DEVELOPMENT OF AN EXPERIMENTAL APPROACH TO MEASURE VITAMIN B₁₂ PRODUCTION AND ABSORPTION IN SHEEP

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by

M. R. Ludemann

Lincoln University
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Development of an experimental approach to measure vitamin B\textsubscript{12} production and absorption in sheep

Abstract
Clinical diagnosis of vitamin B\textsubscript{12}/cobalt (Co) deficiency is difficult due to the unspecific nature of the clinical symptoms. The apparent increase in vitamin B\textsubscript{12} deficiency in New Zealand in the late 1990's made it clear that health providers were very reliant on plasma reference ranges to diagnose deficiency. However, the lack of quantitative data of what these reference ranges represent in terms of supply of vitamin B\textsubscript{12}, has prevented a better understanding of the metabolism of vitamin B\textsubscript{12} within sheep. This thesis describes the development of an experimental approach to measure vitamin B\textsubscript{12} production and absorption in sheep. The model was then used to investigate whether the type of carbohydrate source affects vitamin B\textsubscript{12} production and/or absorption.

In the first trial (Chapter 4), an adaptation of the repletion technique of Suttle (1974) for copper was used. Previously vitamin B\textsubscript{12} depleted sheep were maintained on a diet of 400 g DM meadow hay and 250 g DM crushed barley and which provided a daily intake of 0.03 mg Co. Sheep were intravenously infused with known quantities of vitamin B\textsubscript{12} (0 – 200 nmol hydroxycobalamin/d) to determine whether a relationship exists between plasma vitamin B\textsubscript{12} concentration and rate of entry of the vitamin into the bloodstream and to quantify the relationship. Eight ewes fitted with rumen and abomasal cannulae were used in a paired 4x4 Latin square design. Four levels of vitamin B\textsubscript{12} were infused into the jugular vein for 4 d followed by recovery period of 17 d and the plasma responses measured during this period. Control
animals maintained relatively stable plasma levels while levels in treated animals rose sharply within 4 h after the start of infusion. The responses were proportional to infusion rate with a highly significant difference in concentrations observed between treatments (P<0.001). Appearance of vitamin B\textsubscript{12} in the plasma was quantified by the relationship $Y(\text{pmol/l}) = 238 + 102x^{0.74}$ where $x = \text{infusion rate(nmol B}_{12}/\text{d})$, $R^2 = 0.945$. Plasma levels declined rapidly on cessation of infusion and had returned to original pre-treatment levels by the end of the recovery period.

The second trial (Chapter 5) quantified plasma responses in relation to dietary Co supply to the rumen. The same eight rumen and abomasal cannulated ewes were used in a paired 4x4 Latin square with 4 levels of intra-ruminal Co infusion in amounts ranging from 0 – 1 mg Co/d as CoSO\textsubscript{4}, infused for 4 d followed by a 19 d recovery period. Plasma vitamin B\textsubscript{12} response was measured as were concentration of vitamin B\textsubscript{12} in abomasal digesta supernatant and digesta liquid flow, the latter using polyethylene glycol as liquid phase marker. Plasma concentration increased from approximately 300 pmol/l to 750 – 800 pmol/l, peaking at day 5 after commencement of infusions. Control animals infused with water maintained base-line plasma vitamin B\textsubscript{12} levels around 250 pmol/l. Digesta liquid flow was estimated at 14.6 l/d.

The results from Chapters 4 and 5 allowed estimates of vitamin B\textsubscript{12} production and absorption. These results are discussed and efficiency of capture of Co into vitamin B\textsubscript{12} from dietary Co intake was estimated to decrease from 8.4% to 0.74% as Co intake increased from 0.03 to 1.11 mg Co/d. Absorption coefficient was estimated from the relationships between abomasal vitamin B\textsubscript{12} flow and
plasma response. The absorption coefficient was calculated to increase from 7 to 10% as abomasal vitamin B\textsubscript{12} flow increased from 108 to 142 nmol/d.

The current animal model was subsequently used in the final trial (Chapter 6) to investigate whether the type of carbohydrate supplied in the diet influences vitamin B\textsubscript{12} production and absorption. The same eight ewes fitted with rumen and abomasal cannulae were used in a 2x4 cross-over design. Half of the ewes were maintained on the basal diet and crushed barley while, for the remainder, the barley was replaced by 250 g of fructose:sucrose in a 80:20 mix during a 7 d period of adaptation. While on their respective diets, both groups were then infused with 0.3 mg Co/d for 6 d, a level chosen to optimise plasma vitamin B\textsubscript{12} response. All ewes were then returned to the basal diet and barley for a 15 d recovery period after which treatments were reversed and the procedure repeated. Rumen pH, valeric, propionic, butyric, succinic, and acetic acid and ammonia concentrations were not significantly affected by diet. Digesta flow was 30% greater on a diet enriched with starch compared to sugar-enriched diet. There was an observed difference in vitamin B\textsubscript{12} production. However there was no significant diet x time effect on plasma vitamin B\textsubscript{12} concentration so any difference in production was negated by a difference in absorption. Carbohydrate supplied as water soluble carbohydrate or starch does not appear to influence plasma vitamin B\textsubscript{12} concentration.

