reduced:		
Homocysteine	NH_3^+ $^-OOCCHCH_2CH_2$–SH	1 %
Oxydized:		
Homocysteine	NH_3^+ $^-OOCCHCH_2CH_2$–S $^-OOCCHCH_2CH_2$–S NH_3^+	5-10 %
Mixed-disulfides:		
Protein-bound Homocysteine	NH_3^+ $^-OOCCHCH_2CH_2$–S (Protein)–S	80-90 %
Cysteine-Homocysteine	NH_3^+ $^-OOCCHCH_2CH_2$–S $^-OOCCHCH_2$–S NH_3^+	5-10 %

Figure 2.1 Structural forms of Hcy present in plasma. From Tewari *et al.*, (2004)

Dietary methionine is converted by transmethylation to Hcy, with the transfer of the methyl group to other species. Homocysteine occupies a metabolic site at the

intersection of remethylation and trans-sulphuration pathways where its biochemical fate is linked to vitamins B_{12} and B_6 and the various reduced folate coenzymes. Homocysteine is metabolised to either cysteine or methionine. In the vitamin B_6 dependent trans-sulphuration pathway, Hcy is irreversibly catabolised to cysteine via the rate – limiting enzyme cystathionine *β* – synthase. A major part of Hcy is remethylated to methionine, using CH_3 – H_4 folate as the source of methyl groups and catabolised by a cobalamin-dependant enzyme methionine synthase, with folate 5, 10 – methylenetetrahydrofolate (MTHF) acting as an essential co – factor. Homocysteine can also be remethylated by the betaine methyl group betaine-homocysteine methyltransferase (BHMT) (a Cbl – independent enzyme). Regeneration of MTHF from tetrahydrofolate (THF) is catabolised by the enzyme MTHF reductase.

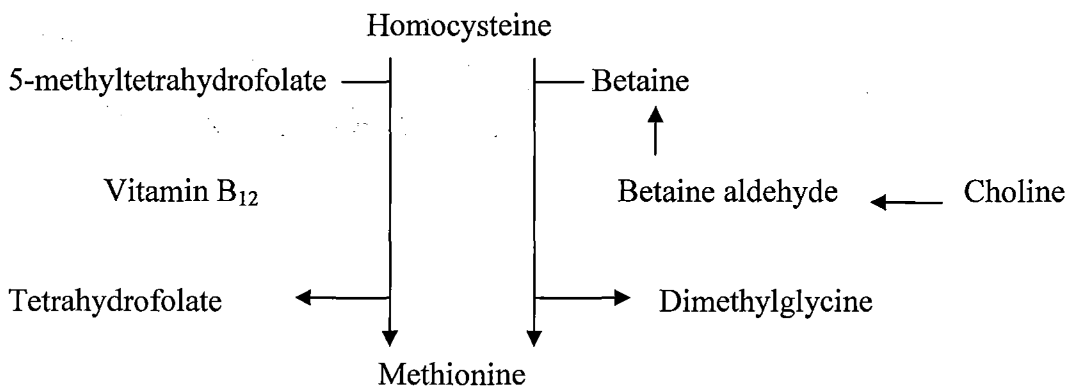


Figure 2.2 Reproduced from Lewis, (2003). Metabolic pathway for the conversion of homocysteine into methionine

Homocysteine is an obligatory co-substrate in the metabolism of both tissue folate and choline. Finkelstein & Martin, (1984) carried out *in vitro* studies mimicking rat liver and measured the simultaneous product formation by three enzymes which utilise Hcy. In the control system, 5-methyltetrahydrofolate homocysteine methyltransferase, betaine homocysteine methyltransferase and cystathionine *β*-synthase accounted for 27, 27, and 46% respectively of the homocysteine consumed. Subsequent studies demonstrated that the adaptation from a high protein diet to a low protein diet is achieved by a significant increase in betaine homocysteine methyltransferase, an 83% reduction in cystathionine synthase, and a total decrease of 55% in the consumption of Hcy. Guttormsen *et al.*, (1996) suggested there is large individual variability in tHcy clearance in humans and variable

adaptation to impaired methionine synthase function may be explained through increased Hcy flux through alternate metabolic pathways.

2.3.2 Function

Methionine is an essential amino acid in mammals and can be used for protein synthesis or can be adenylated by ATP to form a methyl donor *S* – adenosylmethionine (SAM) by the action of adenosylmethionine synthetase. This serves as a methyl donor to a wide variety of important biomolecules such as DNA and RNA bases, amino acid side chains in proteins and is involved in the biosynthesis of such compounds as creatine and epinephrine (Matthews, 1999). In the process SAM is converted to *S* – adenosylhomocysteine (SAH), which is subsequently hydrolysed back to adenosine and Hcy by the action of adenosylhomocysteine hydrolase to commence a new methyl transfer cycle. In this manner, methyl groups ultimately derived from glucose allow methionine to participate in multiple cycles of methylation and demethylation. This is the only known route of Hcy formation in vertebrates (Lucock *et al.*, 1996). The SAM/SAH ratio is an important determinant of one carbon metabolism. Kennedy *et al.,* (1992, 1994) determined in Co deficient sheep that a lower hepatic SAM concentration produced a ratio that was reduced only in the liver, 5.4 ± 0.8 nmol/g vs 2.7 ± 0.6 nmol/g, 5.38 ± 0.76 vs 2.50 ± 0.68 nmol/g wet wt tissue, whereas other organs (kidney, brain, spinal cord) appeared to be unaffected. A decreased ratio is associated with decreased methyltransferase activity. Cobalt deficiency did not affect hepatic SAH concentrations.

Steady methionine and Hcy concentrations in plasma appear to be the net result of subtle competitive mechanisms between re-methylation and trans-sulphuration pathways together with adaptability in the overall homeostasis of methionine, depending on its bioavailability. A metabolic balance between remethylation and transsulfuration is therefore dependent upon SAM levels, concentration of the *de novo* methyl group acceptor Hcy, and specific dietary factors, particularly folate and methionine, but also vitamins B_{12} and B_6 (Lucock *et al.,* 1996). Although mammals cannot synthesise Hcy, they can remethylate it, and in this way, they are able to conserve limiting amounts of methionine in the diet (Matthews, 1999).

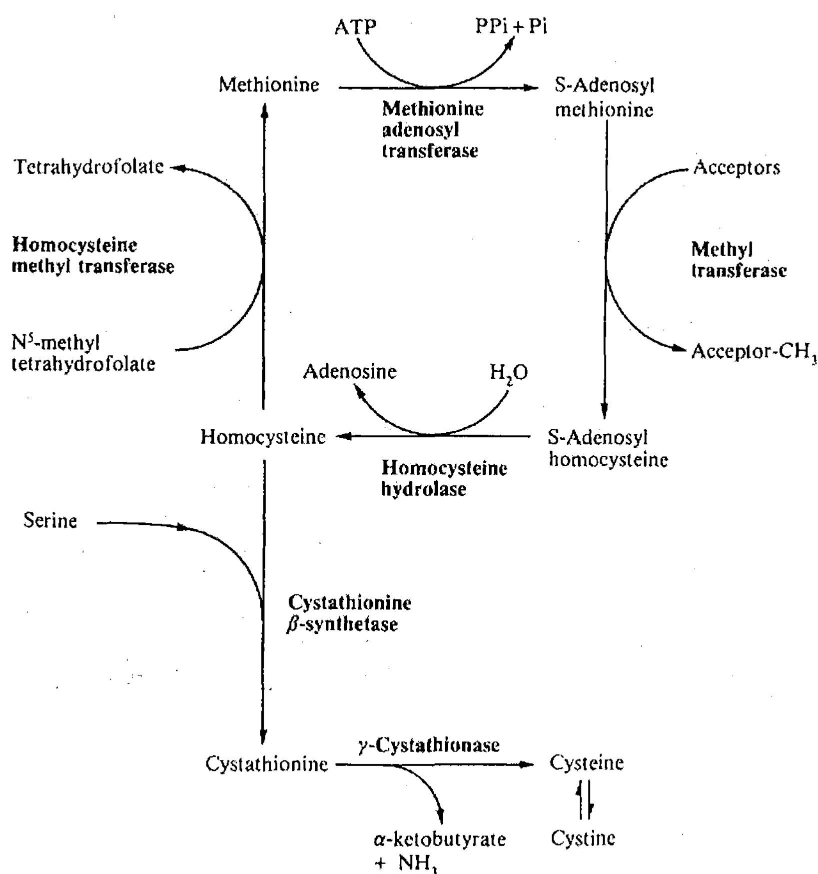


Figure 2.3 Pathways for the metabolic interconversion of methionine to cysteine and cystine. From Thomas & Rook, (1983).

2.3.3 Concentrations in plasma

Few studies have been conducted showing the effects of Cbl deficiency on plasma tHcy concentrations in sheep. Experiments using nine five month old Suffolk Cross lambs (5 Co – deficient and 4 Co – sufficient) fed a whole barley – based diet containing 0.0045 and 1µg/g Co, housed in indoor pens with wire flooring have been conducted by Kennedy *et al.,* (1992). They found elevated tHcy plasma concentrations in lambs from 18 weeks until the end of the experiment, with values rising above 70 µmol/l at its highest and in association with Cbl levels below 220 pmol/l. The mean plasma tHcy concentration in the control animals was 10.1 ± 0.7 µmol/l. The live weight of the animals fed the Co deficient diet rose at a slower rate compared to controls (122 ± 7 g/d vs. 210 ± 10 g/d over the same period) and were only 83% of the weight of controls at week 28 compared to week 0. It

was also stated that inhibition of methionine synthase causes an increase in the plasma concentration of Hcy. Whether some of these results are a consequence of Cbl deficiency *per se* or in combination with appetite reduction is difficult to say. Dysfunction in one-carbon metabolism came after retarded growth was seen; methylcobalamin may not, therefore, have been the first rate-limiting function.

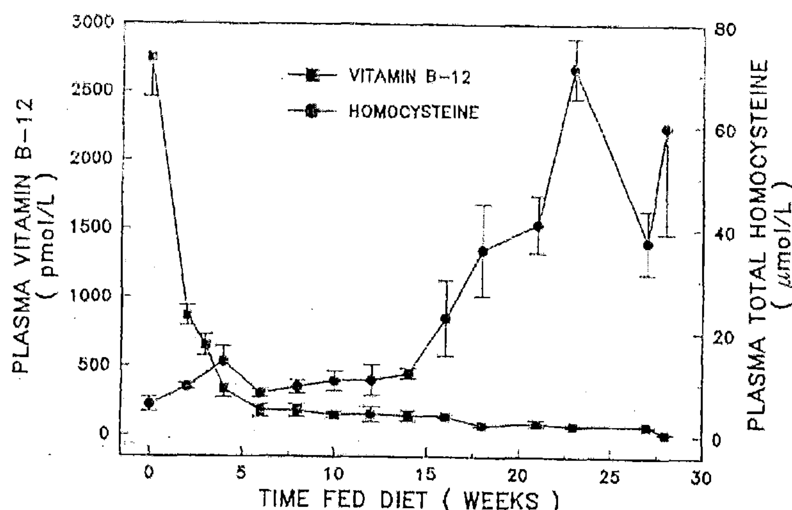


Figure 2.4 Effect of Co deficiency on plasma concentrations of vitamin B-12 and total homocysteine. Lambs were fed the Co-deficient whole barley diet for 28 wk. Jugular blood plasma was collected by venipuncture and assayed for vitamin B-12 by radioassay and for total homocysteine by HPLC with fluorescence detection. Data are means ± SEM of five animals. From Kennedy *et al.*, (1992)

Kennedy *et al.*, (1994) showed significant rises in plasma Hcy in sheep suffering from OWLD in a study using eight five-month-old Suffolk cross lambs (one subsequently died). Major defects in lipid metabolism involving both Cbl-dependent pathways have been proposed on the basis of studies in animals fed on barley-based diets. The livers of the OWLD group were found to have markedly reduced activities of both holo-methionine synthase and holo-methylmalonyl CoA mutase enzymes compared to control animals. In the presence of micromolar concentrations of copper, Hcy can cause the formation of hydrogen peroxide (Starkebaum & Harlan, 1986). The possibility that raised Hcy levels, as a result of decreased methionine synthase activity, could act as a trigger for the lipid peroxidation in OWLD due to hydrogen peroxide reacting with iron II ions to produce hydroxyl radicals was mentioned. Kennedy *et al.*, (1997) also considered elevated Hcy concentration in association with lipid peroxidation leading to the damage of

mitochondrial structure, although Stangl *et al.,* (2000) failed to demonstrate changes in the oxidative/antioxidative balance due to elevated tHcy levels in cattle.

Table 2.1 Reproduced from Kennedy *et al.,* (1994).

Biochemical markers of cobalt deficiency in lambs with ovine liver disease and in cobalt sufficient controls.[1]

Analyte	Matrix	Control	OWLD
Vitamin B_{12}	plasma	2382 ± 359	17 ± 1[5] pmol/l
Homocysteine	plasma	37.8 ± 3.7	90.4 ± 6.4[5] μmol/l
Vitamin B_{12}	liver	395.5 ± 9.7	14.5 ± 4.1[5] pmol/g
Holomutase activity	liver	30.8 ± 2.15	4.4 ± 0.6[5] Units/g
Holosynthase activity	liver	869 ± 102	105 ± 26[4] Units/g
SAM: SAH ratio	liver	5.38 ± 0.76	2.50 ± 0.68[2]
PC: PE ratio	liver	1.45 ± 0.04	1.05 ± 0.09

1. Data are means ± SEM of four sheep (control group) and three sheep (OWLD group).
   Student's t-test was used for statistical analysis.
2. Significant effect of OWLD $P<0.05$
3. Significant effect of OWLD $P<0.01$
4. Significant effect of OWLD $P<0.002$
5. Significant effect of OWLD $P<0.001$

In cattle Stangl *et al.,* (1998) found long term moderate cobalt deficiency (0.083 μg Co/g DM) induced a 53% reduction of methionine concentration (4.87 ± 0.51 vs. 2.30 ± 0.73 mg/l) in plasma relative to their controls and a 4.8-fold increase in Hcy levels (1.00 ± 0.25 vs. 4.80 ± 0.87 mg/l). Impairment of the Cbl dependent remethylation pathway was thought to be of primary consequence, uniquely related to the deficiency. The increase in circulating Hcy was said to emphasize its use as a sensitive index for the diagnosis of Co deficiency in cattle. Stangl *et al.,* (2000) experimentally reproduced cobalt deficiency induced hyperhomocysteinemia in cattle producing plasma tHcy levels of 35.5 μmol/l (B_{12} - 218 pmol/l), compared with 7.39 μmol/l (B_{12} - 905 pmol/l) in the control animals fed a maize-silage based diet containing 0.083 μg Co/g DM.

In an experiment using piglets Stangl *et al.,* (2000a) found an association between Cbl metabolism and the trace elements nickel and cobalt. The piglets were fed a Cbl – free diet without additional folate, combined with a relatively high dietary methionine concentration as methionine loading has been shown to exacerbate hyperhomocysteinemia.

High concentrations of iron (300 mg/kg), copper (30 mg/kg), cobalt (1 mg/kg) or nickel (6 mg/kg) were added to the diet. The lowest Hcy concentration was found in serum of pigs fed the Cbl adequate diet. The highest Hcy concentrations were found in serum of Cbl deficient pigs fed normal trace element amounts and the iron and copper –fortified diets, see Figure 2.5. It was suggested that Cbl and Hcy may alter processes relating to trace element mobilisation and transport and that trace element supplementation could influence the outcome of symptoms including hyperhomocysteinemia. However the dietary manipulations had no detrimental effects on variables symptomatic of oxidative stress.

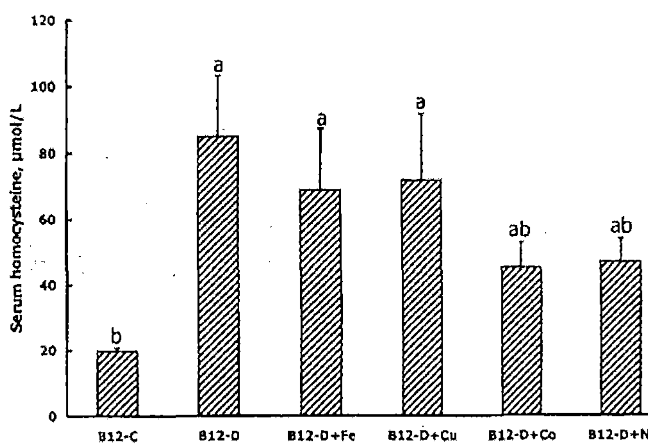


Figure 2-5 Serum homocysteine concentration of vitamin B-12 deficient pigs fed iron, copper, cobalt or nickel. Values with different superscripts differ significantly. $P < 0.05$ (SNK test). Mean ± SEM, n = 8. B12-C, vitamin B-12-adequate control group; B12-D, vitamin B-12_deficient group fed normal concentrations of iron, copper, cobalt and nickel; B12-D – Fe, vitamin B-12-deficient, high-iron group; B12-D + Cu, vitamin B-12-deficient, high copper group; B12-D +Co, vitamin B-12-deficient, high-cobalt group; B12-D +Ni, vitamin B-12-deficient high nickel group. From Stangl *et al.,* (2000a).

Vellema *et al.,* (1999) conducted a three-year field study using Texel twin lambs grazing Co-deficient pasture with Co content in the years 1991, 1992, and 1993 being 0.10, 0.06, and 0.05 mg/kg DM, respectively. The non-supplemented lambs in 1992 & 1993 consistently had higher Hcy concentrations than their supplemented siblings along with higher MMA concentrations, see Figure 2.6. Homocysteine values, although elevated, were much lower and the differences between groups much smaller than values reported in the experiments of Stangl *et al.,* (1998, 2000) & Kennedy *et al.,* (1994) previously, even though MMA levels rose significantly, indicating a possible difference between species and/or diet in relation to Hcy response. Comparisons were unable to be made between

MMA and Hcy concentration in the experiments of Stangl *et al.,* (1998, 2000) & Kennedy *et al.,* (1994) as they were not measured jointly.

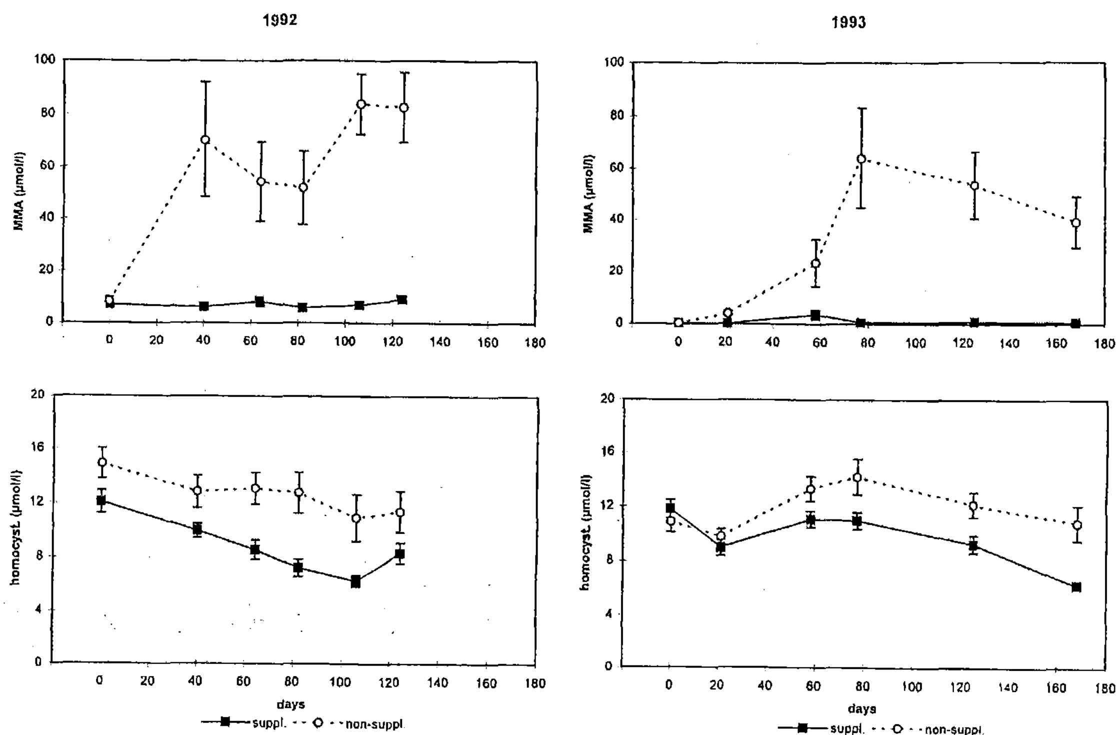


Figure 2.6 Plasma methylmalonic acid (MMA) [µmol/l] and homocysteine (µmol/l) concentrations in supplemented and non-supplemented lambs in 1992 and 1993. From Vellema *et al.*, (1999).

The importance of homocysteine in human health has recently been recognised following demonstrations of elevated plasma levels of the amino acid in patients with coronary, cerebrovascular, end-stage renal disease, and peripheral arterial occlusive disease. Substantial elevations of plasma Hcy can also result from genetic abnormalities such as severe deficiencies of cystathionine ß-synthase. In addition, the role of Hcy in embryonic development may have implications in animal reproduction (D'Mello, 2003). It is suggested that in humans the hyperhomocysteinemia of Cbl deficiency is caused by enhanced tissue export of Hcy, whereas in renal disease hyperhomocysteinemia is related to reduced plasma clearance. The kidney probably plays an important role in Hcy clearance and metabolism (Friedman *et al.,* 2001).

The ranges of tHcy concentrations in plasma from healthy human adults are given below.

Table 2.2 Total plasma homocysteine concentrations sourced from various references.

Total plasma homocysteine: working ranges.

	Jacobsen, (1998)	*Friedman et al., (2001)*	*Refsum et al., (1996)*
Normal range	5-15 μmol/l	6-12 μmol/l	10.92 μmol/l (males)
Desirable (?)	<10 μmol/l		9.61 μmol/l (females)
Hyperhomocysteinemia			
Mild	15-25 μmol/l	12-30 μmol/l	
Intermediate	25-50 μmol/l	31-100 μmol/l	
Severe	50-500 μmol	>100 μmol/l	

2.4 Methionine

2.4.1 Metabolism

Methionine plays a crucial role in mammalian metabolism: first as an essential amino acid; second, as a precursor of cysteine biosynthesis; third, as the key intermediate in methyl group transfer. The blockage of cystathionine ß-synthase activity serves as a salvage mechanism allowing methionine to maintain functional properties of continued existence and allowing for appropriation of Hcy. Methionine is sometimes referred to as a 'methyl donor' and is the sole precursor of *S* – adenosylmethionine (SAM). Over 100 methylation reactions involving methionine are known (Lobley *et al.,* 1996). The methyl group flux through SAM has been calculated, in humans, as more than double the methionine intake and, therefore, implies considerable remethylations are required. Methionine flux through the substrate cycle may be increased to maintain adequate supplies of SAM. Methionine entry into the TCA cycle is at the same point as that of propionate metabolism leading to the formation of succinate.

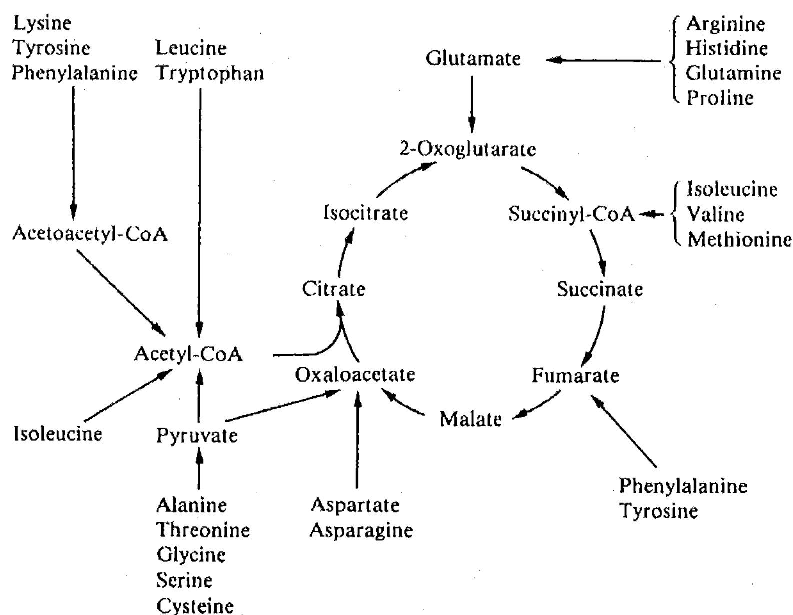


Figure 2.7 Pathways by which the carbon skeletons of the different amino acids enter the tricarboxylic acid cycle. From Vernon & Peaker, (1983).

2.4.2. Dietary intake

Unlike non-ruminants the amino acid composition of the digesta supplying the ruminant is markedly different to the dietary protein consumed. Protein supply depends on a number of factors: the susceptibility of dietary protein to microbial degradation in the rumen, and the efficiency of capture of degraded nitrogen (N) for microbial protein synthesis. There is a clear change in the ranking of limiting amino acids with slight changes in diet thus making the identity of the first-limiting amino acid difficult although it is still widely thought that methionine and lysine are particularly limiting (Chamberlain & Yeo, 2003) and the microbial protein methionine content is relatively low. Ruminants such as sheep have a very small amount of methyl group nutrients available from the diet as the major dietary sources of methyl groups in non – ruminants, such as choline and betaine, are degraded in the rumen. The MeCbl pathway, therefore, might be particularly important in ruminants (Kennedy *et al.,* 1992).

2.4.3. Regulation of methionine

Mammalian livers contain a sensitive regulatory mechanism that allows either conservation or catabolism of methionine with the enzymes that utilise Hcy also found there. Both methionine and Hcy in large quantities are toxic to cells and accumulation of either may be prevented by two alternative catabolic routes: the methionine transamination sequence and Hcy entry into the trans-sulphuration pathway. Consequently, temporal imbalances in the supply of either methyl group acceptors or donors may result in stimulation of net methionine catabolism and, therefore, contribute to the finding of the amino acid often being limiting for ruminants fed on forages (Lobley *et al.,* 1996). The conservation of the Hcy moiety of methionine is an important function of methionine recycling with the methyl groups for Hcy methylation derived from either one of the methyl groups of betaine, or from the one-carbon pool. Thus the utilisation of methionine is balanced either by dietary intake or by Hcy methylation.

Studies comparing methionine synthesis in sheep and rat tissues *in vitro* (Xue & Snoswell, 1985) found the capacity of hepatic BHMT for methionine synthesis relative to bodyweight to be much smaller in sheep than in rats. On the other hand it was suggested that the total body capacity for methionine synthesis by 5, MTHF in sheep is considerably greater. A higher ratio of SAM to SAH was also observed in sheep liver together with a much lower rate of hepatic methionine recycling (2.8 times less) than that in rats. Hepatic methionine recycling was stimulated by the addition of betaine. The results indicated that in sheep 5-MTHF and BHMT play significant roles in hepatic methionine synthesis with BHMT only having a minor role in total body methionine synthesis, whereas the rat is virtually dependent on BHMT.

2.4.4. Methionine loading

Dietary methionine-containing protein loads increase plasma levels of Hcy and cysteine. Finkelstein & Martin, (1986) failed to observe evidence of toxicity until the level of methionine was 3.0% in rat livers, confirming the existence of adaptive processes. Excessive dietary methionine led to the depletion of both betaine and serine with a 12 – fold increase in the synthesis of cystathionine, a 150% increase in flow through the betaine

reaction, and a 550% increase in total metabolism of homocysteine; the concentrations of these substrates limit their respective reactions. Increasing dietary methionine from 0.3% to 1.0% was also found to increase cystathionine synthase activity by 35% (catabolising a toxic excess of Hcy to cysteine). In contrast Lambert *et al.,* (2002) reported that sulphur amino acid metabolism might be regulated differently in cattle than in other tested species. Hepatic cystathionine synthase activity was unresponsive to methionine infusion into the abomasum, therefore indicating a lack of methionine-sparing cysteine conversion activity. Hepatic methionine synthase decreased linearly with increasing methionine supply, and hepatic BHMT activity responded quadratically ($P = 0.04$) to increasing methionine supplementation with 0 or 10 g/d resulting in higher activity than that with 5 g/d. Overall the activities of hepatic BHMT were approximately two-fold those reported in sheep and half those in rats (Xue & Snoswell, 1985) indicating that remethylation of Hcy in cattle may predominate, independent of cysteine supply.

Radcliffe and Egan, (1978) reported no change in the hepatic cystathionine synthase activity of sheep when abomasal methionine supply was increased from 1.4 to 4.2 g/d. A build up of either Hcy or Hcy-cysteine mixed disulphide in plasma and urine was not seen, with BHMT being the only hepatic enzyme that had increased activity in response to methionine supplementation, therefore possibly preventing the accumulation of Hcy. It could be speculated that protection against Hcy accumulation occurs through an increase in the BHMT pathway, which may be of physiological importance. Gawthorne & Smith, (1974) in studying the effects of injected L-methionine in the liver of Cbl deficient sheep found failure in folate metabolism due to the impairment in the rate of transport of folates into the liver in sheep fed a Cbl deficient diet with methionine injections stimulating the transport of folates into the cell, offsetting the effect of deficiency. Hepatic SAM concentration was also found to be about one half (10.24 ± 0.60 vs. 17.16 ± 0.91 nmol/g) in deficient animals compared to normal animals and was restored to normal by either Cbl or L-methionine.

It is known that cattle have a lower sensitivity to Co deficiency than sheep therefore methionine supply may be of particular importance in the management of Cbl regulated pathways, especially as sheep have a high requirement of sulphur amino acids for wool synthesis. The clinical significance of the effect of dietary methionine in relation

to Hcy concentrations requires further consideration. Little work has been carried out in this regard with particular reference to sheep.

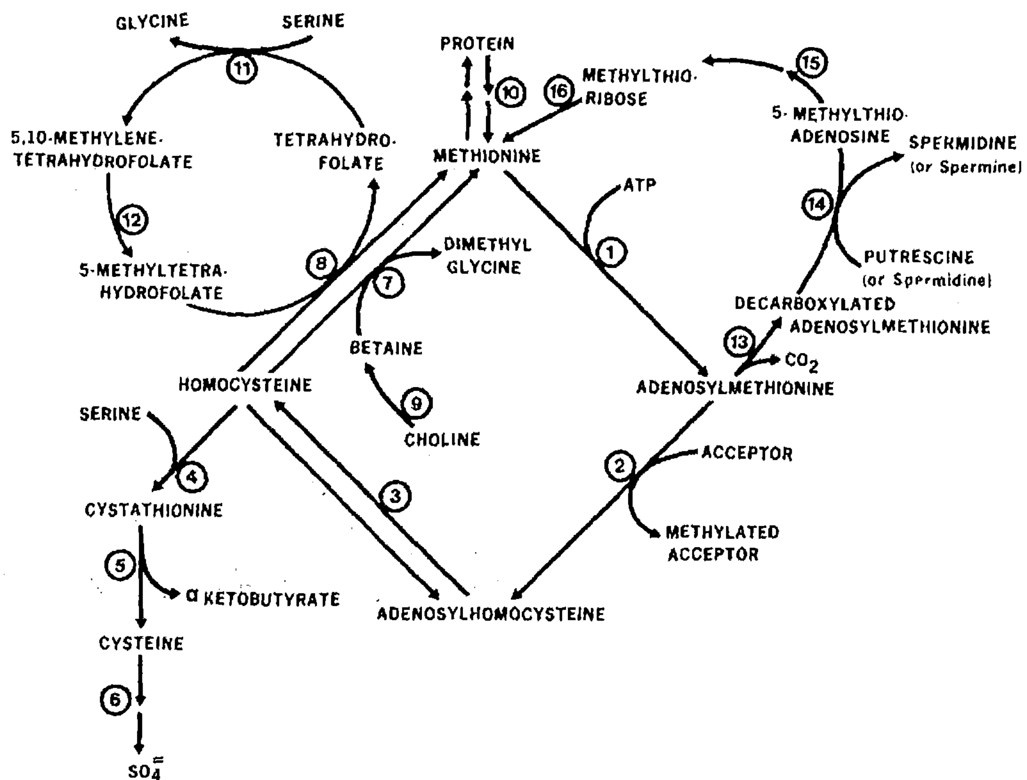


Figure 2.8 Pathways of methionine metabolism in mammalian tissues. The numbers represent the following specific enzymes or enzymatic sequences: 1, methionine adenosyltransferase; 2, representative transmethylation reaction; 3, adenosylhomocysteinase; 4, cystathionine *B*-synthase; 5, cystathionse; 6, multiple reactions leading from cysteine to sulphate; 7, betaine-homocysteine methyltransferase; 8, methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase); 9, choline dehydrogenase + betaine-aldehyde dehydrogenase; 10, the equilibrium between protein methionine and free methionine; 11, serine hydroxymethylase; 12, methylenetetrahydrofolate reductase; 13, adenosylmethionine decarboxylase; 14, spermidine (or spermine) synthase; 15, methylthioadenosine phosphorylase; 16, conversion of methylthioribose 1-phosphate to methionine. From Finkelstein & Martin, (1986).

2.5 The betaine metabolic pathway

Betaine (a zinc metalloenzyme) is a product of choline oxidation by an irreversible two-step reaction that occurs in the liver and kidney of humans and pigs but only in the liver of rats, with concentrations of betaine in human serum ~ 20 – 60 μmol/l (Schwab *et al.,* 2002). Betaine can be an important alternative methyl donor for Hcy methylation. The betaine-homocysteine methyltransferase pathway operates independently of the Cbl and

folate-dependent reaction. N, N-dimethylglycine is formed and then catabolised to N-methylglycine, glycine and, finally, CO_2 and NH_3, in which the last three reactions result in the transfer of a one-carbon unit group tetrahydrofolate (H_4 folate) to form 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4 folate). N-methylglycine (sarcosine) can also be formed by glycine N-methyltransferase, which utilises SAM to methylate glycine. *S* – adenosylmethionine (SAM) is important in the activity of betaine-homocysteine methyltransferase (BHMT). Betaine-homocysteine methyltransferase has an important role in the maintenance of hepatic methionine concentration in mammals especially when dietary intake of methionine is limited. Comparatively little attention has been placed on the BHMT methylation pathway as opposed to methionine synthase and its potential influence on Hcy concentrations. Betaine levels are normal in Cbl and most folate – deficient patients (Allen *et al.,* 1993; Stabler, 1999); although ruminants are notoriously poor in betaine due to degradation of betaine by rumen microbes.

Slow *et al.,* (2004) conducted experiments using rats and found the mean baseline plasma Hcy concentration for 44 animals was 8.5 ± 0.2 µmol/l. A fall in plasma Hcy concentration was seen when three known substrates of BHMT - glycine betaine 0.8 ± 0.4 µmol/l (P = 0.064), dimethylsulfoniopropionate 1.0 ± 0.5 µmol/l (P = 0.041), and dimethylthetin 1.5 ± 0.7 µmol/l (P = 0.033) - were administered. Conjecture was made as to whether Hcy/methionine cycling could explain why circulating Hcy concentration decreased by 30% even though a massive excess of BHMT substrate was available *in vivo.* Schwahn *et al.,* (2004) found plasma Hcy and BHMT activity both showed a strong negative correlation (r = -.8558, P = .0004, n = 12; r = -.7494, P = .0050, n = 12, respectively) with liver betaine in mice with mild hyperhomocysteinemia, therefore suggesting that increased plasma Hcy can be seen as an indicator of limited betaine supply for remethylation, especially when Hcy metabolism is impaired.

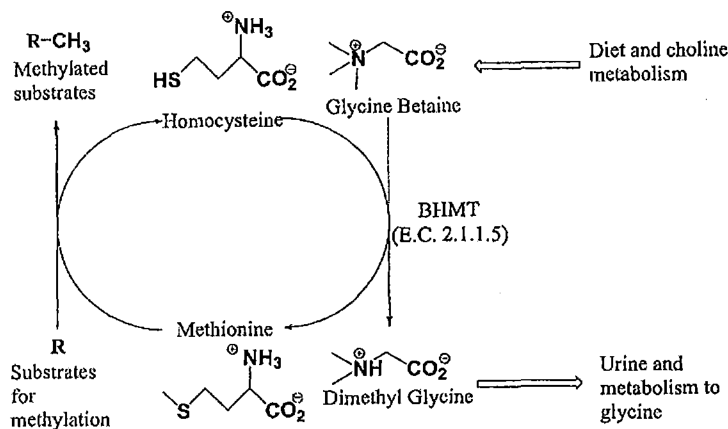


Figure 2.9 Betaine-homocysteine methyltransferase cycle (simplified). Methionine is a source of methyl groups used to methylate various substrates (such as DNA, phospholipids, and proteins), ultimately forming homocysteine, which can be remethylated by betaine-homocysteine methyltransferase (BHMT) using glycine betaine obtained from the diet or from choline metabolism. From Lee *et al.*, (2004).

As sheep progress from the pre-ruminant to ruminant state there may be a vital change in methyl group nutrition. Dietary choline, for example, a major preformed methyl group nutrient, in which milk is rich, is an important source for the pre-ruminant, but is almost unavailable to the post-ruminant sheep as dietary choline. Xue & Snoswell, (1986) investigated postnatal increases in the activities of choline oxidase and BHMT in pre-ruminant lamb liver and noted decreases in both substances when the lambs reached the ruminant state, which was considered a consequence of adaptation to the reduced availability of dietary choline through degradation by rumen microbes. The availability of dietary choline, therefore, appears to regulate the activity of choline oxidase as well as BHMT in sheep. The developmental change in the activity of hepatic 5, MTHF is negatively correlated with those of hepatic BHMT in sheep enabling the physiological level of methionine to be maintained. In conclusion, adaptation of post-ruminant sheep to the low availability of dietary preformed methyl nutrients is achieved by a markedly reduced rate of methyl group catabolism and an elevated rate of *de novo* synthesis of methyl groups from a one-carbon pool. Indeed, an increase in methionine synthesis, particularly in lactating ewes, provides a means to replenish methyl groups used for milk choline, creatine and carnitine synthesis (Xue & Snoswell, 1985a). Thus conservative metabolic features of methyl groups in adult sheep may indicate the need to preserve the MeCbl pathway in times of Cbl deficiency.