**Keywords:** sheep, cobalt, vitamin B\textsubscript{12}, supplementation, infusion, production efficiency, absorption efficiency
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CHAPTER 1: Introduction

The wasting disease or ill-thrift of a vitamin B₁² deficient animal has been recognised since 1831 in parts of Scotland. However it was not until the early 1930’s that such wasting was attributed to cobalt (Co) *per se* (Andrews, 1971). In New Zealand, cobalt (Co) deficiency has also been known regionally as ‘bush sickness’ in Nelson (Askew & Dixon, 1936), ‘Mairoa dopiness’ in the King Country (Wright & Taylor, 1931) and ‘Morton Main disease’ in Southland (Dixon, 1936).

Cobalt is required in ‘trace’ quantities by ruminants for the synthesis of vitamin B₁₂ and its analogues by rumen microorganisms (Marston, 1970; Kennedy *et al.*, 1994; Clark, 1998; Underwood & Suttle, 1999). Since there are no known requirements for Co *per se*, the clinical signs of cobalt deficiency are ultimately a result of vitamin B₁₂ deficiency. It was not until 1948, when two independent research groups, one in the United States and one in Great Britain (Andrews, 1971) discovered vitamin B₁₂, that an understanding of how Co eliminated the wasting of deficient animals was obtained.

The problem of clinical diagnosis of Co/vitamin B₁₂ deficiency is the unspecific nature of the clinical symptoms. While some symptoms such as poor thrift are also seen with other diseases, particularly parasitism and malnutrition, Co deficiency is typically described by loss of appetite, resulting in ill-thrift, anaemia, excessive lachrymation, increased perinatal mortality, decreased fertility and a fatty infiltration of the liver giving it a pale and friable appearance (Grace, 1994; Underwood & Suttle, 1999).
susceptibility is typically seen in lambs followed by mature sheep, calves and adult cattle (Andrews, 1971).

In the late 1990’s, the New Zealand farmer-funded Meat and Wool New Zealand Boards (then named Meat New Zealand and Woolpro respectively) were concerned with an apparent perceived increase in incidence of vitamin B$_{12}$ deficiency in the South Island of New Zealand. It became clear that farmers and veterinarians were very dependent on marginal reference ranges of vitamin B$_{12}$ in plasma to diagnose deficiency without knowing exactly what these reference ranges represented in terms of supply of vitamin B$_{12}$. The reference range was utilized by animal health laboratories derived from data collated by Clark et al. (1989). Generally, as long as plasma values were within the marginal range (336 – 500 pmol vitamin B$_{12}$ /l for weaned lambs) supplementation was recommended. This was not the intended purpose of the reference curves which were meant to aid “veterinarian, consultants and farmers to make more informed decisions about cobalt supplementation” (Clark & Wright, 2005) rather than an absolute supplementation range. No knowledge exists on how much vitamin B$_{12}$ needs to be absorbed to maintain plasma levels and how variable individual rates of absorption are.

As an aid to diagnosis of deficiency, measurement of the vitamin in tissues such as plasma and liver has traditionally been adopted, initially using microbiological assays. These assays do not differentiate well and uniformly between true vitamin B$_{12}$ and analogues which are also produced by rumen bacteria and absorbed (Stabler, 1999). More recently a competitive binding assay has been developed which only binds true vitamin B$_{12}$ through a specific
vitamin B\textsubscript{12} binder and should provide a more reliable and repeatable method (Stabler, 1999). However obtaining liver tissue samples is invasive and expensive to conduct and the extent to which they reflect current Co intake is difficult to judge. Obtaining a blood sample is much less invasive, easily obtained and analysis of vitamin B\textsubscript{12} is less expensive. The reference range for diagnosis of deficiency is understandably broad, having been based on data derived from a range of methodologies with varying specificity and variation between trial weight gain responses to vitamin B\textsubscript{12}/cobalt supplementation (Clark \textit{et al.}, 1989). The interpretation is possibly also very broad due to a lack of precise understanding of the animal requirement for vitamin B\textsubscript{12}, and of what a plasma value represents in terms of daily supply of the vitamin. The vitamin does not appear to have an active role in the bloodstream hence the blood is considered to be a transport pool. Due to the complex nature of metabolism of vitamin B\textsubscript{12}, quantitative aspects of cobalt / vitamin B\textsubscript{12} metabolism are poorly understood. As a consequence, plasma reference ranges are broad due to a wide range of plasma values where weight gain response is greater than 10 g/d are of low probability. There is a need for better quantification of the transformation of cobalt to vitamin B\textsubscript{12} within sheep. The more specific assays available make this line of research much more feasible. Further to this, quantification of flow of vitamin B\textsubscript{12} in digesta is now possible, and opens up the possibility of measuring the influence of rumen conditions on vitamin B\textsubscript{12} synthesis, efficiency of absorption and therefore supply to the tissues.