Although the significance of BHMT in Hcy homeostasis is not clear, it is known that the relative contribution of BHMT to Hcy remethylation can be influenced by diet. Limited amounts of methionine in the diet may cause activation of the BHMT pathway and therefore lower tHcy levels. In studies using Angora and Alpine kids, Puchala *et al.,* (1995) observed an increase in methionine concentration in plasma as a result of intravenous betaine injection in both sets of kids. It was hypothesized as to whether it was not only elevated Hcy remethylation that contributed to the increased levels of methionine, but direct utilisation of the betaine methyl group. This would conserve methionine (SAM) and result in a further increase in methionine levels. Partly replacing methionine - SAM as a methyl donor with betaine or even replacing methionine in other physiological body processes was considered. Such replacement could account for the elevated methionine levels after supplementation with betaine. In *in vitro* studies involving rats, Finkelstein & Martin, (1984) reported that adaptation to a low protein diet produced a significant flow through the BHMT reaction despite a marked reduction in the content of this enzyme suggesting that the percentage of Hcy remethylated could vary according to the dietary concentration of methyl groups such as methionine and choline.

High plasma Hcy levels are a risk factor for cardiovascular disease in humans and can be lowered through supplementation with 6 g/d of betaine (Schwab *et al.,* 2002). Olthof *et al.,* (2003) noted in humans that the normal dietary intake of betaine is ~0.5-2 g/d. Investigations into the effect of betaine supplements within this range found reductions in fasting and post-methionine loading plasma tHcy concentrations. A person consuming a diet rich in betaine (~2 g/d of betaine) had a 1.3 µmol/l (12%) lower plasma tHcy concentration than a person who consumed a diet poor in betaine (0.5 g/d). A betaine – rich diet might, therefore, lower cardiovascular disease risk. These results emphasise the importance of the liver in mediating the lowering of elevated circulating Hcy by the betaine pathway. On the other hand Kim *et al.,* (2003) suggest caution in using betaine supplementation and careful evaluation of the use of high betaine doses (1000 mg/kg body weight) in clinical situations. Although Hcy concentrations in liver and plasma were decreased in studies using rats and mice, rapid alterations in the hepatic concentrations of the critical metabolites in the trans-sulphuration pathway such as SAM and GSH that have diverse biological functions in the body could be induced. Furthermore, a subsequent

decline in betaine concentration would be accompanied with an abrupt increase in homocysteine level.

In supplementation trials using pigs it was found that in contrast to previous findings with rats and chicks, hepatic and renal BHMT activity in pigs was not influenced substantially by methionine deficiency, or by surfeit levels of choline or betaine, although tHcy concentrations appeared not to have been accounted for. (Emmert *et al.,* 1998).

Interest has been expressed in the potential of betaine as a feed supplement in enhancing animal performance by improving the methionine status in the body by substituting for choline and functioning as an effective methyl donor when dietary methionine is insufficient, although further investigation is required. Betaine is also a substance that is capable of reducing lipid accumulation in the liver. Fernández *et al.,* (1998) indicated that dietary supplementation with betaine (2g kg^{-1}) produced a reduction in subcutaneous fat of lamb carcass and intramuscular lipids, with a limited effect on fatty acid composition. Betaine – homocysteine methyltransferase activity can be measured directly and kinetically by H-nuclear magnetic resonance spectroscopy (Lee *et al.,* 2004)

2.6 Rumen fermentation

The main source of energy for ruminants is volatile fatty acids produced by fermentation in the rumen. Concentrations of these in the peripheral blood are influenced by numerous factors, which include the nature of the diet, rate of absorption from the rumen, endogenous production of acids and a series of metabolic reactions. (Marston *et al.,* 1972).

There is a lack of understanding on the effect of Co deficiency on rumen fermentation, which warrants further investigation. Cobalt is important for rumen microbial function allowing them to synthesise Cbl important in their own metabolism. Bacteria require and synthesise their own Cbl for conversion of succinate to propionate as part of their use of plant carbohydrate, in fact the reverse of the conversion in the animal. The volatile fatty acid profile may well alter as the Co deficiency progresses in response to the requirement of the animal and microbes metabolic pathway.

Kennedy *et al.,* (1991) determined that failure of microbes to produce Cbl when lambs transferred to a barley-based diet, extremely low in Co, was associated with large increases in succinate concentrations in rumen liquor suggesting a reduction in ability to support the succinate – propionate pathway. Kennedy *et al.,* (1991a) reported significant impairment of propionate metabolism at an early stage in the progression of Cbl deficiency in sheep, however, although the impairment was significant, it was not extensive and gluconeogenesis remained unchanged. Whether impaired propionate metabolism is the primary metabolic effect in ovine Cbl deficiency is still unclear as glucose metabolism remained apparently unaltered by conditions that lead to sub optimal weight gain and an accumulation of MMA in plasma. Although this effect operated at dietary Co concentrations of 0.0042 µg/g, these are below concentrations that would be seen in farming practice. It remains to be seen if these changes in rumen fermentation could be repeated on grass fed sheep.

As Co deficiency progresses a shift in the requirement for Cbl in the animal may occur through a reduction in propionate. The incorporation of succinate directly into the tricarboxylic acid (TCA) cycle may explain why changes in glucose metabolism were not seen. These changes in the rumen volatile fatty acid profile may lower the importance of Cbl in the animal to gluconeogenesis and therefore make the MMA pathway of secondary importance to the animal's survival, although this is tentative. This leads on to the question as to whether the methyl donor pathway becomes the essential pathway and therefore the pathway of metabolic necessity.

Lambs are particularly susceptible to Co deficiency. It has been revealed that Co deficiency progresses more seriously in lambs on some pastures than on others with similar grass Co content. On some pastures low Cbl values in the lambs are not reflected in reduced productivity with the lambs having good weight gain and thrive well. Ulvund & Pestalozzi, (1996) conducted a six year study involving lambs on two different Co deficient pastures; S (disease susceptible – Særheim) = 0.12 mg Co/kg DM, H (not disease susceptible – Høyland) = 0.10 mg/kg DM. Lambs on pasture S had poor weight gain compared to supplemented lambs with Cbl plasma concentrations below 300 pmol/l throughout grazing. The lambs on pasture H, however, grew normally and were 12 kg heavier in live weight than the untreated lambs on pasture S with plasma Cbl levels below 150 pmol/l throughout grazing. Methylmalonic acid levels were elevated in both sets of

untreated lambs, although to a larger extent on pasture S. Elevated serum Hcy levels were found after two months on pasture. Elevation of folate, GLDH and serum bile acids were found with the occurrence of bile acid elevation more prominent on pasture S.

The amount of water-soluble carbohydrates (fructosan) in the grass at the time of rapid lamb growth and heavy Cbl need may be significant. The fructosan content was found to be higher on pasture S with high levels that may be responsible for initiating hepatic lipodystropy, leading to hepatic insufficiency, growth reduction and OWLD. The lack of information on short crop rotation in New Zealand highlights the need for more research in this area.

2.7 Homocysteine assay development

2.7.1. Methodologies

Methodologies to determine total plasma (or serum) homocysteine were first developed in the mid to late 1980's (Jacobsen, 1998), with measurement widely carried out by High Performance Liquid Chromatography (HPLC) (Araki & Sako, 1987; Jacobsen *et al.,* 1994)), with electrochemical or fluorometric detection. As plasma contains both reduced and oxidised species of Hcy, the chemical reduction of disulphide bonds to create free Hcy in plasma must first be achieved. This is of great importance and commonly used reductants include 2-mercaptoethanol, dithiothreitol, sodium borohydride, *n*-tributylphosphine and more recently, the water-soluble phosphine tris (2-carboxyethyl) phosphine. Secondly, the proteins present in the sample matrix are precipitated from the solution using such agents as trichloroacetic acid, sulfosalicylic acid, methanol, perchloric acid, and meta-phosphoric acid and centrifuged. Homocysteine can then be resolved from other low molecular weight thiols (cysteine, cysteinylglycine, and glutathione) by reversed-phase HPLC. Total homocysteine is then determined directly by electrochemical detection, or by pre column derivatisation with a fluorochromophore, such as ammonium 7-fluorobenzo-2-oxa-1, 3-diazole-4-sulfonate (SBD-F) (Vester & Rasmussen, 1991), resolution by HPLC, and detection by fluorescence detection. Homocysteine can also be derivatised, after reductive generation, for gas chromatography and detected by mass

spectrometry and also separated by capillary electrophoresis (Nekrassova *et al.,* 2003). A summary of different techniques is shown below.

Table 2.3 Modified from Nekrassova *et al.,* (2003)

The analytical parameters obtained for the various detection methodologies of homocysteine after a separating technique has been applied to the sample.

Matrix	Reduction	Derivatisation	Separation	Detection
Plasma	MCE	OPA	HPLC	FL:NG
Plasma urine	$NaBH_4$	mBrB	HPLC	FL:365, 475 nm
Blood	TPP	mBrB	HPLC	FL300, 470 nm
Plasma	TBP	SBD-F	HPLC	FL:385, 515 nm
Plasma	MCE	OPA, MCE	HPLC	FL:340, 450 nm
Plasma	DTT	mBrB (MEOH)	HPLC	FL:270, 474 nm
Cell cultures	TCEP	OPA	HPLC	FL370, 480 nm
Cell cultures	DTT	4,4′-dithiopyridine	HPLC	UV: 327 nm
Urine	TBP	CMPI	HPLC	UV: 312 nm
Plasma	TBP	CMPI	HPLC	UV: 312 nm
Plasma	$NaBH_4$	CQMT	HPLC	UV: 350 nm
Plasma	$NaBH_4$		HPLC	EC: Graphite
Plasma	$NaBH_4$	None	HPLC	EC: GC
Plasma	TCEP	None	HPLC	EC: Au
Plasma	$NaBH_4$	None	HPLC	EC: NG
Plasma	TBP	ECF	GC	MS
Serum urine	2-MCE	MTBSTFA	GC	MS
Plasma	DTT	MTBSTFA	GC	MS
Plasma	DTT	ECF	GC	MS
Plasma	TBP	ABD-F	CE	UV: 220 nm
Plasma	TCEP	None	CE	EC; Au/Hg
Plasma	DTT	FM	CE	LIF:488,520nm
Serum	TCEP	6-IAF	EC	LIF: NG
Blood	None	SBD-F	CZE	FL: NG

NG, not given; **HPLC,** high performance liquid chromatography; **FL,** fluorescence detection; **UV,** ultraviolet detection; **EC,** electrochemical detection; **GC,** gas chromatography; **MS,** mass spectrometry; **CE,** capillary electrophoresis; **LIF,** laser induced fluorescence detection; **CZE,** capillary zone elctrophoresis; **MCE,** 2-mercaptoethanol; **OPA,** *o*-phthaladehyde; **mBrB,** monobromobimane; **TPP,** triphenylphosphine; **TCEP,** tris(2-carboxyethyl)phoshine; **TBP,** tri-*n*-butylphosphine; **SBD-F,** 7-fluorobenzo-2-oxa 1,3-diazole-4-sulfonate; **DTT,** dithiothreitol; **TCEP,** tris(2-carboxyethyl)phosphine; **CMPI,** 2-chloro-1-methylpyridinium iodide; **CGMT,** 2-chloro-1-methylquinolinium tetrafluoroborate; **ECF,** ethylchloroformate; **MTBSTFA,** N-(*tert*-butyldimethylsilyl)-N- methyltrifluoroacetamide; **ABD-F,** 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole;**FM,** fluorescein-5-maleimide; **6-IAF,** 6-iodoacetamidofluorescein.

It has been reported by Shipchandler & Moore, (1995) and Frantzen *et al.,* (1998) that tHcy can be measured through immunoassay techniques, such as the Abbott IMx (fluorescence polarization) analyser or the Bayer ADVIA Centaur® chemiluminescent assay (Tewari *et al.,* 2004). Both these assays use a mouse monoclonal antibody directed against S-adenosylhomocysteine, which is formed when Hcy is allowed to react with adenosine in the presence of S-adenosylhomocysteine hydrolase. These are considered to

be high-throughput assays particularly suitable to biological laboratories where routine analysis and automation is required and where advances in this type of assay development are likely to continue.

2.7.2. Standardisation of laboratory reference ranges

Differences in laboratory methodologies used for the determination of tHcy along with efficiency of disulphide bond reduction and use of different internal standards and calibrators allows for some discrepancies in agreements between laboratories, although they do correlate well (Ueland *et al.,* 1993). Therefore, there are no standardised references or consensus on the use of a standardised calibrator (Jacobsen, 1998). Pfeiffer *et al.,* (1999) in an international laboratory study found that some of the methods tested could be used interchangeably but an improvement can be aided by the introduction of standard reference materials and more external quality assessment programmes. Analytical results must always be cautiously examined and sometimes the affirmation of hyperhomocysteinemia is not easy to state, in view of the differences between the mean Hcy levels in controls and in patients, linked to the inter-method and inter-laboratory variability (Ducros *et al.*, (2002).

Although there are a plethora of techniques available for the measurement of tHcy, sheep pose particular problems as assays used have been developed essentially for human diagnostic purposes. The development and validation of an assay with particular specificity to sheep plasma (or serum) becomes essential when running a trial requiring a selective, sensitive, accurate measurement of tHcy concentrations with the ability to quantitate 1 μM differences and less.

2.7.3. Quantification of tHcy

Although the use of external calibration alone is commonly used for the quantification of tHcy, the importance of finding a suitable internal standard such as cysteamine hydrochloride (Kuo *et al.,* 1997), ensures a greater degree of accuracy, a control for the derivatisation reaction and as a standard for the size of the Hcy peak. High

Performance Liquid Chromatography assays incorporating mercaptopropionylglycine (Vester & Rasmussen, 1991) and N-acetylcysteine (Fortin & Genest, 1995) as internal standards have also been described. The standards used should be prepared in suitable matrices e.g. nanopure water, pooled plasma using the same anticoagulant as the sample, to allow for any matrix effects. Kuo *et al.,* (1997) found the calibration slopes for Hcy diluted in different matrices were indistinguishable from each other when cysteamine was included as an internal standard and the calibration slopes were calculated with Hcy/cysteamine peak area ratios. It was found that when Hcy was diluted with either water or pooled plasma, no difference in the calibration slopes was obtained. The addition of cysteamine was found to improve the within- and between-assay precision.

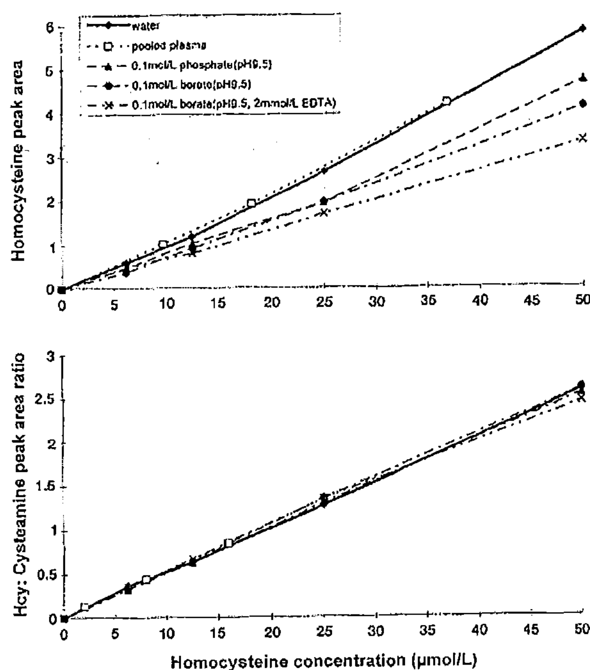


Figure 2.10 Calibration slopes for homocysteine calibrators constituted in different matrices calculated by reference to the external calibrator only (top) or by using an internal standard (bottom). From Kuo *et al,* (1997)

Vester & Rasmussen, (1991) found calibrators prepared in potassium borate pH 9.5 to be different to pooled plasma (15% difference in slopes) and therefore recommend calibrators to be performed with plasma/serum to overcome matrix effects that could be due to the presence of proteins, ionic strength, or some other species catalysing the derivatisation step.

2.7.4. *Collection of blood samples*

A standardisation of blood sampling procedures is required in order to prevent false tHcy concentrations being recorded. Studies investigating the stability of Hcy in whole blood before separation have shown considerable increases in Hcy concentration (Vester & Ramussen (1991); Ubbink *et al.,* (1992)). The increase was found to be linear for at least four hours and amounted to 9-10% per hour. The increase in Hcy concentration may stem from the continuing production or Hcy conversion in red blood cells. Therefore cooling or centrifuging immediately after blood sampling becomes important. Vester & Ramussen, (1991) found the stability on storage of plasma samples was good without any measurable change in the Hcy concentrations. Furthermore, one sample was thawed and frozen nine times during one week and aliquots taken and analysed; no difference in Hcy concentration was seen. Ducros *et al.,* (2002) in a Hcy review states that after centrifugation and separation of plasma, Hcy is stable for four days at room temperature, several weeks at 0-2°C, and several years at -20°C. Haemolysis does not influence plasma Hcy concentration (Bayle *et al.,* 2002). Ubbink *et al.,* (1992) found that food consumption in humans also affected Hcy results with a small decline of tHcy levels after consumption returning to normal levels after eight hours. Ueland *et al.,* (1993) suggests that food intake may affect the concentration of plasma Hcy and that its effect, although small, may persist for several hours.

Almost all determinations of Hcy in blood are performed on plasma rather than serum with the most used anticoagulant being EDTA. It has been shown that there are slight variations of plasma Hcy concentrations according to blood drawn into EDTA, sodium citrate, sodium/lithium heparin or dry tubes. Stability trials should be conducted in order to eliminate any discrepancies in the collection and handling of blood samples.

2.8 Discussion

It has been shown that serum Cbl concentrations have limitations in specificity and sensitivity in diagnosing deficiency and in predicting response to supplementation (Stabler *et al.,* 1996). Since cobalt deficiency adversely affects animal productivity, there is a continuing interest in evaluating indicators of Cbl deficiency. Although MMA and Hcy concentrations are indicators of Cbl deficiency only rises in MMA serum concentrations are specific of Cbl deficiency *per se.* Elevated Hcy concentrations alone are not a true indicator of deficiency as it is also linked to folate metabolism, genetic defects and/or chronic renal insufficiency and therefore needs to be correlated with serum MMA/Cbl to aid diagnosis. Serum MMA and Hcy concentrations have been shown to be elevated in almost every patient who has a clinical response to Cbl and suggests that the primary cause for most of the elevated Hcy concentrations in the elderly is Cbl deficiency (Stabler *et al.,* 1996). Kennedy *et al.,* (1994) concludes that Hcy concentrations elevate during Cbl deficiency in sheep and suggests that determination of Hcy levels in plasma or serum may serve as a diagnostic tool in assessing cobalamin status. Normal concentrations of both metabolites virtually exclude clinical Cbl deficiency.

The identification of the first limiting of the two Cbl – dependent pathways creates a more sensitive approach to diagnosis. It is argued that AdoCbl may be the first limiting of the two metabolic pathways as growth retardation in Co deficient lambs has been seen before plasma Hcy had begun to elevate (Underwood & Suttle, 1999). Price, (1991) observed that propionate metabolism in sheep was found to be impaired earlier than one – carbon metabolism during Cbl deficiency, induced by feeding sheep a low cobalt diet (hay containing 0.03mg Co/kg DM). Price, (1991) supports the contention that plasma methylmalonate is a sensitive indicator for cobalt deficiency in this species and found changes linked to the MeCbl pathway to be much more resistant to Cbl depletion than that of the AdoCbl/ propionate pathway. Methylcobalamin deficiency may not therefore be the first rate-limiting function in sheep.

In this respect, though, sheep could be more vulnerable to MeCbl deficiency than cattle, because of the high requirement for sulphur amino acids for wool synthesis. Kennedy *et al.,* (1995) in studies using cattle confirmed that cattle are less susceptible to

the effects of cobalt deficiency than sheep and concludes that prolonged cobalt deficiency had little significant effect on tissue metabolism.

A rapid effect on appetite raises the question of a rumen – based dysfunction. Marston *et al.,* (1972) hypothesises that it is the failure to metabolise propionate at the normal rate (brought about by the lack of AdoCbl) that leads to the progressive loss of appetite in Cbl – deficient sheep. It could, therefore, be concluded also that this indicates AdoCbl to be the first rate – limiting affect with the production of high levels of MMA.

The relative importance of the AdoCbl and MeCbl dependent pathways may be different for barley and grass fed sheep. The abnormal biochemistry found in ovine white liver disease in barley fed lambs may well be very different to that of forage – fed lambs. Quantities of branch chain fatty acids (BCFA) were found to occur to a lesser extent naturally when grass rather than barley is fed, due to the lower production of propionate in the rumen (Wahle *et al.,* 1979). This leads on to the question as to whether fermentation in the rumen and the subsequent production of succinate from propionate and its incorporation into the TCA cycle, could affect the extent to which abnormal lipid accumulation occurs and any degeneration of the liver seen as a consequence of Cbl deficiency. This could lead to differences seen in the biochemical anomalies found in cobalt deficient sheep and the importance placed on the two Cbl dependent pathways, although this is very speculative.

If at very low Co concentrations a change in rumen fermentation occurs it could produce a switch from propionate to a higher succinate production. Thus the production of methionine may therefore be down regulated as requirement lessens. A build up of Hcy levels may then occur (no need to remethylate Hcy) as seen in trials with very low Co content i.e. Kennedy *et al.,* (1992). Therefore, an increase in Hcy concentration may be secondary to reduced propionate and reduced demand for methionine. Whether this would be the case in pasture fed lambs remains to be seen.

The BHMT pathway has been given very little attention until recently in the regulation of circulating Hcy concentrations in mammals. In relation to sheep and Cbl deficiency there appears to be no work done to date on the role of BHMT and its effect on Hcy levels as a consequence of Cbl deficiency. Along with the tHcy concentrations in

sheep serum very little information exists on the importance of this metabolic pathway in relation to Cbl deficiency, diet, rate limiting amino acids and production losses. It is suggested that the effects of betaine may be to spare methionine by providing labile methyl groups for the synthesis of methylation products, and to reduce abdominal fat by increasing carnitine synthesis and beta-oxidation of fatty acids as well as providing energy for cell metabolism as suggested in studies of chicks (Xu & Zhan, 1998)

2.9 Summary

The influence of diet on rumen fermentation and pattern of Cbl deficiency in sheep may be integrated in such a way that requirements for particular nutrients may bear heavily on the metabolic pathway concerned. The amino acid and volatile fatty acid profile may affect the concentrations of Hcy and MMA in plasma/serum through rumen microbial production. The importance of one metabolic pathway over another may well be reflected in such dietary changes. Therefore investigation into levels of Hcy in pre weaned and pasture fed weaned lambs (given the importance of the sulphur amino acid supply) in relation to MMA, Cbl levels and live weight will further enhance the understanding of Cbl deficiency in sheep and highlight which metabolic pathway is of greater diagnostic importance when determining the severity of Cbl deficiency. In particular it has been noted that lambs with high MMA values and low Cbl levels can still continue to thrive, (Ulvund & Pestalozzi, 1996), therefore raising the questions as to what part diet and metabolic pathway division has influence over the progression of the deficiency and would Hcy concentrations therefore give a better indication of Cbl status over time in combination with growth rates. If lambs are protected while still suckling, despite high MMA concentrations, could this be linked to the supply of Cbl, glucose (lactose) and choline (methyl generators) in milk therefore influencing the progression of the deficiency and the metabolic pathway concerned?

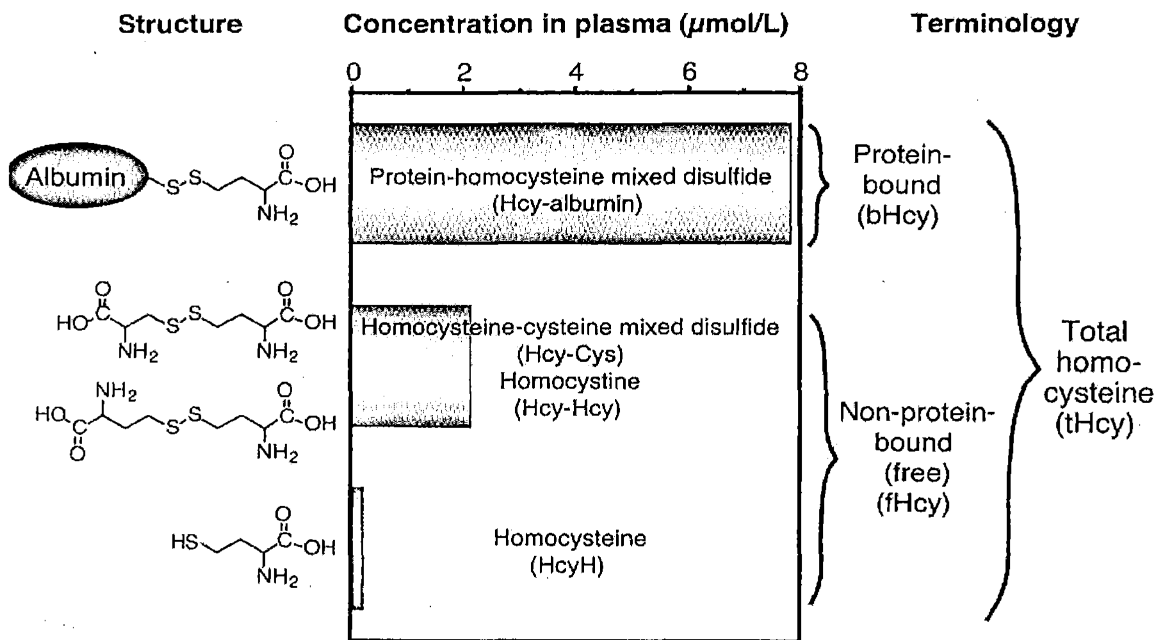
The following study requires the investigation of tHcy concentrations in plasma as a determinant of Cbl deficiency and metabolic consequence. The development of a sensitive and precise method of analysis for the quantification of tHcy in sheep plasma will also be implemented.

CHAPTER THREE

Analysis of Total Homocysteine in the plasma of sheep by High-Performance Liquid Chromatography with Fluorescence Detection

3.1 Introduction

Although there are a wide variety of methods available for the measurement of tHcy in plasma, (Araki & Sako 1987; Stabler *et al.,* 1996), high-performance liquid chromatography (HPLC) with fluorescence detection being the most utilised due to its high sensitivity and reliability, they have on the whole been essentially developed for human diagnostic purposes. See Figure 3.1 for diagrammatic explanation of various analyses. The calibrators, standards, matrices, or antibodies used, as in the case of immunoassay techniques (Shipchandler & Moore, 1995; Frantzen *et al.,* 1998), may not be specific to sheep and therefore cause problems in accuracy of analysis. It is therefore important to develop such a method of analysis specific to sheep plasma. Here described is a simple, sensitive, but selective isocratic HPLC method for the determination of tHcy in the plasma of sheep using pre-column derivatisation with 7-fluoro-2, 1,3-benzoxadiazole-4-sulfonamide (ABD-F), separation using a C18 reversed phased HPLC column and fluorescence detection. Improvement was achieved by the use of a sodium acetate buffer and methanol (Tröbs *et al.,* 1998), and implementation of the internal standard cysteamine to compensate for variation in thiol derivatisation and any losses incurred during analysis (Kuo *et al.,* 1997). Stability trials optimised assay variation and sensitivity enabling shorter run times to be achieved along with accurate measurement of tHcy concentrations with the ability to quantitate differences of < 1 μM.



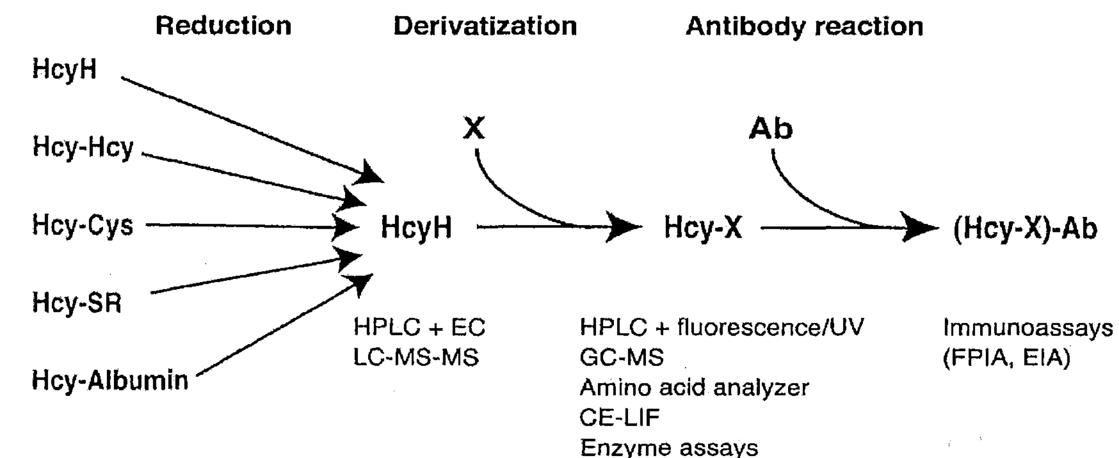


Figure 3.1 Hcy and the related disulfides in human plasma, and the principles for determination of plasma tHcy. (Top), Hcy in plasma rapidly becomes oxidised and therefore exists in multiple forms: most exist as a mixed disulphide with albumin, the remainder as free circulating disulphide forms. Only a small proportion remains as the sulphhdryl form, HcyH. (Bottom), in all tHcy assays, the disulphides, including protein-bound Hcy, are cleaved by treatment with a reducing agent, yielding a single form, HcyH, which then is either determined directly or after derivatisation. Hence, tHcy is the sum of all Hcy species (14, 25, 287). Ab, antibody; CE-LIF, capillary electrophoresis with laser-induced fluorescence detection; EC, electrochemical detection; EIA, enzyme immunoassay; Hcy-SR, Hcy-mixed disulphide; LC, liquid chromatography; UV, ultraviolet. From Refsum *et al.*, (2004)

3.2 Materials and Methods

3.2.1 Reagents

DL-Homocysteine, L-cysteine, cysteamine, and ABD-F were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Tris-(2-cyanoethyl)phosphine was obtained from Molecular Probes. (Eugene, Oregon, USA). Methanol (LiChrosolv) was obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

3.2.2 Standard solutions

DL-Homocysteine (5 mmol/l), L-cysteine (5 mmol/l), and the internal standard cysteamine (5mmol/l) were dissolved in 0.1 mol/l HCl and stored at -20°C. These stock solutions were further diluted in nanopure water to a working solution of 50 µmol/l, aliquoted and kept at -20°C until required.

3.2.3 Instrumentation

Separation and quantification was achieved on an Agilent 1100 series HPLC, (Ca, USA) (degasser G1322A, binary pump G1312A, autosampler and thermostat G1329A/30A, column thermostat G1316A, and fluorescence detector G1321A). Separations were performed on a Phenomenex C18 Luna, (Ca, USA) ODS 250mm x 4.6mm, 5 µm reversed phase column incorporating a Phenomenex securiguard guard column.

3.2.4 Chromatographic conditions

HPLC was carried out by an isocratic system modified from that of Araki & Sako, (1987), Vester & Rasmussen, (1991), and Tröbs *et al.,* (1998). These modifications involved changes in the mobile phase pH, flow rate, column temperature, and autosampler compartment temperature. The fluorescence intensities were measured with excitation at

385 nm and emission at 515 nm. The mobile phase consisted of an acetate buffer (0.1 M acetic acid – 0.1 M sodium acetate (600:1500, v/v), containing 20 ml methanol, adjusted to pH 4.35 and filtered through a 0.45 μm Sartorius filter, (eluent A); Methanol, filtered through a 0.22 μm Millipore filter (eluent B). The isocratic system was run for 20min at 85% (A) 15% (B). The column temperature was maintained at 29 °C with a flow rate of 0.7-ml/min and injection volume of 10 μl.

3.2.5 *Sample preparation*

The method has been modified from that of Ubbink *et al.,* (1991) on the basis of the chemical description provided by Araki & Sako, (1987) using ABD-F as the derivatising reagent. Whole blood was collected into Vacutainer tubes containing Na heparin from Cbl deficient sheep grazing pasture and cooled immediately on ice. The blood tubes were centrifuged as soon as possible at 3000 rpm for 10 min at 4 °C and plasma obtained. Samples were stored at –20 °C until required. All standards were prepared in pooled plasma from Cbl sufficient sheep (Na heparin anticoagulant) to avoid any possible matrix problems due to the presence of proteins, ionic strength or some other species catalysing the derivatisation step (Vester & Rasmussen, 1991). Cysteamine was added to the plasma sample or standards to achieve a final concentration of 10.0 μmol/l (30 μl of 50 μmol/l cysteamine plus 120 μl sample).

The plasma sample or standard (150 μl) was incubated with of 0.078 g/ml tris- (2-cyanoethyl)phosphine in dimethyl formamide (15 μl) for 30 min at 4 °C to complete the reduction and release of thiols. Deproteinisation with 10% trichloroacetic acid containing 1 mmol/l EDTA (150 μl) was achieved and solution centrifuged. An aliquot of supernatant (50 μl) was mixed with sodium hydroxide solution 1.5 mol/l (10 μl), borate buffer 0.2 mol/l containing 4 mmol/l EDTA, pH 9.23 (125 μl) and ABD-F 1mg/ml in borate buffer (50 μl). The solution was incubated for 5 min at 50 °C and then immediately put on ice to cool.

3.2.6 *Quantification*

A two point internal standard method was used from analysis of the plasma pool (Vester & Rasmussen, 1991) and also the pool spiked with 50 µmol/l of Hcy. The slope of the linear regression line was determined in which x = the added homocysteine and y = the ratio of the homocysteine peak area to that of the internal standard, cysteamine. The concentration of Hcy in the samples was then determined by dividing the ratio between the area of the homocysteine peak and the cysteamine peak by the slope of the linear regression line.

3.3 Results

3.3.1 *Optimisation*

Improvement was achieved by the use of an isocratic elution incorporating an acetate buffer and methanol as the mobile phases. With the inclusion of cysteamine as an internal standard it was possible to gain a much shorter run time than the previously used N- (2-mercaptopropionyl)-glycine, which eluted much later than the Hcy peak. Failure to detect a peak at the cysteamine position when omitted from plasma samples assured its suitability. Setting the column compartment temperature to 29 °C stabilised peak retention times along with an optimised injection volume of 10 µl for greater sensitivity. Stability trials on ABD-F derivatisation were conducted and it was found that a shorter incubation period than previously mentioned i.e. 5 min instead of 10 min was achieved to accomplish the complete derivatisation of Hcy and other plasma thiols.

The stability of samples (after deriviatisation with ABD-F) on exposure to light was examined and it was found that sample only remain stable if kept in the dark. Dudman *et al.,* (1996) and Tröbs *et al.,* (1998) also found similar results. If exposed to light a lowering of tHcy concentrations occurred, as noted by Ubbink *et al.,* (1999), therefore all samples were protected from light during HPLC analysis. A methanol/water programme was introduced after every sample set to clean and preserve the life of the column along with sample set run times of less than twelve hours and refrigeration of the autosampler compartment at 4 °C to maintain the integrity of the samples.

3.3.2 Specificity

Cysteine, cysteamine and homocysteine were separated with near-baseline resolution. The retention times of cysteine, cysteamine, and Hcy were 9.493, 12.072, and 13.636 min respectively. The individual peaks were identified separately by adding homocysteine, cysteine, and cysteamine to a plasma sample and observing an increase in peak area. The peak at 11.101 min is tentatively identified as cysteinyl-glycine by comparison to work carried out by Ubbink *et al.,* (1991) and Araki & Sako, (1987). See Figure 3.2 for plasma standard concentrations and Figure 3.3 showing the homocysteine concentration of a Cbl deficient sheep.

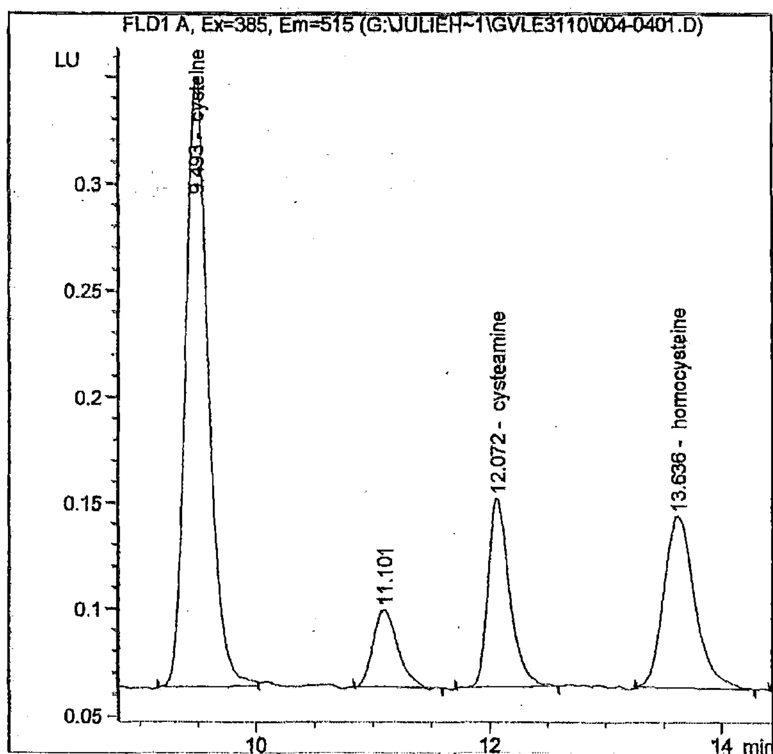


Figure 3.2 HPLC chromatogram of ABD-F derivatised plasma standard with cysteamine as an internal standard. Cysteine, cysteamine, and homocysteine standards had final working concentrations of 10 μmol/l.

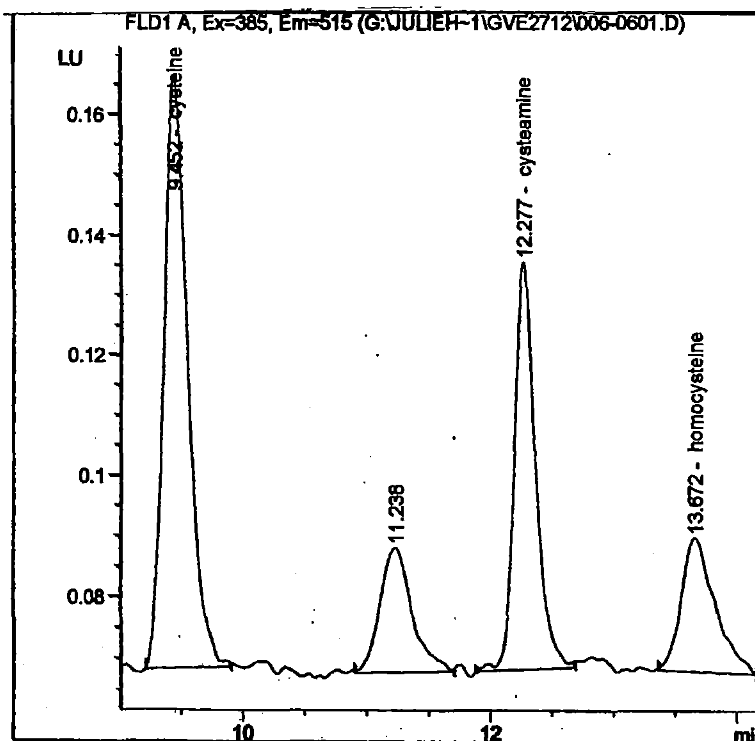


Figure 3.3 HPLC chromatogram of ABD-F derivatised plasma sample from a Cbl deficient sheep with a homocysteine concentration of 2.16 μmol/l. and cysteamine as the internal standard.