Given the abovementioned issues, the goal of the present research was to adapt and extend the experimental approach developed by Suttle (1974) originally for
copper metabolism, to more accurately measure vitamin B<sub>12</sub> production and absorption in sheep. Having developed such a model, the aim was to test factors other than cobalt, such as diet, for their influence on vitamin B<sub>12</sub> production and/or absorption.
CHAPTER 2: Literature Review

2.1 History of vitamin $B_{12}$

In New Zealand, cobalt (Co) deficiency has also been known by the area in which it was first found, such examples as 'bush sickness' (Askew & Dixon, 1936), ‘Mairoa dopiness’ (Wright & Taylor, 1931) and ‘Morton Main disease’ (Dixon, 1936). The first official report in New Zealand of the disease occurred in Tauranga in 1893 (Andrews, 1971). This wasting disease or ill-thrift of deficient animals, see Plate 2.1, has been recognised since 1831 in parts of Scotland (Andrews, 1971), but not until the early 1930’s was such wasting related to Co per se.

Plate 2.1: Cobalt deficient sheep (left) and cobalt supplemented sheep (right) (Clark & Millar, 1983).

The understanding of how cobalt supplementation rectified the ill-thrift of deficient animals was unclear until 1948 when two independent research groups, one in the United States headed by Edward Rickes and Karl Folkers, and the other in Great Britain with E. Lester Smith (Andrews, 1971) isolated vitamin $B_{12}$. The researchers subsequently demonstrated that vitamin $B_{12}$ contained Co and was the factor explaining remission of all clinical signs.
2.2 Structure of vitamin B$_{12}$

Vitamin B$_{12}$ is one of a group of cobalamins and has a general formula of C$_{63}$H$_{88}$N$_{14}$O$_{14}$PCo, molar mass of 1355 and contains 4.4% Co (Underwood & Suttle, 1999). Micro-organisms in the rumen produce vitamin B$_{12}$ also called cobalamin (cbl), together with a series of vitamin B$_{12}$-like but inactive analogues. Vitamin B$_{12}$ has a complex molecular structure, (Figure 2.1) consisting of a tetrapyrrol group (corrin ring), ribose-3-phosphate group and 5,6-dimethyl-benzimidazole group and amino-1-propan-2-ol as Co$\alpha$-ligand, and a ‘X’ (Co$\beta$ ligand) group. The $\alpha$-ligand determines metabolic activity between active ‘true’ vitamin B$_{12}$ and inactive analogues. Analogues have a modified $\alpha$-ligand (termed cobamides) or no ligand at all (termed cobinamides). The $\beta$ ligand determines the specific biological activity and can occur as a hydroxyl, methyl, 5-deoxyadenosyl, cyanide or sulphuryl group. The two coenzymes of major importance for biological processes in the ruminant animal are considered to be methyl-cobalamin (Me-cbl) and 5-deoxy-adenosyl cobalamin (Ado-cbl) (Suttle, 1986). Together, these co-enzymes of vitamin B$_{12}$ have been estimated to make up approximately 80% of the true vitamin B$_{12}$ production in sheep (Gawthorne, 1970).

**Figure 2.1:** Structure of cobalamin (vitamin B$_{12}$).
2.3 Function of vitamin B$_{12}$
Inter-conversion between the different forms of vitamin B$_{12}$ can occur. Methylcobalamin (Me-cbl) and 5-deoxyadenosyl-cobalamin (Ado-cbl) are the biologically active forms of vitamin B$_{12}$. It is believed that conversion to the appropriate coenzyme forms occurs after transportation into the cells (Gimsing et al., 1982).

2.3.1 5-deoxyadenosyl-cobalamin (Ado-cbl)
Short-chain volatile fatty acids (VFA) (acetate, propionate and butyrate) are the major end products of fermentation in the rumen. Adenosyl-cobalamin (Ado-cbl) is a co-enzyme for methyl-malonyl mutase in the conversion of methylmalonyl CoA to succinyl CoA (Figure 2.2). Succinyl CoA in turn, enters the tricarboxylic acid (TCA) cycle to yield glucose and energy (ATP) via full oxidation. In ruminants this process is particularly important as it is the main pathway for utilization of propionate in the provision of their glucose requirements. Metabolism of propionate in the liver it is considered to provide 50-80% of glucose needs in roughage-fed animals (Brockman, 1993).