3.3.3 Assay precision

Different concentrations of Hcy were added to a plasma pool so as to increase its concentration by 0.95, 4.76, 9.52, 19.04 and 47.62 μmol/l. A linear regression analysis yielded y = 0.1372x – 0.0157 with a correlation coefficient of 0.9993. See Figure 3.4.

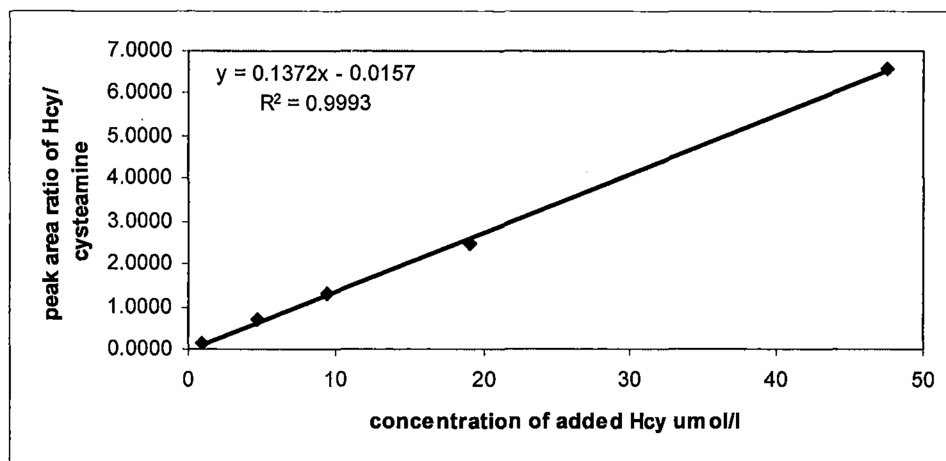


Figure 3.4 The response of the detector to increased amounts of Hcy showed linearity over the range. Where *y* is the peak area and *x* is the added amount of Hcy.

The within run precision (intraassay) was determined by dividing a plasma sample into 9 aliquots and determining the tHcy value in each aliquot. The CV value was 6.44% (mean = 2.48 ± 0.10 μmol/l Hcy). Individual plasma samples (n=4) were also taken and analysed by repeat injections x five within one run giving a CV of 6.98% (mean = 1.06 ± 0.07 μmol/l Hcy). The between run (interassay) precision of the method was estimated from repeated analysis of a pooled plasma sample (n=14) with a CV value of 6.29% (mean = 3.24 ± 0.11 μmol/l Hcy). The signal to noise ratio was determined as 0.67 for the homocysteine peak with a limit of detection of < 0.3 μmol/l.

3.3.4 *Method comparison*

Random samples from 10 sheep were analysed by the HPLC method and by an independent method based on a fluorescence polarization immunoassay incorporated into the IMx system at Canterbury Health Laboratories in Christchurch. Results showed good agreement with mean values of 3.0 and 2.8 μmol/l respectively over the range of samples analysed. A population of another set of plasma samples (n = 5), from a previous trial, within a small range of concentration were also analysed and evaluated by both laboratories. A summary of the study is shown in Table 3.1.

Table 3.1 Correlation of plasma samples (n=5) analysed by both IMx and HPLC methodology

Number of observations	Intercept	Slope	Correlation Coefficient
5	- 0.17	0.963	0.955

3.3.5 *Stability studies*

A supplementary trial was conducted to evaluate the stability of Hcy in whole blood during collection and subsequent handling focusing on issues encountered during a field experiment. The type of anticoagulant tubes used for blood collection was also examined. It has been noticed that homocysteine levels continue to rise after the sample has been taken due to ongoing Hcy metabolism in red blood cells. (Ubbink et al., 1992). The type of anti-coagulant used in blood collection tubes along with the effects of temperature, storage conditions and time of centrifugation could adversely affect the results of analysis. Therefore, considering the practical limitations of a field experiment, a

standardised protocol was devised as a result of this trial, to optimise the blood handling of samples for tHcy determination.

Trial: - Three groups containing one sheep in each group were used. Different types of anti-coagulant tubes were examined as follows using a total of thirty tubes: -

Group A tubes 1 to 10 (EDTA)

Group B tubes 1 to 10 (Sodium heparin)

Group C tubes 1 to 10 (Lithium heparin)

Blood samples were collected from the sheep into the above tubes and a protocol was designed to expose designated tubes to various temperature and centrifugation time treatments. The collected plasma samples were then analysed for tHcy concentration using the method for tHcy by HPLC analysis. See Table 3.2.

Table 3.2 Analysis of plasma samples collected into different anti-coagulant tubes and assessed for stability with time.

Tube ID	Room temp, time after collection (Hours)	Time on ice (Hours)	Centrifuge 3000rpm 10mins 4°C	Freeze plasma
Tube 1	0	0	Immediate	Yes
Tube 2	0	4	Then spin & separate	Yes
Tube 3	0	8	"	Yes
Tube 4	0	24	"	Yes
Tube 5	1	0	"	Yes
Tube 6	2	0	"	Yes
Tube 7	4	0	"	Yes
Tube 8	6	0	"	Yes
Tube 9	24	0	"	Yes
Tube 10	48	0	"	Yes

Results: - As shown in Figure 3.5, very small differences were seen between the three anti-coagulants. A rise in Hcy concentrations was seen in EDTA tube 9 and in all three types of anti-coagulant in tube 10 indicating that prolonged exposure to ambient temperature does increase tHcy levels.

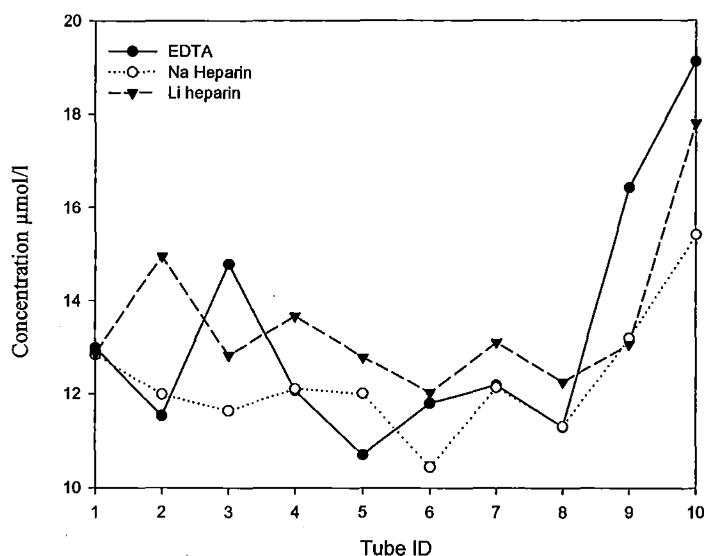


Figure 3.5 Homocysteine concentrations of plasma samples after exposure to different handling conditions.

Discussion: - As a result of these findings sodium heparin anti-coagulant tubes were chosen for blood collection along with keeping the samples on ice and centrifuging them as soon as possible to obtain plasma.

3.4. Summary

An improved method for measuring tHcy in the plasma of sheep was achieved. Optimisation of the assay included the use of an internal standard with properties close to that of Hcy with a retention time of ~12 min compared to ~30min for the previously used N- (2-mercaptopropionyl)-glycine), which ensured a significant contribution to the precision, accuracy and performance of the method. A much improved peak shape from the latter, which presented as a very broad peak, also allowed for greater exactness of quantification. The use of a sodium acetate buffer and methanol (Tröbs *et al.,* 1998) instead of a previously used phosphoric acid buffer and acetonitrile mobile phase resulted in a shorter run time of resolved peaks from 40.00 min down to 20min allowing for greater

sample throughput and improved baseline linearity and signal to noise ratio. Wavelength changes to an excitation wavelength of 385 nm and an emission wavelength of 515 nm enhanced the signal output. These enhancements ensured an improved detection limit which enabled the accurate quantification of low concentrations of tHcy found to be present in the subsequent samples. The assay was found to be robust and repeatable with stability over time.

Stability of sample storage over time was considered and six samples were analysed by IMx FPIA to look at any sample deterioration coinciding with method comparison. It was found that the samples had remained stable throughout a storage time of at least 5 years at -20°C with a correlation coefficient of 0.955 to previous results.

In developing this method deliberation of analytical range was considered with low imprecision being particularly important. Refsum *et al.,* 2004 recommended that the interassay and intraassay variation in tHcy, the bias should be <10% and imprecision CV <5%, although many methods do not fulfil these criteria. The data presented here given the low concentrations of tHcy measured, compared well with these recommendations.

In conclusion, the method performed well and meets the requirements for the accurate measurement of tHcy in the plasma of sheep. Attention to the collection, handling and storage protocol for the samples to be analysed ensured continuity in the subsequent analysis.

CHAPTER FOUR

ON-FARM SUPPLEMENTATION TRIALS

4.1 Introduction

Homocysteine (Hcy) is inextricably linked to methionine metabolism via two metabolic transmethylation pathways, one of which is dependent on cobalamin (Cbl) supply. A major part of Hcy is remethylated to methionine, using CH_3-H_4 folate as the source of methyl groups and catabolised by a cobalamin-dependent enzyme, methionine synthase; the other pathway involves the remethylation of Hcy by betaine-homocysteine methyltransferase (BHMT). Homocysteine is converted to cysteine via a vitamin B_6 dependent transsulphuration pathway, which catabolises Hcy to cysteine by way of the rate-limiting enzyme cystathionine ß - synthase. Homocysteine is exported into plasma where it circulates, mostly in its oxidised form, bound to plasma proteins.

It is known that Hcy is increased markedly in the plasma of patients with Cbl or folate deficiency and is also considered an independent risk factor for premature cardiovascular disease and homocystinuria (Ueland *et al.,* 1993), as mentioned in previous chapter. The importance of Hcy in human health is therefore recognised and the measurement of Hcy in the clinical setting becomes essential.

The recognition of Cbl deficiency in sheep is of major importance as the signs and symptoms are non-specific. Loss of production due to weight loss and anaemia, with probable compromised immune function can severely affect the animal and non-treatment may eventually culminate in death. The early diagnosis of deficiency requires investigation into the metabolic consequences of the disease to predict a response to supplementation. It is suggested that tHcy determinations could prove useful as a sensitive index for the diagnosis of Cbl status in sheep (Kennedy *et al.,* 1994), and necessary for understanding the role of Hcy in the development of Cbl deficiency.

This chapter describes for one experiment and the use of samples from a previous experiment to test the usefulness of Hcy for diagnosis of Cbl deficiency in grazing animals

4.2 Animals

The animals used for this research were sheep and their lambs from a farm with a reported cobalt deficiency. The trial was conducted *in situ* on the farm in the South Island of New Zealand. The experiments were carried out under the authority of the Lincoln University Committee on Ethics of Animal Experimentation, Lincoln, New Zealand for Trial A and the Crown Research Institutes Animal Ethics Committee, Palmerston North, New Zealand for Trial B.

4.3 Experimental design

4.3.1 Trial A

A property located in Southland, New Zealand (45°50' latitude south and 168°50' longitude east) with Mataura silt soil and known to be cobalt-deficient was used for this experiment. The pastures were traditional perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). Eighty two pregnant three year old Romney ewes were allocated into two groups of 43 and 39 ewes. The first group remained unsupplemented (UE) as controls whereas the latter had been supplemented (SE) with a Co bullet during late pregnancy twelve months previously (Gruner *et al.,* 2004). The whole mob was grazed in the same paddock.

Supplementation of lambs began one month after lambing on the 31st October 2001 (day 0) when 135 lambs were allocated into four groups in a 2 x 2 factorial design consisting of: -

1. unsupplemented ewes/unsupplemented lambs (UEUL)
2. unsupplemented ewes/supplemented lambs (UESL)
3. supplemented ewes/unsupplemented lambs (SEUL)
4. supplemented ewes/supplemented lambs (SESL)

All the lambs were weighed and 64 identified animals, equally distributed amongst four treatment groups, were blood sampled at approximately one monthly intervals until 1st May 2002 (day182). The lambs were weaned at day 89.

4.3.2 *Trial B*

Blood samples were available from a trial conducted on the same property in 1999 when it was first identified as a farm on which a response to Co or Cbl supplementation may be expected. Five week old lambs were randomly allocated into three treatment groups of thirty animals each along with an untreated group of forty seven animals. At approximately monthly intervals, 10 monitor lambs in the treated groups and the 47 lambs in the untreated group were blood sampled and all lambs weighed until 26th July 2000 (day 242). Lambs were weaned on 24th January 2000 (day 59). Treated lambs received one of three rates of intramuscular injection of microencapsulated vitamin B_{12} (SMARTShot™ B12; Stockguard, Auckland, NZ) in the following groups; - (SL 6) 6 mg, (SL 4.5) 4.5 mg, (SL 3) 3 mg on the 25th November 1999(day 0). The whole mob was grazed together in the same paddock. Data other than Hcy has been reported elsewhere (Gruner *et al.*, 2004b, Grace *et al.*, 2003).

4.4 Statistical analysis

Statistical analysis was carried out using GenStat Release 7.2 software, Lawes Agricultural Trust 2004. Trial A data was analysed for repeated measures using restricted measures likelihood with estimates of missing values. Trial B data was also analysed for repeated measures using restricted measures likelihood with estimates of missing values and data $\log_{10}$ transformed before analysis with back-transformed means presented.

4.5 Sample collection

4.5.1 Blood

Sample collection was conducted monthly by a local veterinarian. A strict sampling protocol was adhered to in order to preserve sample integrity. Animals were yarded within 15 min of sampling and 10ml of blood was collected by jugular venipuncture into sodium heparin vacutainers (140 USP Na heparin/10ml), (Becton Dickinson, USA). These samples were placed immediately on ice in an insulated box. The samples were then transported to Lincoln University for separation the following day when they were centrifuged at 3000 rpm for 10min at 4°C. The plasma was then aliquoted and stored at -20°C until analysis of tHcy, Cbl, & MMA concentration.

4.5.2 Pasture cobalt

Plucked herbage samples were taken on all occasions by stratified random sampling from the paddock which the animals were grazing for Co content determination. Any soil or root material was removed and the sample stored in a plastic bag.

Trial A

4.6 Supplementation

Cobalt bullets (PermaCo, Schering-Plough, Animal Health Ltd., Upper Hutt, New Zealand), 10g pellets each containing 2g Co as Co_3O_4, were used as the Co supplement to ewes.

Lambs in supplemented groups received an intramuscular injection of 3mg microencapsulated Cbl (SMARTShot™ B_{12}, Stockguard, New Zealand: 1 ml contains 6 mg vitamin B_{12}). The Cbl was injected into the anterior half of the neck.

4.7 Assays

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

4.7.1 Homocysteine

As described in Chapter three.

4.7.2 Vitamin B_{12} (Cobalamin)

Approximately 1ml of the plasma sample was sent via courier to the animal health laboratory at Labnet Invermay Ltd., (Mosgiel, New Zealand). The Cbl content was determined by radio-isotope dilution assay (RIDA) based on the method of Green *et al.*, (1974).

4.7.2.1 Reagents and solutions

The cyanocobalamin (Cbl) standard was prepared by dissolving CN-cbl (10mg, Sigma, V 2876, St Louis, USA) in 25 % v/v ethanol (500 ml) to obtain a 20 mg/l solution, which was then stored at 4°C in an amber glass bottle. A 5 mg/l solution was made by a 1 in 4 dilution of the above standard with 25 % (v/v) ethanol and stored at 4°C. When required a 10 μg/l solution was prepared fresh by diluting the 5 mg/l standard to 500 ml with 0.9 % (w/v) sodium chloride solution and adding a 1 % (w/v) aqueous potassium cyanide solution (50 μl). From this a 0, 1, 2, 4, 10 and 20ml aliquot were pipetted into separate 100 ml volumetric flasks and diluted to volume with a 0.9 % sodium chloride solution. The resulting concentrations were 0, 74, 148, 295, 738, and 1476 pmol/l. Aliquots of these (0.5 ml) were frozen until required for use.

The extraction buffer was a solution of 0.4 M sodium acetate titrated to pH 4.0 with (0.4 M) hydrochloric acid. The neutralising buffer was a 0.8 M solution of glycine made up in sodium chloride (0.8 M). This was titrated to pH 10.0 with 0.8 M sodium hydroxide.

The chick serum binder (CS binder) was made up in 0.9% (w/v) sodium chloride solution to determine the appropriate dilution for a 40 to 60 % binding based upon the determined unsaturated Cbl-binding capacity of the serum. The serum is diluted to the following dilutions; 1:500, 1:1000, 1:1500, 1:2500, 1:3000, 1:3500, 1:4000. Two total count, two blank and two zero standard tubes are set up for each dilution and the test procedure carried out as usual (see below) omitting the boiling stage. The total counts are read and the values calculated as a % binding of each dilution as follows;

$$\% \text{ binding} = \frac{(\text{Zero std count} - \text{Blank counts})}{\text{Total counts}} \times 100$$

The dilution giving a 40 - 60 % binding was selected and 11 ml aliquots of this dilution frozen until required.

A charcoal mixture was made by adding 40 g of activated charcoal (Sigma, St Louis, USA) to 800 ml of water, mixed and stored in a plastic bottle at room temperature until required. Mix thoroughly before use. A 1 % (w/v) bovine-albumin solution (fraction 5, Boehringer, Manheim, Germany) was prepared fresh and stirred gently until dissolved. 10 ml of this solution was added to 10 ml of activated charcoal mixture (under constant stirring) to obtain an albumin/charcoal suspension.

The dithiothreitol (DTT) (Sigma, D0632, St Louis, USA) solution was prepared by dissolving 5 g in water and making up to volume (100 ml). 1ml aliquots are prepared and frozen until required. The tracer solutions was made by combining extraction buffer with distilled water in a 1:1 ratio and for each 10 ml mixed add 600 μl of ^{57}Co-cyanocobalamin stock tracer (10 μl, 10μCi/ml, Amersham, code CT2), 10 μl of potassium cyanide (1% solution), and 100 μl of DTT (5% solution). Prepare on day of use.

4.7.2.2 Procedure

Duplicate test tubes were labelled with 'total', 'blank', and standards 'A' to 'E'. Further tubes were labelled according to the samples to be analysed. The 'blank' tubes received 200 μl of 0.9% sodium chloride solution; 200 μl of standards or sample were pipetted into the other tubes.

1000 μl (1 ml) of tracer solution was pipetted into every test tube 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 except 'total' and 'blank' which received 0.9% sodium chloride solution (200 μl) instead. The test tubes were vortexed and incubated for 1 hour at room temperature.

1000 μl (1ml) of charcoal mixture 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 measured using a Gamma counter (LKB Wallace, 1470 Wizard, Finland). Readings were recorded to the nearest 10 pmol/l for serum.

4.7.2.3 Detection and accuracy.

Detection limits for the assay were 57 pmol/l for serum and the 95% Confidence interval was ± 17 pmol/l.