Figure 2.2: Role of 5-deoxyadenosyl cobalamin (Ado-cbl) in propionic acid metabolism in the liver.
A consequence of vitamin B\textsubscript{12} deficiency is considered to be reduction in energy and glucose from glycolysis, with increase in energy from gluconeogenesis or oxidation of lipids. The conversion of methyl malonyl CoA to succinate slows, and methylmalonic acid (MMA) is hydrolysed from methylmalonyl CoA. Methylmalonic acid accumulates in tissues and the bloodstream and is excreted in the urine. Periods of low feed intake (as seen in vitamin B\textsubscript{12} deficiency) also stimulate the mobilisation and oxidation of fatty acids of lipids (β-oxidation) in the liver to supplement the short fall in energy from glycolysis (Figure 2.3). However under vitamin B\textsubscript{12} deficiency, the reduction in the conversion of methylmalonyl-CoA to succinyl-CoA prevents glycolysis by failing to produce the succinic acid for the TCA cycle to yield ATP. In turn, the build up of methyl malonyl-CoA also inhibits β-oxidation. The inhibition of β-oxidation in the liver is one possible explanation for the accumulation of fatty acids in the liver of vitamin B\textsubscript{12} deficient sheep, causing ovine-white liver disease (Kennedy et al., 1994).

**Figure 2.3:** Diagram of β-oxidation in the liver of ruminants caused by reduced propionate metabolism from vitamin B\textsubscript{12} deficiency (Sykes, A.R. lecture material, Lincoln University 2000).
Kennedy et al., (1991) suggested that large amounts of propionate in the rumen can be absorbed at excessive rates. This would place pressure on the Ado-cbl dependent methylmalonyl CoA mutase pathway, as propionate is normally metabolised rapidly in the liver to succinate. However in the rumen, bacteria utilise Ado-cbl to convert succinate to propionate, the opposite direction to the pathway which occurs in the liver. This Ado-cbl-dependent pathway may be capable of being by-passed to reduce the bacterial dependence on Ado-cbl. The result can be high rumen concentrations of succinate and high rates of absorption of succinate. This theory was investigated further by Northern Ireland researchers (Kennedy et al., 1991) who hypothesised that increased rumen succinate production may ameliorate the effects of vitamin B\textsubscript{12} deficiency by reducing the pressure on the propionate (B\textsubscript{12}-dependent) pathway. Logically, it could be assumed that an increase in rumen succinate production as a result of change in rumen fermentation may reduce the pressure on the vitamin B\textsubscript{12}-dependent propionate pathway in the animal since succinate is readily absorbed (Kennedy et al., 1991). Succinate can then enter the TCA cycle directly, bypassing the requirement for conversion of propionate to succinate. Further, this may ameliorate the energy/glucose-deficit effects of vitamin B\textsubscript{12} deficiency. However, Kennedy et al., (1991) found no difference in mean plasma MMA concentration even though rumen succinate production was increased using severe and moderate Co deficient diets (Kennedy et al., 1996) as discussed in more detail in section 2.9.
2.3.2 Methyl-cobalamin (Me-cbl)
Methyl-cobalamin is a co-enzyme for the enzyme methionine synthetase for transfer of methyl groups from 5-methyl tetrahydrofolic acid (Me THF) to homocysteine to form methionine, an amino acid required for wool and body growth. Methyl-cobalamin is required by a number of methyl transferase enzymes, acting as a donor of a methyl group (Underwood & Suttle, 1999) (Figure 2.4). Vitamin B₁₂ deficiency leads to an accumulation of homocysteine and forminoglutamic acid (FIGLU) due to the failure of conversion of homocysteine to methionine and THF to 5-formimino THF respectively. Both of these accumulated products have been used for diagnostic purposes and are discussed later.

![Figure 2.4: Role of methyl-cobalamin in methyl transferase enzymes (Gruner, 2001).](image)

2.4 Absorption
Limited research data exist on the amount of vitamin B₁₂ that is produced or absorbed by ruminants or the mechanisms involved. The majority of research has been conducted with humans or rodents. Traditionally, aspects of this research have been applied under the assumption that human or rodent data will be applicable to sheep.
Ruminants utilise Co contained within pasture for the synthesis of vitamin B\textsubscript{12} by ruminal bacteria. This is then bound with vitamin B\textsubscript{12} binding proteins, and is eventually absorbed from the small intestine. Salivary secretions of non-ruminants contain significant amounts of haptocorrin (HC), a vitamin B\textsubscript{12}-binding protein (also commonly known as R-proteins or R-binder). This glycoprotein binds with cbl and inactive analogues (Guèant & Nicolas, 1990). The isoproteins found in HC are most stable at pH 2.8 - 4.7 (Guèant & Nicolas, 1990) which would make them unstable in the rumen, but potentially the main vitamin B\textsubscript{12}-binding protein in the anterior part of the post-ruminal digestive tract. HC also occurs in plasma, where it is commonly termed transcobalamin I and III (TC I or TC III, respectively) (Hansen, 1990; Seetharam & Alpers, 1994).

Intrinsic Factor (IF) is another vitamin B\textsubscript{12}-binding glycoprotein which is secreted by gastric parietal cells (Glass, 1974; McKay & McLeay, 1979) and is stimulated by the presence of food in the stomach (Jacob et al., 1980). However the exact physiological mechanism of regulation of secretion is unclear. The binding between IF-cbl and the receptor may be Ca\textsuperscript{2+} and pH dependent (Underwood & Suttle, 1999) and optimal between pH 6.4 and 8.4 (Jacob et al., 1980) or up to pH 10.0 (Glass, 1974).

Vitamin B\textsubscript{12} is considered to be preferentially bound to HC in the acidic pH of the stomach, the affinity of which is considered to be 50 times that of the IF - cbl complex (Allen et al., 1978). Haptocorrin-cobalamin complex and unbound IF may proceed to the small intestine where HC is degraded in the presence of pancreatic enzymes at the more neutral pH of the duodenal/jejunal section of
the small intestine (Guèant & Gräsbeck, 1990), to yield free cbl (Figure 2.5). Unbound IF is considered to have a greater affinity for cbl than degraded HC (dHC) in the neutral conditions of the small intestine and binds with cbl on a one-to-one basis and is carried to the ileum for absorption.

Unbound human IF is considered to be partially degraded by pepsin and by chymotrypsin when at high concentrations, but is relatively stable when bound to a vitamin B₁₂ molecule (Jacob et al., 1980) (Figure 2.5). This allows the IF-cbl complex to be presented to the intestinal receptor in the terminal ileum. Some of the IF that escapes degradation has the potential to bind with cbl produced by bacteria in the lower gut or cbl present in bile, the latter being available for re-absorption in the ileum of the ruminant (Guèant & Gräsbeck, 1990).

### 2.5 Transport

Once the cbl is presented to the bloodstream, a proportion is bound to one of two main serum proteins; transcobalamin I (TC I, also termed TC III) or transcobalamin II (TC II). Transcobalamin I and TC III are basically considered to be immunologically identical, only varying in carbohydrate content, particularly sialic acid (Glass, 1974) and fucose (Jacob et al., 1980). TC
III may well be an artefact of the isolation process (Burger et al., 1975a). It is therefore grouped with TC I hereafter. TC I is a specific serum HC.

Transcobalamin I may also act protective way for the body in regard to dealing with potentially toxic cbl analogues. Seetharam & Aplers (1994) have alluded to evidence that cbl analogues may gain entry into the bloodstream via IF independent mechanisms. Because of the strong affinity of TC I for vitamin B_{12}-like compounds, one hypothesis developed is that it provides a potential screening process, particularly against inactive analogues that are potentially toxic and compete with true cbl for binding and absorption sites (Kanazawa & Herbert, 1983). Preferential uptake of analogues by TC I may allow them to be transported to the hepatocytes for excretion (Burger et al., 1975b). In the liver, analogues are transported into the bile and excreted via the faeces, preventing any potential accumulation in metabolically active sites. A summary of the transport and absorption process is presented in Figure 2.6.

**Figure 2.6:** Schematic representation of vitamin B_{12} production and binding through the gastro-intestinal tract and cellular uptake. (adapted from (Alpers & Russel-Jones, 1999)).
2.6 Storage
While the liver is commonly referred to as the storage pool for vitamin B\textsubscript{12}, this may be a misunderstanding. A definition of a storage pool is ‘the reserve of a mineral or vitamin that depletes once the transport pool is depleted’ (Grace, 1994), as seen in copper homeostasis. However when sheep are depleted of Co (hence vitamin B\textsubscript{12}), liver concentration of vitamin B\textsubscript{12} depletes in similar fashion to serum (Suttle, 1986). Repletion of deficient animals sees serum levels increase within hours (Field et al., 1988) but an 8-day lag has been observed before liver concentrations increase (Grace, 1998). Since it is considered that the liver is the functional site of vitamin B\textsubscript{12}, it may be more appropriate to term the liver as the functional pool, while the blood is considered as the storage pool, except during absorption, when it also acts as the transport pool. Such differences can help explain the different affinities and actions of transcobalamin I and II (TC I and TC II respectively), as explained above, with TC I-cbl complexes operating as a storage pool of true and analogue vitamin B\textsubscript{12} while TC II-cbl complexes act as a transport pool of true vitamin B\textsubscript{12}.

2.7 Excretion
Human excretion of cbl via urinary losses is considered to be negligible under normal vitamin B\textsubscript{12} status (Underwood & Suttle, 1999). In the rat, urinary excretion of cbl via the kidney only appears to occur when the total binding capacity of the blood is exceeded (Cooksley et al., 1974) and surplus cbl (bound and unbound) is filtered through the kidneys and excreted, such as after injections of vitamin B\textsubscript{12}. Urinary losses of vitamin B\textsubscript{12} were estimated at only
1% of that excreted in the faeces in sheep fed a Co deficient (0.02-0.04μg Co/kg) diet (Dawbarn & Hine, 1955).