4.7.3 Methylmalonic acid

The plasma samples were assayed for MMA using a modification of the method of McMurray *et al.*, (1986). This was a direct capillary gas chromatography (GC) method with adaptations in column type, injection volume and temperature ramp as described below.

4.7.3.1 Reagents and solutions

The ethylmalonic acid (EMA) internal standard (ISTD) (Sigma, E 8758, St Louis, USA) was prepared by dissolving 1.32 g/l in acetone to make a 10 mmol/l stock standard.

This was diluted with acetone to 1 mmol/l and stored at 4°C. The methylmalonic acid standard (Sigma, M 2633, St Louis, USA) was prepared in the same way, dissolving 1.18 g/l in acetone to make a 10 mmol/l stock standard. This was further diluted with acetone to 0.5 mmol/l and stored at 4°C.

A 0.5 M sulphuric acid solution saturated with sodium chloride was prepared along with a mixture of one part acetyl chloride added slowly to ten parts of butan-1-ol; prepare fresh.

A sheep injected with 2 mg hydroxycobalamin (Prolaject, Bomac Laboratories Ltd, Auckland, New Zealand) 48 hours previously provided a blood sample in order to obtain a pooled plasma sample, which was aliquoted and frozen at -20°C until required for standards.

4.7.3.2 Procedure

Plasma (250 μl) was placed into glass quickfit tubes (3 ml) and EMA (50 μl) ISTD added. Acetone (250 μl) was added to the tube whilst vortexing followed by 0.5 M sulphuric acid (1.0 ml) in order to denature and precipitate the protein. Ethyl acetate (0.5 ml) was added to extract EMA and MMA into the ethyl acetate phase and all tubes stoppered and vortexed for 25 sec.

The samples were then centrifuged at 3000 rpm for 10 min at 4°C and the ethyl acetate layer pipetted off into a glass quickfit tube (1 ml). The ethyl acetate extraction was then repeated and the two extracts combined. The ethyl acetate was evaporated to dryness under nitrogen (oxygen free), using a multi-needle manifold. The acetyl chloride/butan-1-ol mixture (50 μl) was added to the residue, the samples vortexed and then placed in a water bath (70°C) for 20 min.

After cooling hexane (150 μl) and nanopure water (400 μl) were added, the tubes stoppered and vortexed. When the layers had separated the upper layer was transferred to a glass autosampler vial fitted with a limited volume insert, capped and analysed on the GC as soon as possible. All plasma samples were done in duplicate.

Alongside the plasma samples three standards and a solvent blank were prepared. For the standards, MMA (50 μl) standard and 200 μl nanopure water were used and another MMA (50 μl) and 200 μl pooled plasma; ISTD (50 μl) was added to these standards. An EMA standard (50 μl) was also prepared diluted with pooled plasma (200 μl). The solvent blank contained nanopure water (250 μl) with no ISTD added. The standards are taken through the complete analytical procedure.

4.7.3.3 GC analysis

Changes in the GC methodology from the original paper by McMurray *et al.*, (1986) were initiated. These included a reduction in injection volume from 10 μl to 5 μl, GC column change from a 100% dimethylpolysiloxane to a 5%-phenyl-95%-dimethylpolysiloxane (slightly more polar), increased flow rate from 1.5 ml/min to 4.2 ml/min, and changes in the temperature ramp from 150°C to 160°C with initial temperature hold withdrawn.

Standard or sample (5 μl) were injected onto a HP-5MS (5%-phenyl-95%-dimethylpolysiloxane) cross- linked capillary column 30 m x 0.32 mm x 0.25 μm (Agilent Technologies, USA) using an autosampler fitted to a HP6890 gas chromatograph. The injector had a split ratio of 10:1 with a temperature of 200°C. Flow rate was 4.2ml/min in constant makeup mode with a nominal initial pressure of 146.7 kPa. The initial oven temperature was 120°C with a temperature ramp rate of 5°C/min to a final temperature of 160°C with a post run of 5 minutes at 280°C. The detector was a flame ionisation detector with a temperature of 280°C.

At the beginning and end of each analytical run a set of standards of combined MMA and EMA for calculation of response factor and to quantify methylmalonic acid using the GC system's own integrator and report generator were placed. The standards not only monitor the performance of the method and inter-run precision but the addition of an internal EMA standard compensates for losses and variations accumulated during analysis. The use of pooled plasma for the standards was to alleviate any matrix problems. The mean of the standards was used to find the relative response factor which was then used to calculate the amount of MMA μmol/l in each sample as follows.

Calculation.

a. Response factor: EMA area/MMA area x 0.5 = RRF

RRF x 200 = multiplication factor

b. MMA in sample: MMA area/EMA area x multiplication factor

= amount MMA μmol/l

Methylmalonic acid was added to a plasma sample so as to increase its concentration by 0.5, 1, 5, 10, 20, 50, 100,150 and 200 μmol/l. Linear regression analysis of the response gave $y = 0.9314x + 0.8046$, with $R^2 = 0.9994$, where y is the ratio of the MMA peak area to internal std peak area, and x is the added MMA (endogenous MMA was subtracted from the MMA measured). The standard deviation of estimation was 0.88 μmol/l and the detection limit defined as 0.31 μmol/l.

4.7.4 Cobalt

The pasture samples were analysed by Hill Laboratories, (Hamilton, New Zealand) using inductively-coupled plasma source mass spectroscopy (ICP-MS). The plant material was oven dried at 62°C overnight and ground to pass through a 1.0 mm screen. Aliquots (1 g) were then weighed into 30 ml digestion tubes and a nitric/perchloric acids digestion carried out. This involved 12 ml of 5:1 HNO_3:$HClO_4$, three to five anti-bumping granules and one drop of kerosene added to the sample and agitated to thoroughly wet the plant material. The tubes were then transferred to the digestion block where they were heated for 5 hours using a temperature ramp up to 220°C. The samples were cooled to room temperature then 18.5 ml of water was added to each tube and then vortexed. Analysis was then carried out via ICP-MS. This method was based on the ones described by Clinton, (1979) and the Analytical Methods Committee, (1979) and has a coefficient of variation (COV) of 7%.

Trial B

4.8 Supplementation

Lambs in supplemented groups received an intramuscular injection of 3, 4.5 or 6 mg of microencapsulated Cbl (SMARTShot™ B_{12}, Stockguard, New Zealand: 1 ml contains 6 mg vitamin B_{12}). The Cbl was injected into the anterior half of the neck.

4.9 Assays

Assay protocols for MMA and Cbl were the same as described for Trial A.

4.9.1 Homocysteine assay

The plasma samples were assayed for tHcy based on the method developed by Vester and Rasmussen, (1991) for HPLC and precedes the method developed for Trial A.

4.9.1.1 Reagents and solutions

The N-(2-mercaptopropionyl)-glycine, ISTD, (7 mmol/l) and DL-homocysteine (7 mmol/l), both from Sigma-Aldrich Co. (St. Louis, MO, USA), were dissolved in 0.1 mol/l HCL and stored at -20°C until required. They were prepared fresh monthly. Tris-(2-cyanoethyl)phosphine (0.078 g/ml), Molecular Probes. (Eugene, Oregon, USA) was dissolved in dimethyl formamide and prepared fresh daily. Trichloroacetic acid (TCA) 10% containing ethylenediaminetetra- acetic acid disodium salt (Na_2EDTA 1 mmol/l) was prepared and stored at 4°C along with a borate buffer (pH 9.23) containing Na_2EDTA (4 mmol/l) with equal volumes of boric acid (0.1 mol/l) and sodium tetraborate (borax) (0.1 mol/l).

ABD-F (1 mg ml) dissolved in borate buffer, Molecular Probes. (Eugene, Oregon, USA) was light sensitive in solution and required to be protected from light and stored under refrigeration. This solution had a limited shelf life of approximately one week.

Two HPLC solvents were prepared. Solvent A consisted of phosphoric acid (0.15 mol/l) containing triethylamine (50 mmol/l) pH 2.1 and solvent B, which was acetonitrile.

4.9.1.2 Procedure

A solution of Hcy and ISTD (70 μmol/l) was prepared by diluting the previously prepared stock standards with borate buffer (100 μl of each in 10 ml). An ISTD (70 μmol/l) was also prepared in the same way.

Plasma (125 μl) was placed into eppendorf tubes, four of which were identical plasma for standards. Into two of the plasma standards pipette 50 μl of Hcy and ISTD mix. Into the other two plasma standards and each of the samples pipette 50 μl ISTD. Tris (12.5 μl) was added and the samples vortexed and then placed at 4°C for 30 min.

Trichloroacetic acid (125 μl) was added to all tubes, vortexed, then centrifuged at 12,000 rpm for 5min. Clear supernatant (25 μl) was taken and borate buffer (50 μl) added to each tube. ABD-F (25 μl) was added, vortexed, then incubated (50°C) for 10 min and afterwards cooled in crushed ice.

The samples were filtered into a limited volume HPLC vial insert, using a Phenomenex (AFO-3368) filter (0.45 μm).

4.9.1.3 HPLC analysis

Standard or sample (15 μl) were injected onto a Phenomenex C18 Prodigy, (Ca, USA) ODS 250 mm x 4.6 mm, 5 μm reversed phase column incorporating a Phenomenex securiguard guard column using an Agilent 1100 series HPLC as described in Chapter 4. A flow rate of 1 ml/min was employed using a solvent composition of 85% A, 15% B and the isocratic system had a run time of 40 min. Fluorescence detection was used with

excitation at 380 nm and emission at 510 nm. Column temperature and autosampler temperature were not controlled.

At the beginning and end of each analytical run a set of standards were included for calculation of Hcy using the HPLC system's own integrator and report generator. Quantification was achieved as described in Chapter 3.

4.9.1.4 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%. The detection limit for a sample with a signal-to-noise ratio of 4:1 was 1 µM. Different concentrations of Hcy were added to serum samples so as to increase its concentration by 0, 1, 10, 50, 100 and 500 µM. A linear regression analysis gave $y = 0.024x - 0.0824$, $R^2 = 0.9952$, where y is the ratio of the homocysteine peak area to internal standard peak area, and x is the added Hcy.

4.10 Results

Trial A

4.10.1 Cobalt

Herbage cobalt concentrations are given in Figure 4.1. Concentrations showed large variation during the experimental period. On day 0 Co levels were 0.12 μg/g DM but quickly fell to half that during the first month of the trial. Large fluctuations were recorded thereafter with increases to 0.21 μg/g DM at day 89, before decreasing to 0.05 μg/g DM at day 126. At the end of the trial Co concentrations returned to levels recorded on day 89.

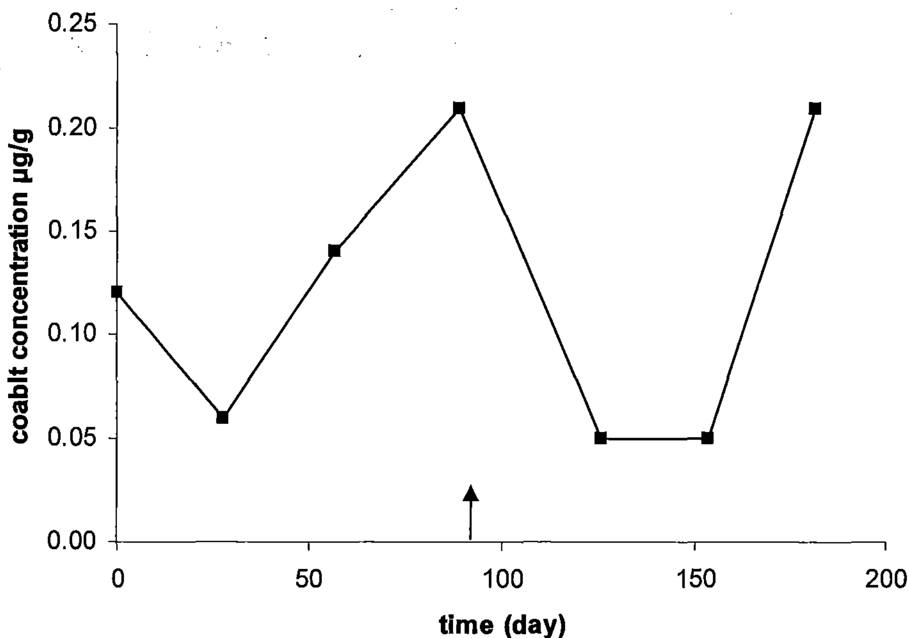


Figure 4.1 Herbage cobalt concentration during days 0 to 182. Lambs were weaned on day 89 (↑).

4.10.2 Liveweight

Mean liveweights of the lambs are given in Figure 4.2. There was a highly significant lamb treatment x time interaction ($p < 0.001$) reflecting a greater increase in liveweight in supplemented lambs, compared to their unsupplemented counterparts, regardless of ewe treatment. Differences between the four groups were non significant from day 28 to day 89 except for a significant difference between groups SESL and UEUL ($p > 0.005$) on day 89. Highly significant differences in liveweight between supplemented and unsupplemented lambs ($p < 0.001$) were seen from day 126 onwards. The liveweight of supplemented lambs increased linearly during this period compared to the lack of weight increase in unsupplemented lambs.

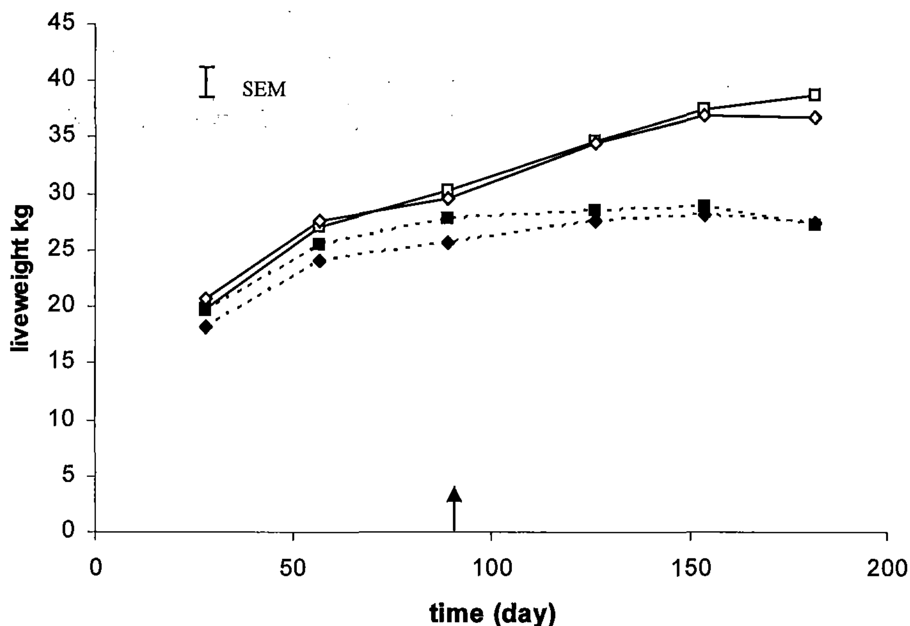


Figure 4.2 Mean liveweights of supplemented lambs suckling supplemented ewes (SESL, □), unsupplemented lambs suckling supplemented ewes (SEUL, ■), supplemented lambs suckling unsupplemented ewes (UESL, ◇), and unsupplemented lambs suckling unsupplemented ewes / (UEUL, ◆). Lambs were weaned on day 89 (↑).

4.10.3 Vitamin B_{12} (cobalamin)

Changes in mean Cbl concentration in the lambs are shown in Figure 4.3. There was a significant ewe treatment x time interaction ($p < 0.05$) and a highly significant lamb treatment x time interaction ($p < 0.001$) reflecting the increase in Cbl concentration in lambs both influenced by ewe and subsequent lamb supplementation and return of values to pre-supplementation levels. On day 0 lambs from supplemented ewes had significantly higher Cbl concentrations ($p < 0.05$). On day 28 SESL lambs had greater ($p < 0.001$) concentrations of Cbl in plasma than their UESL contemporaries (2775.7 vs. 837.6 pmol/l). Although concentrations had declined by day 28 supplemented lambs still had significantly ($p < 0.05$) higher Cbl concentration compared to their unsupplemented counterparts, irrespective of ewe treatment. No significant difference between any treatment groups was seen from day 57 onwards.

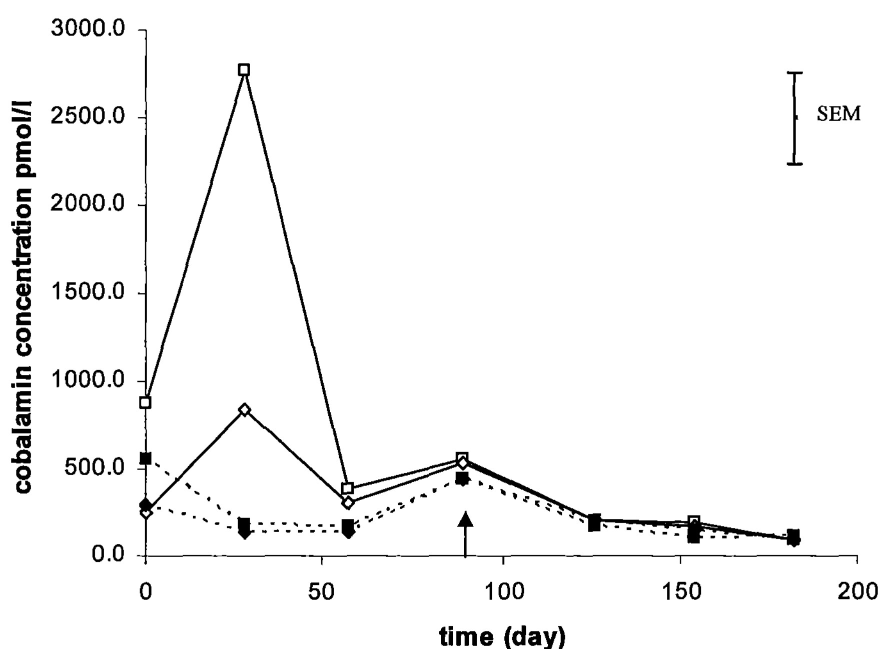


Figure 4.3 Mean plasma vitamin B_{12} (Cbl) concentrations in supplemented lambs suckling supplemented ewes (SESL, □), unsupplemented lambs suckling supplemented ewes (SEUL, ■), supplemented lambs suckling unsupplemented ewes (UESL, ◇), and unsupplemented lambs suckling unsupplemented ewes / (UEUL, ◆). Lambs were weaned on day 89 (↑).

4.10.4 Methylmalonic acid

Mean plasma MMA concentrations are given in Figure 4.4. There was a highly significant ewe treatment x lamb treatment x time interaction ($p < 0.001$) reflecting a greater increase in plasma MMA in unsupplemented lambs, compared to their supplemented lamb counterparts, with unsupplemented lambs from unsupplemented ewes showing the greatest increase. There was no difference between the four groups on day 0 with MMA concentrations being between 1 and 2 μmol/l. On day 28 a significant difference was seen ($p < 0.05$) with a lamb treatment effect apparent due a rise in MMA levels in the unsupplemented groups. However, on day 57 a highly significant ewe treatment effect was detected ($p < 0.001$) between UEUL and SEUL reflecting a larger increase in mean MMA concentrations (to values of 42.5 vs.16.0 μmol/l) in lambs from unsupplemented ewes. A significant ewe treatment x lamb treatment interaction ($p < 0.05$) was seen across the four groups with minimal MMA increases seen in supplemented lambs, whereas the greatest treatment effect ($p < 0.001$) was found between the UEUL and SESL groups, with a mean difference of 39 μmol/l at day 57.