Excretion of vitamin B₁₂ is initially via the biliary route. It is difficult however, to estimate true daily endogenous losses of vitamin B₁₂ via faeces as synthesis of the vitamin occurs by bacteria in the lower gut (Kercher & Smith, 1956). Smith and Marston (1970) showed 95% of radio-labelled ⁶⁰Co was excreted in the faeces within 14 days after oral dosing and 2.5% appeared in the urine. However, no attempt was made to determine if the ⁶⁰Co was present as the Co ion or incorporated into the corrin ring of vitamin B₁₂.

### 2.8 Requirements

Very few studies have investigated the amount of cobalt required for synthesis of vitamin B₁₂ or the amounts of vitamin B₁₂ absorbed and utilised. The traditional factorial approach for determining nutrient requirement is generally not applicable, due to the lack of production and absorption data resulting from the complexity of Co/vitamin B₁₂ metabolism (Grace, 1994). The factorial approach allows the measurement of the amount of mineral fed and the amount excreted; the difference being apparent absorption. However with vitamin B₁₂, sheep consume Co and produce the vitamin internally. Unlike humans, which require a regular supply of vitamin B₁₂, ruminants need a regular supply of Co for microbial synthesis of vitamin B₁₂ in the rumen. Fermented products such as silages and brewers grains contain trace amounts of vitamin B₁₂, but otherwise the ruminant is totally dependent on Co intake for its vitamin B₁₂ supply. Supplementation by frequent injection of vitamin B₁₂ is
possible and effective. Oral dosing of vitamin B\textsubscript{12} tends to be ineffective due to degradation of the vitamin by rumen micro-organisms.

Estimated minimum pasture Co concentrations for provision of adequate vitamin B\textsubscript{12} for sheep, determined in supplementation trials or from observations of occurrence of clinical symptoms, have ranged from 0.06 mg Co/kg DM (Millar & Albyt, 1984) to 0.11 mg Co/kg DM (Grace, 1994). Trials with Romney lambs have shown live weight loss and death on pastures containing 0.04 – 0.08 mg Co/kg DM (Grace, 1994). The requirements of weaned lambs are considered to be higher than those of adult sheep, possibly due to their higher growth rate and/or an undeveloped rumen with inadequate capacity for vitamin B\textsubscript{12} synthesis. Suckling lambs may have a lower requirement for vitamin B\textsubscript{12} as they receive a supply of lactate from milk and are therefore not as dependent on propionate as a source of energy and glucose substrate. Lactate can enter the TCA cycle directly, bypassing the vitamin B\textsubscript{12}-dependent propionate pathway. Suckling lambs also receive a supply of vitamin B\textsubscript{12} from the milk which by-passes the rumen and is available for absorption from the small intestine. Grace et al., (1986) concluded that milk probably provides the majority of suckling lambs vitamin B\textsubscript{12}, as hepatic stores were low until 3 or 4 weeks of age when lambs began to graze pasture and develop their own rumen function and synthesise vitamin B\textsubscript{12}.

Previous methods used to estimate vitamin B\textsubscript{12} production in the rumen have been indirect and crude. The historical method used for estimating true vitamin B\textsubscript{12} activity in the rumen was by specific microbial assays. This method is time consuming and does not detect low levels of activity with accuracy. The
efficiency of conversion of Co into vitamin B\textsubscript{12} in the rumen is thought to be dependent on the concentration of Co in rumen fluid, and has been estimated to decrease from 13 to 3% with increasing Co intake from 0.07 μg to 1.0 mg Co/d (Smith & Marston, 1970). However, these latter estimates were based on vitamin B\textsubscript{12} production data measured by microbiological assay using the ratio of vitamin B\textsubscript{12} to lignin. Lignin, an internal marker of forage, in rumen fluid 4 h after feeding was multiplied by daily intake of lignin. A number of problems are associated with this method of internal marking. Water turnover in the rumen, with which vitamin B\textsubscript{12} - a water soluble vitamin could be expected to travel, could be expected to be greater than that of solids such as lignin, and no account is taken of the effect of pattern of feed intake. Therefore, the rates of production estimated are considered to be minimum values. Initially it was thought the microbe used to determine true vitamin B\textsubscript{12} activity (\textit{Ochromonas}) was specific for vitamin B\textsubscript{12}. However, later discoveries found \textit{Ochromonas} responded to other vitamin B\textsubscript{12}-like factors so that results from such assays probably overestimate true vitamin B\textsubscript{12} (Dawbarn & Hine, 1955). There is evidence that vitamin B\textsubscript{12} production is possibly 50% lower on high concentrate starch diets (Sutton & Elliot, 1972; Hedrich \textit{et al.}, 1973). Logically, it can be hypothesised that the lower fractional outflow rate, and greater cellulose fermentation with roughage diets, allows more time and substrate for microbial synthesis to capture limited available Co within the diet.