At weaning, day 89, MMA concentrations in all four groups had fallen to levels similar to those on day 28 and then showed a gradual rise through to day 126. A highly significant ewe treatment x lamb treatment interaction ($p < 0.001$) was seen on day 154 with SEUL having a greater increase in MMA than the UEUL counterpart. At this time a significant lamb treatment effect ($p < 0.05$) was seen with greater MMA concentrations in unsupplemented (73.3 & 32.1 μmol/l) compared to supplemented lambs (19.7 & 9.4 μmol/l). By day 182 a fall in MMA concentration had occurred although a significant difference ($p < 0.05$) still remained between unsupplemented and supplemented lambs.

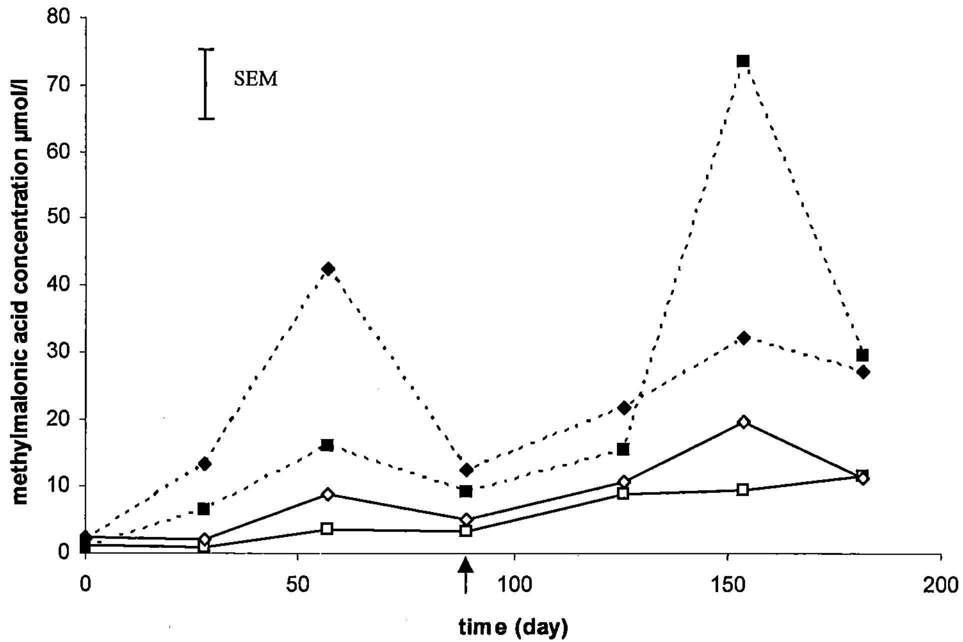


Figure 4.4 Mean plasma methylmalonic acid (MMA) concentrations in supplemented lambs suckling supplemented ewes (SESL, □), unsupplemented lambs suckling supplemented ewes (SEUL, ■), supplemented lambs suckling unsupplemented ewes (UESL, ◇), and unsupplemented lambs suckling unsupplemented ewes/ (UEUL, ◆). Lambs were weaned on day 89 (↑).

4.10.5 Homocysteine

Mean plasma tHcy concentrations are given in Figure 4.5. There was a highly significant ewe treatment x lamb treatment x time interaction ($p < 0.001$) due to higher ($p < 0.001$) concentrations of Hcy in lambs from supplemented ewes on day 0 than in lambs from unsupplemented ewes (4.0 vs. 2.0 µmol/l) and a trend for unsupplemented lambs to have higher tHcy values than supplemented lambs in the later stages of the trial. At other stages of the trial there were no consistent differences between groups and, throughout, concentrations of all groups remained within the narrow range of 1.5 – 3 µmol/l.

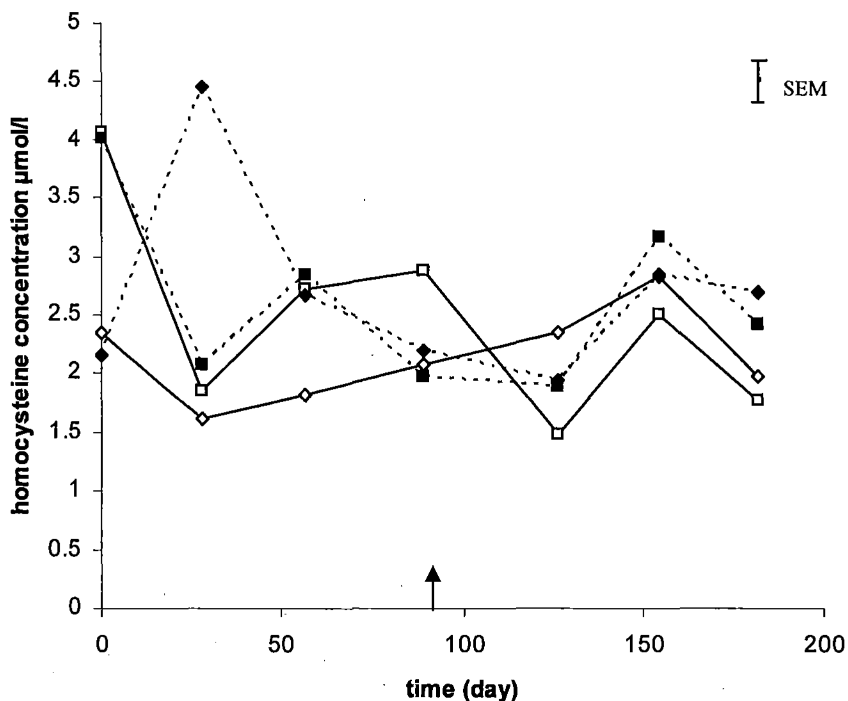


Figure 4.5 Mean plasma total homocysteine (tHcy) concentrations in supplemented lambs suckling supplemented ewes (SESL, □), unsupplemented lambs suckling supplemented ewes (SEUL, ■), supplemented lambs suckling unsupplemented ewes (UESL, ◇), and unsupplemented lambs suckling unsupplemented ewes / (UEUL, ◆). Lambs were weaned on day 89 (↑).

Trial B

4.10.6 Liveweight

Mean liveweights of the lambs are given in Figure 4.6. There was a highly significant lamb treatment x time interaction ($p < 0.001$) reflecting a greater increase in liveweight in animals from the supplemented groups, with a difference of ~ 10.0 kg between supplemented and unsupplemented lambs at the end of the trial. Liveweights were similar on day 0, although from day 25 onwards liveweight of supplemented groups tended to rise faster than that of their unsupplemented counterpart. At weaning, from day 59 onwards, liveweight gain in supplemented groups continued as weight gain in the unsupplemented group became static, with a liveweight gain of 177 and 11 g/day in supplemented and unsupplemented groups, respectively, thereafter.

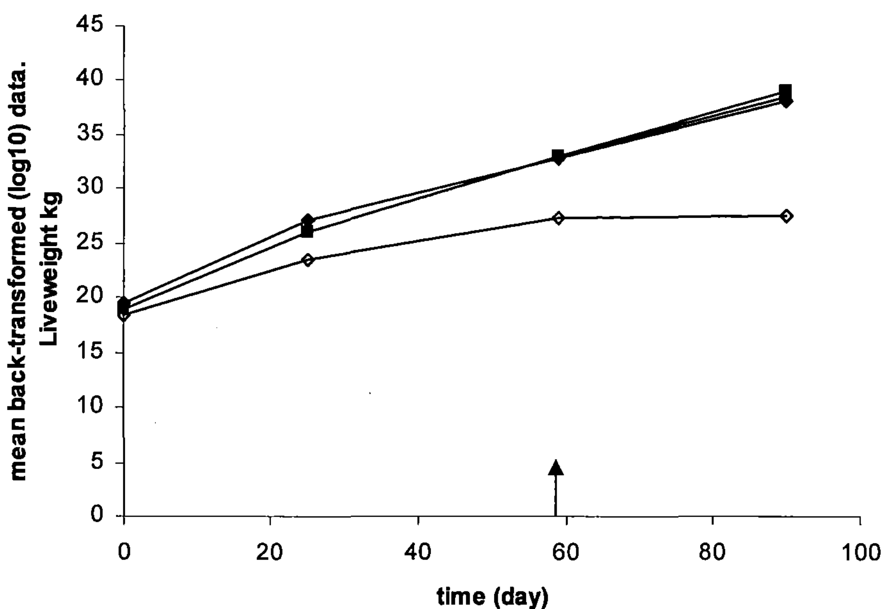


Figure 4.6 Mean liveweights of lambs supplemented on day 0 with 6 mg SMARTShotTH Cbl (SL6, ■), lambs supplemented with 4.5 mg SMARTShot^{TM} Cbl (SL4.5, □), lambs supplemented with 3mg SMARTShot^{TM} Cbl (SL3, ♦), and unsupplemented lambs (UL, ◊). Lambs were weaned on day 59 (↑).

4.10.7 *Vitamin B_{12} (cobalamin)*

Changes in mean Cbl concentrations in the lambs are shown in Figure 4.7. There was a highly significant lamb treatment x time interaction ($p < 0.001$) reflecting the initial increase then subsequent decline in concentrations in supplemented lambs to between 163.0 and 251.0 pmol/l. Concentrations in the untreated group remained between 117.0 and 177.0 pmol/l. No significant difference was seen between groups at day 0 with Cbl concentrations at similar levels. At day 25 animals from the supplemented groups had significantly higher Cbl values as compared to their unsupplemented counterpart, which fell slightly. The SL3 group had lower Cbl concentration than the other two supplemented groups (SL6 & SL4.5), reflecting their higher dose of Cbl. By weaning, day 59, Cbl concentrations in the supplemented groups had tended to fall as Cbl levels in the unsupplemented group rose. Concentrations of Cbl across all four groups by day 90 were similar with the unsupplemented lamb group values remaining the lowest.

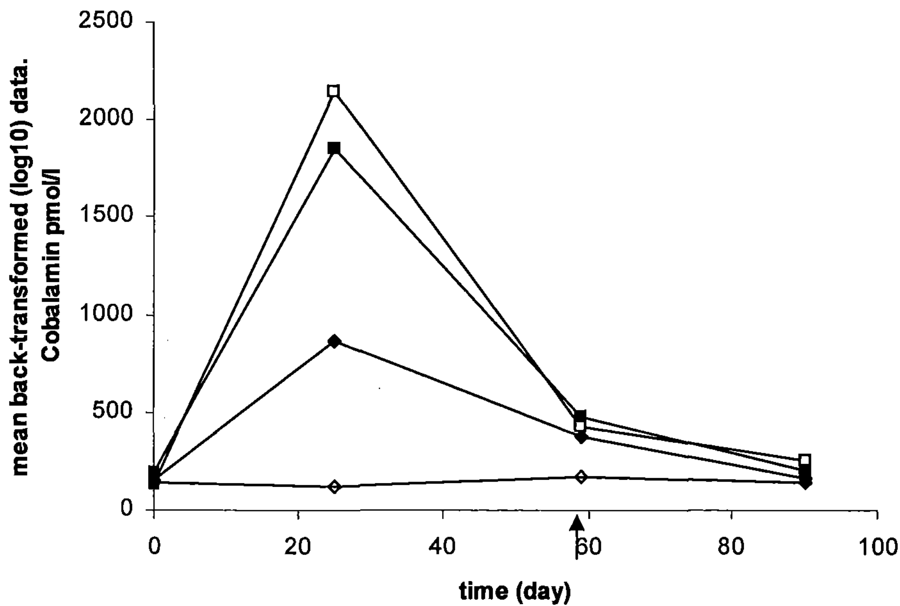


Figure 4.7 Mean plasma vitamin B_{12} (Cbl) concentrations in lambs supplemented on day 0 with 6 mg SMARTShotTH Cbl (SL6, ■), lambs supplemented with 4.5 mg SMARTShot^{TM} Cbl (SL4.5, □), lambs supplemented with 3mg SMARTShot^{TM} Cbl (SL3, ♦), and unsupplemented lambs (UL, ◊). Lambs were weaned on day 59 (↑).

4.10.8 Methylmalonic acid

Mean plasma MMA concentrations are given in Figure 4.8. There was a highly significant lamb treatment x time interaction (p <0.001) reflecting the initial rise of concentration in untreated lambs from 9.6 through to 26.2 µmol/l by day 90, compared to a decrease in treated sheep. Concentrations of MMA were similar across all four groups on day 0. On day 25 MMA values in the unsupplemented group were 18.0 µmol/l and greater than their supplemented counterparts (0.7, 0.8 & 0.9 µmol/l), with MMA levels in the supplemented groups remaining between 0.7 and 6.0 µmol/l throughout the trial with no significant difference seen between the three supplemented groups.

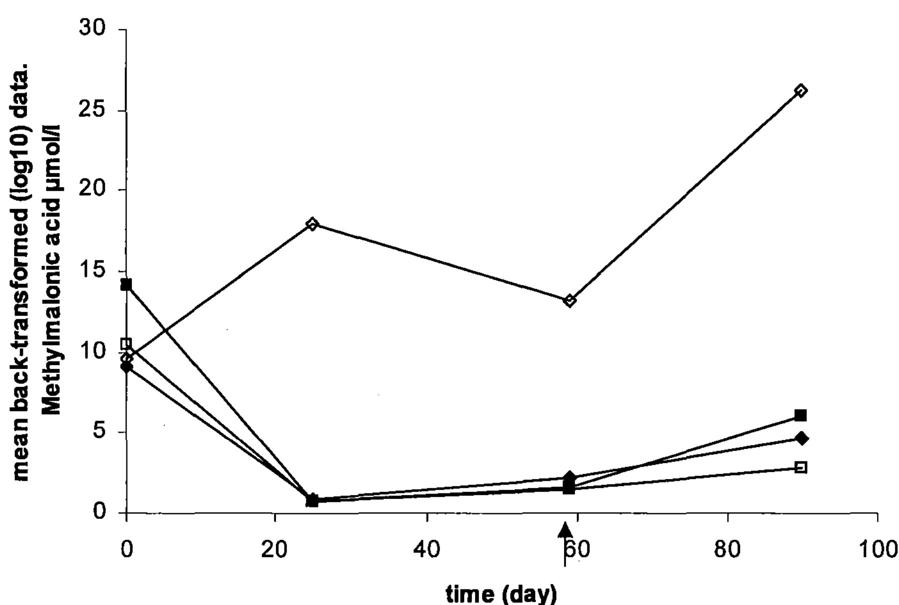


Figure 4.8 Mean plasma methylmalonic acid (MMA) concentrations in lambs supplemented on day 0 with 6 mg SMARTShotTH Cbl (SL6, ■), lambs supplemented with 4.5 mg SMARTShot^{TM} Cbl (SL4.5, □), lambs supplemented with 3mg SMARTShot^{TM} Cbl (SL3, ♦), and unsupplemented lambs (UL, ◊). Lambs were weaned on day 59 (↑).

4.10.9 Homocysteine

Mean plasma tHcy concentrations are given in Figure 4.9. There was a highly significant lamb treatment x time interaction (p <0.001) reflecting an effect of supplementation with unsupplemented lambs having higher tHcy concentrations than their supplemented lamb counterparts from day 0 until day 90. At day 0 lambs from each of the four groups had varying tHcy concentrations with unsupplemented lambs having the highest tHcy values followed by animals from SL3 group (12.7 & 9.0 µmol/l respectively, compared to 5.3 & 4.0 µmol/l for the remaining supplemented lambs groups). Concentrations of tHcy in plasma from lambs in the unsupplemented lamb group remained higher than their supplemented counterparts through to day 90. At day 90 the unsupplemented lamb group tHcy values declined to similar values as their supplemented contemporaries i.e. 6.4 vs. 6.4, 4.0, & 5.0 µmol/l in UL, SL3, SL4.5 & SL6 respectively. Although this trial extended to day 242 there were many missing values due to animals leaving the trial due to welfare issues and therefore statistical analysis beyond day 90 was not possible. From day 90 though mean values in the unsupplemented lamb group remained low i.e. 6.0 µmol/l through to the end of the trial with a transient rise to 10

μmol/l seen at day 186. Cobalamin levels in the unsupplemented group reflected this trend and remained below 200 pmol/l until day 242 when an increase to 300 pmol/l was recorded. Methylmalonic acid concentrations rose to 75 μmol/l at day 154 before declining to 12 μmol/l at the end of the trial. Mean tHcy concentration in the supplemented groups remained similar to day 90.

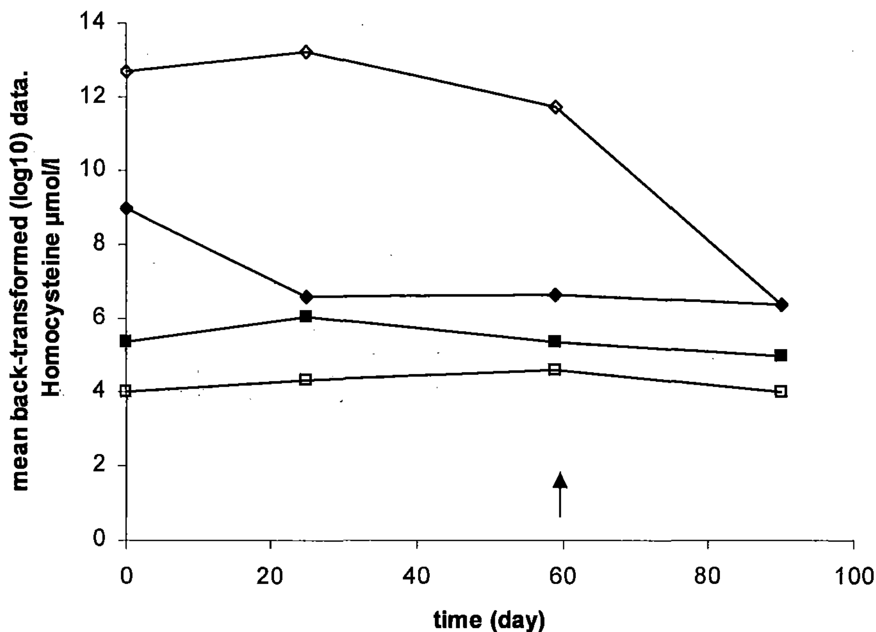


Figure 4.9 Mean plasma total homocysteine (tHcy) concentrations in lambs supplemented on day 0 with 6 mg SMARTShotTH Cbl (SL6, ■), lambs supplemented with 4.5 mg SMARTShot^{TM} Cbl (SL4.5, □), lambs supplemented with 3mg SMARTShot^{TM} Cbl (SL3, ♦), and unsupplemented lambs (UL, ◊). Lambs were weaned on day 59 (↑).

Six animals from the unsupplemented lamb group (UL) were selected and their individual Cbl, MMA and tHcy plasma concentrations examined in order to track the parameters of a Cbl deficient state. These animals had been removed from the trial after day 90 when their weight loss indicated that they were unlikely to survive and were supplemented with a vitamin B_{12} SMARTShot^{TM} injection. Although MMA concentration clearly reveals a response to the severity of the deficiency and therefore recorded elevated levels, tHcy values gradually fell as the severity of the Cbl deficiency progressed. These changes are shown in Figure 4.10.

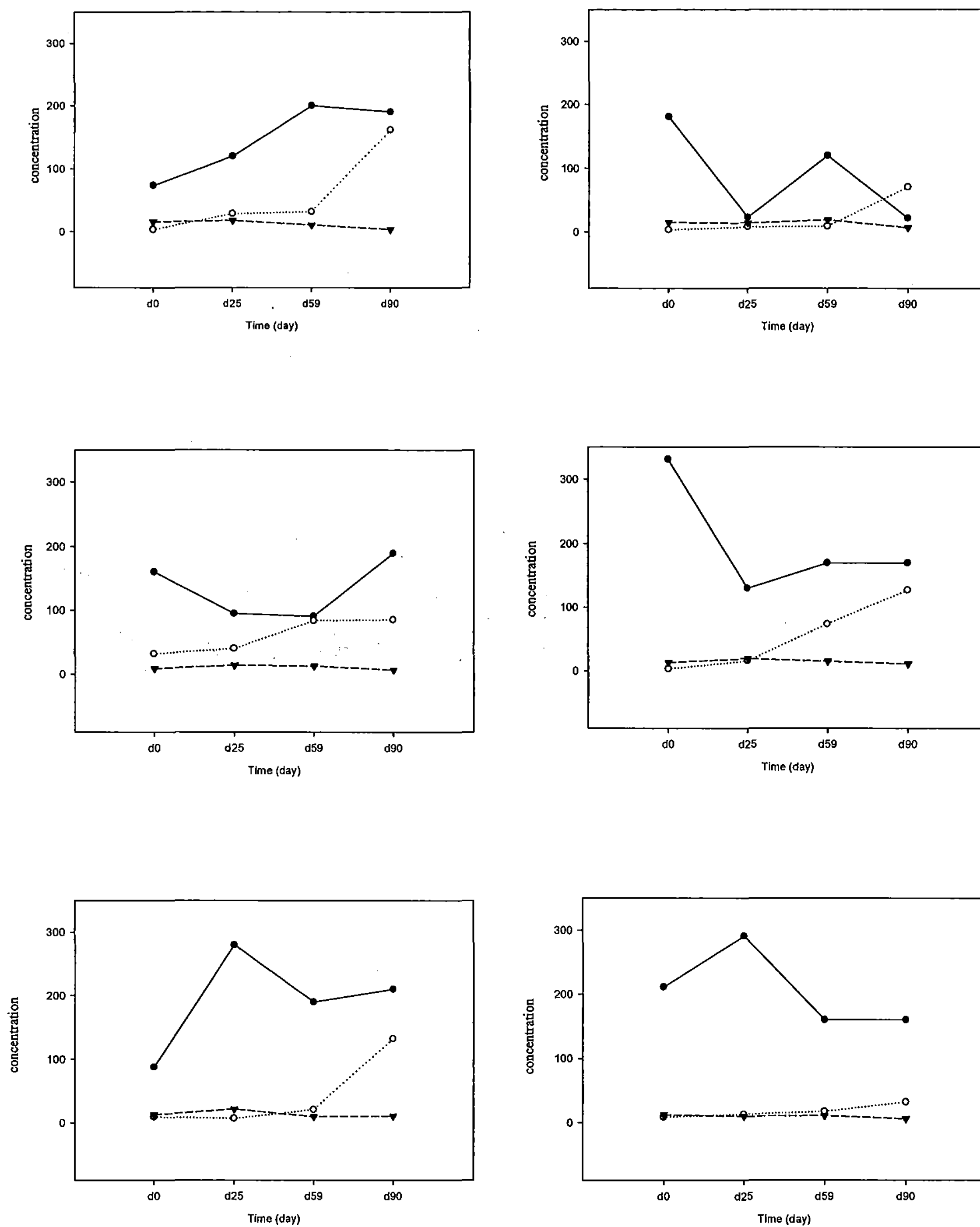


Figure 4.10 Concentrations of Cbl, pmol/l (●), MMA, µmol/l (○), & tHcy, µmol/l (▼) from six animals taken from the unsupplemented group (UL) and removed after d90 due to welfare issues.

4.11 Discussion

The research findings presented here suggest that measurement of homocysteine concentration will not enhance the diagnosis of vitamin B_{12} deficiency in sheep at pasture over that obtained by measurement of herbage Co, plasma MMA, Cbl or even the simple measurement of liveweight change.

Total homocysteine values showed a clear division between lambs from supplemented and unsupplemented ewe groups at the start of Trial A with an almost two fold greater concentration in plasma tHcy in lambs from supplemented ewes suggesting an influence of ewe Cbl status on tHcy values. Small fluctuations thereafter, appeared not to be influenced by the Cbl/MMA status of the lambs and therefore cannot be interpreted as a meaningful guide to the deficient state of the animal. Given these small changes in tHcy concentration it can be assumed that no change was observed through this metabolic pathway. The concentrations observed (ranging from 1.5 to 4.5 µmol/l) did tend to be lower than concentrations observed in the normal animals in the literature of Kennedy *et al.,* (1992) (10.1 ± 0.7 µmol/l), Kennedy *et al.,* (1994) (37.8 ± 3.7µmol/l), and Vellema *et al.,* (1999) (~ 9.4 µmol/l).

This lack of clear change in plasma Hcy was surprising, given the severity of the deficiency experienced by these lambs. Mean Cbl levels in the UEUL (142 pmol/l) and SEUL (148 pmol/l) groups fell well below the suggested Cbl reference range for deficiency of ≤200 pmol/l for ~ 12 weeks and MMA concentration was elevated into the deficient range of >13 µmol/l as suggested by Gruner *et al.,* (2004a) for the majority of the trial. It is acknowledged that the herbage Co levels fluctuated markedly around the time of weaning and at the end of the trial period, but this had only a transitory effect on Cbl and MMA values. Kennedy *et al.,* (1992) detected very high concentrations of tHcy in Co deficient sheep, albeit in a controlled environment. Concentrations exceeded 70 µmol/l towards the end of the trial period of 28 weeks, with Cbl levels below normal levels (220 pmol/l) by week six. Methylmalonic acid concentrations were not measured as a comparison in this trial. In a previous trial (Kennedy *et al.,* 1990) demonstrated increasing levels of MMA to 140 µmol/l by day 140 against Cbl concentrations below 220 pmol/l by about day 53 in sheep on a Co barley-based diet with very low Co concentrations of

0.0045 & 0.0042 μg/g respectively. By these criteria an increase in Hcy concentrations would have been anticipated in the present sheep. See Figure 4.11

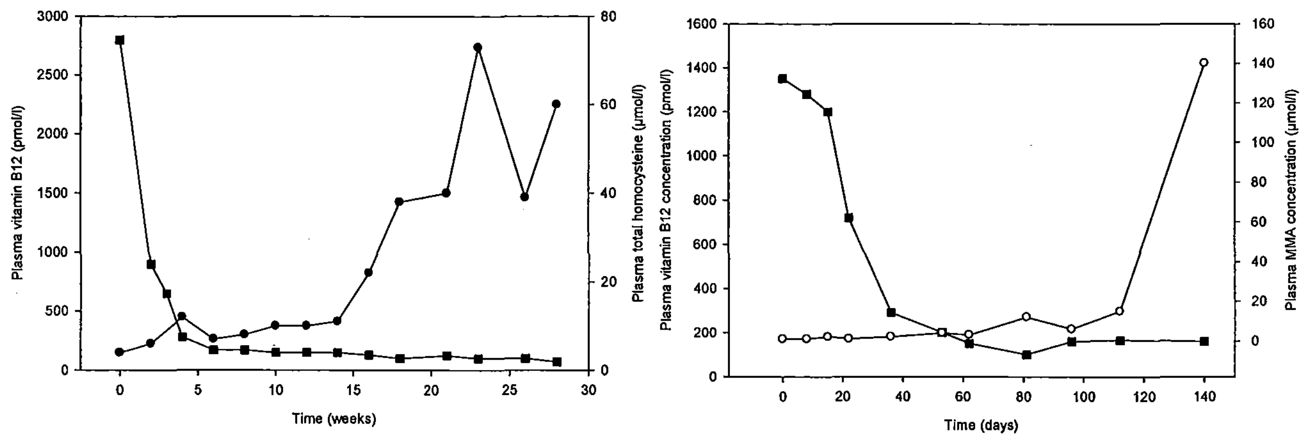


Figure 4.11. Changes in concentrations of plasma Cbl (■), plasma tHcy (●) and plasma MMA (○) in lambs fed on a Co deficient whole barley diet. Values are means. Graphs reproduced from Kennedy *et al.,* (1992 & 1990).

Vellema *et al.,* (1999) also reported elevated Hcy concentrations in Co deficient sheep at pasture, compared to supplemented contemporaries. However, they observed that initialiy Hcy concentration was higher in non-supplemented animals (~15 vs. 12 μmol/l) in a trial conducted in 1992 at a time before a difference in plasma MMA concentration became apparent. As MMA concentration rose in their unsupplemented animals Hcy levels actually fell with the difference between supplemented and unsupplemented groups remaining constant at ~3 μmol/l at the end of the trial. These results are uninterpretable in relation to the development of the Cbl deficiency since these similar reductions occurred at a time when MMA concentrations were rising above 80 μmol/l in unsupplemented sheep. A second experiment they describe and which was carried out in 1993 showed lower initial values of Hcy in an unsupplemented group. Concentrations in both groups were then seen to rise for a short time but Hcy values then declined from 14 to 11 μmol/l even though MMA concentration remained above accepted reference ranges for normality in unsupplemented sheep.

There is no question that severe Cbl deficiency and large response to supplementation occurred during the present research project. This is shown in major

differences in the mean liveweight gain of supplemented and unsupplemented lambs. At the end of the trial the difference in liveweight was 10kg, reflecting average growth rates of 66 and 111 g/day in unsupplemented and supplemented lambs respectively. Previous trial data from this property, which is known for its low pasture Co concentrations, has shown a response in liveweight gain to Cbl supplementation with Grace *et al.,* (2003) stating an increased group mean growth rate of at least 116g/day in lambs supplemented with 3 mg microencapsulated Cbl at tailing compared to unsupplemented controls on pasture concentrations of 0.05 to 0.07 mg Co/kg DM, and this is the data provided in Trial B.

Evidence of the severity of the Cbl deficiency in the unsupplemented animal groups is provided in the changes in plasma MMA and Cbl shown in Figure 4.12. These clearly highlight the inverse relationship that existed between Cbl and MMA concentrations and the duration of the maintenance of MMA and Cbl concentration in plasma outside the accepted normal range. This provides justification that the present unsupplemented animals were indeed severely Cbl deficient for periods of at least three months. The lack of response in tHcy concentration clearly indicates the lack of value of this metabolite in diagnosis of Cbl deficiency.

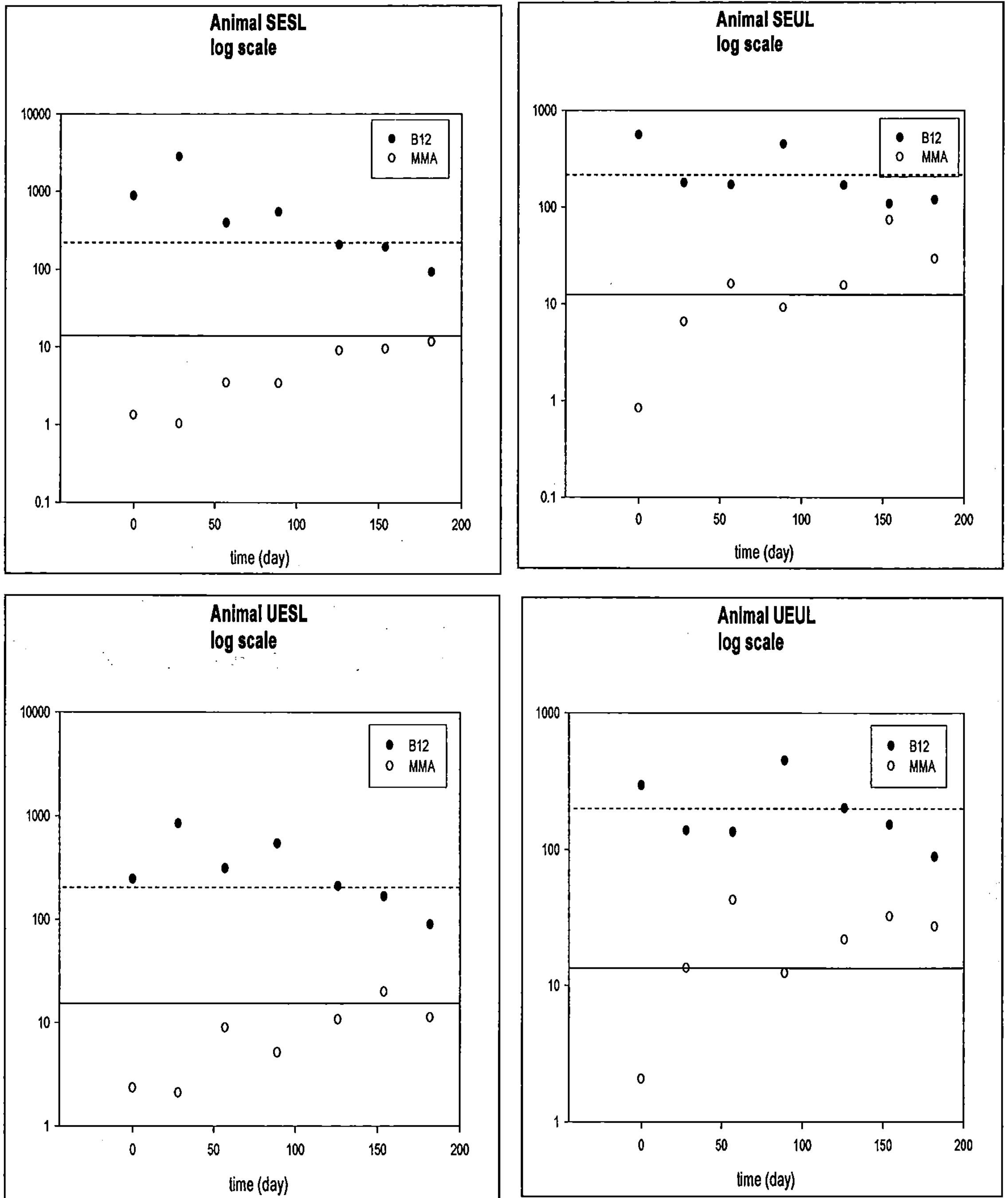


Figure 4.12. Mean cobalamin (B_{12}) ● and methylmalonic acid (MMA) ○ plasma concentration relationships within the four lamb groups namely supplemented ewe/supplemented lamb (SESL), unsupplemented ewe/supplemented lamb (UESL), unsupplemented ewe/supplemented lamb (UESL), unsupplemented ewe/unsupplemented lamb (UEUL).

Represents B12 deficient status reference line (200 pmol/l) ----------

Represents MMA deficient status reference line (13 µmol/l) ————

This, therefore, raises the question as to why this would be the case. Data from the previous 1999 trial supported these findings with no rise in tHcy concentration seen in animals clearly severely Cbl deficient, as judged by response to Cbl supplementation. Initial Hcy values were higher in the unsupplemented lamb group with a mean of 11.0 μmol/l, but this was before any supplementation was undertaken. It suggests a high degree of individual animal variation in normal Hcy concentration, and suggests that randomisation should have occurred after establishment of individual values. As the deficiency progressed and MMA values rose to 18.0 μmol/l by day 25, Hcy values were seen to fall correspondingly over time to similar levels to those found in the supplemented lamb groups by day 90. This variation in Hcy concentration observed between experiments seems to be a feature of Hcy measurement. Values in normal animals have ranged from a mean of 2.3 μmol/l in this present trial, through to ~9.4 μmol/l (Vellema *et al.,* 1999), 10.1 μmol/l (Kennedy *et al.,* 1992), and 37.8 μmol/l (Kennedy *et al.,* 1994). The variation observed in my data seems unlikely to be due to analytical error. The HPLC method developed for the measurement of tHcy in plasma was found to be robust with validation of the method resulting in an excellent mean recovery rate of 93% with high repeatability, determined from analysis of intra and inter assay precision studies. The stability of the samples during handling and subsequent storage was examined carefully and although the samples were stored at -20°C for some time, this proved to be inconsequential with further repeat examination of samples stored for much longer periods, analysed approximately five years previously, still returning similar values, which is confirmed in the literature (Ducros *et al.,* 2002; Vester and Ramussen, 1991; Ubbink *et al.,* 1992). These data suggest that issues of analysis are not a factor in interpretation of the data presented here.

In this context, therefore, the relationship between tHcy and diet requires consideration. There was a clear effect of ewe supplementation on plasma tHcy values in lambs at the start of Trial A. The only variable was the Cbl status of their dams. It was surprising that the higher values were in lambs from the supplemented ewes, which was the reverse of what would have been expected if the effect was caused by deficiency in the lambs from the unsupplemented ewes. This is especially surprising since a Cbl deficiency *per se* is unlikely in suckling lambs (Gruner at al., 2004b) and, moreover, the lambs were not deficient as judged by MMA and Cbl concentrations, nor did they appear to have experienced reduced milk intake as judged by liveweight at six weeks of age.

The question then arises as to whether the general nutritional conditions provided by fresh pasture enhance the ability of the ruminant to maintain the Hcy methylation metabolic pathway. The metabolic pathway related to Hcy regulation can be influenced by factors outside Co supply such as sulphur amino acid availability and the influence of the non-Cbl associated pathway, betaine. Methionine supply to the sheep is crucial for many metabolic processes and it is this requirement along with rumen function that may off-set any elevation in plasma tHcy concentrations. Homocysteine is derived solely from methionine metabolism and relies on methionine supply for its production as part of its metabolic cycle; it is also significantly recycled to conserve sufficient methionine for protein and SAM synthesis. The liver plays a central role with nearly one-half of the daily methionine intake metabolised there. It cannot, therefore be discounted that the betaine-homocysteine methyltransferase (BHMT) reaction has a possible role in preserving methionine supply when plasma Cbl concentrations are limited. The enhancement of methionine recycling may offset rising tHcy values in time of Cbl deficiency where cobalt supply and rumen function are not severely compromised due to abnormal dietary factors. However, it is generally considered that sheep at pasture are particularly sensitive to methionine supply as the ruminant is reliant predominantly on microbial protein, which is considered to be a poor source of methionine (Storm & Orskov, 1984). The influence of dietary methionine on the three metabolic reactions that function to maintain low intracellular concentrations of homocysteine in times of Cbl deficiency, with particular interest in the involvement of the betaine and choline in the BHMT pathway, requires further investigation as they may possibly be strong determinants of plasma Hcy clearance.

In conclusion the homocysteine/methionine pathway may be highly conserved indicating its importance to the animal's survival such that large rises in tHcy concentration may only be found in times of extreme metabolic distress not normally seen in sheep at pasture.

The finding in Trial A that the Cbl status of the ewe may influence the response of the suckling lamb to Cbl supplementation is interesting and confirms the observation of Gruner *et al.,* (2004). Plasma Cbl concentrations were elevated from a common concentration of 245 to 838 pmol/l and 874 to 2776 pmol/l in lambs from ewes unsupplemented and supplemented with a Co bullet. It suggests a priming effect on the tissues of the developing foetus may exist, which on the one hand could alleviate initial

severity of the Cbl deficiency. It also suggests that plasma concentrations in these lambs gave quite misleading estimates of their true vitamin B_{12} status. As argued by Gruner *et al.,* (2004) it suggests the greater plasma response of the lambs suckling the supplemented ewes is that their extravascular tissues were more 'saturated' with the vitamin and therefore transfer of the injected Cbl from the circulation was reduced.

This trial did not prove causality between elevated tHcy concentrations in plasma and Cbl deficiency in sheep at pasture. Homocysteine is not, therefore, considered the primary metabolic dysfunction and a suitable diagnostic aid in determining a Cbl deficient status.

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