Efficiency of absorption of vitamin B\textsubscript{12} produced in the rumen was calculated to be 3 – 5% in adult sheep (Marston, 1970), based on the failure of 100 μg vitamin B\textsubscript{12}/d given \textit{per os} to be as effective as 3 μg vitamin B\textsubscript{12}/d injected intramuscularly, in terms of ability to maintain blood haemoglobin
concentration and body weight. A treatment group on a low Co diet (0.04 mg Co/kg DM wheaten hay chaff) was given 100 μg vitamin B₁₂/d *per os* and began to lose weight during the period 16-19 weeks after the beginning of the trial, at which time a control group receiving the same low Co diet, was also losing weight. Treatment with 100 μg vitamin B₁₂/d *per os* or less did not significantly influence weight change or blood haemoglobin concentration until the first death of a control animal in week 35. By this time it was obvious to the authors that 100 μg vitamin B₁₂/d *per os* retarded weight loss but not significantly enough to prevent loss from the tissues. No account was taken of the possible degradation or modification of vitamin B₁₂ administered *per os*, and the question must be raised as to how much vitamin B₁₂ reached the small intestine? Vitamin B₁₂ administered orally is degraded rapidly by rumen microbes. Smith & Marston (1970) noticed rapid decline in the true vitamin B₁₂ activity in rumen fluid and an increase in vitamin B₁₂–like compound activity during the first 10 h after oral treatment of deficiency with 500 μg cyanocobalamin/d. The decrease in true vitamin B₁₂ reflected rapid degradation of orally administered cyanocobalamin into vitamin B₁₂ analogues. It is possible that small responses to orally administered vitamin B₁₂ may be due to degradation of cyano cobalamin to an extent that makes the Co ion available for true vitamin B₁₂ synthesis by rumen micro-organisms.

Minimum daily requirements of vitamin B₁₂ for normal growth were estimated in wethers fed on Co deficient diets (0.04 mg Co/kg DM wheaten hay chaff) and injected parenterally with 3.12, 6.25, 12.5 or 25 μg cyanocobalamin daily. Long-term (60 weeks) daily DM intakes were recorded. Maximum intakes were obtained when animals were injected with 6.25 μg/d or greater rates of cyano
cobalamin. Appetites of all sheep in the 6.25 μg cyano cobalamin/d group fell within 6 weeks of cessation of injections. It was also suggested that 6.25 μg cyano cobalamin/d was the minimum supplement required to maintain normal metabolism of sheep on Co deficient diets and yet it provided little or no excess for storage in the liver over animals that received 3.12 μg cyano cobalamin/d. Concentrations of the vitamin in the liver were 0.123 and 0.052 μg vitamin B₁₂/g liver respectively, measured 50 weeks after treatments started. In sheep which received 3.12 μg cyano cobalamin/d the onset of clinical symptoms of deficiency were delayed until week 30 when some animals began to lose appetite. Sheep receiving supplements above 6.25 (namely 12.5 and 25 μg cyano cobalamin/d) had significantly higher liver vitamin B₁₂ levels (approximately 0.4 and 1 μg vitamin B₁₂/g liver respectively), suggesting the vitamin was in excess of requirements. However, possible losses of the vitamin via urine following intramuscular injections were not considered and requirement could therefore have been overestimated.

When sheep were confined to a standard Co-deficient diet estimated rate of absorption of vitamin B₁₂/d from the intestinal tract was 5 μg/d. This estimate was based on an extrapolation of the 34 μg vitamin B₁₂/d needed to be injected intramuscularly to achieve an apparent maximum liver concentration of 1.4 μg vitamin B₁₂/g liver after receiving 1 mg (or 10 mg) Co/d in the diet (Marston, 1970) and an estimate of ruminal production of 700 μg vitamin B₁₂/d (Smith & Marston, 1970). The 34 μg vitamin B₁₂/d estimated to be stored in the liver following production of 700 μg vitamin B₁₂/d in the rumen equated to an absorption efficiency of 5%. However, no effort has been made to separate rate
of absorption and utilisation. It was concluded by Marston (1970), that daily maintenance requirement for vitamin B\textsubscript{12} was about 11 μg/d, and represented the sum of the amount needed to be injected to maintain constant serum vitamin B\textsubscript{12} concentration (6 μg/d) in addition to 5 μg estimated to be absorbed from ruminal synthesis, the latter based on data from previous studies by Smith & Marston (1970) in sheep on similar diets. The 11 μg vitamin B\textsubscript{12}/d provided ‘normal’ metabolism, without increasing liver concentrations. There has been no other evidence on requirement but these values have been generally accepted since these earlier studies.

Difficulty in estimating sheep’s requirement for vitamin B\textsubscript{12} has been associated with the rapid clearance from the bloodstream. The conversion of the vitamin to its active forms could be the rate limiting factor (Judson et al., 1988; Judson et al., 2002). The use of soluble vitamin B\textsubscript{12} injections to alleviate Co/vitamin B\textsubscript{12} deficiency is limited by the length of time plasma levels are raised. Under New Zealand conditions, Grace et al., (1998) found 2 mg injection was effective in increasing lamb serum vitamin B\textsubscript{12} levels for only 24 days when grazing pasture with 0.08 mg Co/kg DM. In Australia, Judson & Babidge, (2002) found 2 mg vitamin B\textsubscript{12} prevented metabolic dysfunction for less than 51 days. Long-acting vitamin B\textsubscript{12} injections have also recently been developed. In New Zealand, microencapsulated vitamin B\textsubscript{12} copolymer microspheres suspended in peanut oil have prolonged the release of vitamin B\textsubscript{12}. Lambs have been shown to maintain serum vitamin B\textsubscript{12} levels significantly higher than controls for 210 days when injected with 6mg copolymer vitamin B\textsubscript{12} (Grace & Lewis, 1999). In Australia, using 2 mg vitamin B\textsubscript{12} as cyano-cobalamin tannin complex suspended in a sesame-oil aluminium monostearate
gel elevated plasma vitamin B\textsubscript{12} for more than 10 weeks (Judson \textit{et al.}, 2000). Clearly, the rapid clearance from the bloodstream shows a need for more direct and accurate methods of measuring vitamin B\textsubscript{12} production and absorption from ruminants. Developments in the measurement of vitamin B\textsubscript{12} are now possible without the inherent analytical errors associated with microbial assays used in the 1970’s. The model developed within the present study attempts to address these issues.

2.9 Volatile fatty acid production
Volatile fatty acids (VFA) are the main end product of rumen fermentation after the conversion of carbohydrates to pyruvate, and then pyruvate into VFA, as linked in Figures 2.7 and 2.8. The type of VFA which dominates in the rumen fermentation is dependent on diet (McDonald \textit{et al.}, 1995). Succinate is normally metabolised rapidly by other rumen bacteria, the majority of which are vitamin B\textsubscript{12}-dependent (Kennedy \textit{et al.}, 1991), to produce propionate. Figure 2.8 shows the production of propionate via two pathways; the lactate pathway which dominates from a concentrate-rich diet and the succinate pathway which dominates from a roughage-rich diet. A lack of vitamin B\textsubscript{12} in the rumen prevents the conversion of succinate to propionate and allows succinate to accumulate in the rumen fluid. This proposed pathway is the exact opposite to the pathway which occurs in the liver.
Figure 2.7: Conversion of carbohydrates to pyruvate in the rumen (McDonald et al., 1995)

Figure 2.8: Conversion of pyruvate to VFA in the rumen (McDonald et al., 1995)

Under severely Co deficiency conditions (<0.004 mg Co/kg) Kennedy et al. (1991 & 1996) were able to demonstrate an increased concentration of succinate in the rumen. Investigations were conducted to ascertain whether absorption of this succinate can reduce or ameliorate the effects of vitamin B$_{12}$ deficiency by offsetting the anticipated reduced succinate synthesis in the liver.

Three diets containing 0.004, 0.04 and 1 mg Co/kg DM were fed to sheep for 23 weeks. Ruminal succinate concentration increased 100 fold within 2 days in animals on the 0.004 mg Co/kg DM diet and was matched by an equimolar decrease in propionate concentration. Sheep on 0.04 mg Co/kg DM (or greater) showed no changes in propionate and succinate concentrations. The
hypothesis was that sheep on the more severe Co deficient diet promoted production of succinate which could be absorbed, thus offsetting the requirement for methylmalonyl CoA mutase to convert propionate to succinate. However this hypothesis was discarded as the mean plasma MMA concentrations of animals on the moderate and severely deficient diets were not significantly different, suggesting no reduction in load on the endogenous propionate to succinate pathway, even though the diets did promote variation in succinate production in the rumen (Kennedy et al., 1996). In practical situations, a diet so low in Co is rarely seen.

In this context however, evidence is beginning to accumulate to suggest that fermentation type may affect vitamin B₁₂ production, its absorption or its utilisation. These include observations of reduced growth of lambs on diets providing accepted ‘adequate’ amounts (0.32 - 1.7 mg) of Co per day (Ulvund, 1990d); development of ovine white-liver disease (OWLD) in lambs grazing both ‘deficient’ as well as ‘sufficient’ pastures (Ulvund, 1990c; Ulvund, 1990b). In addition, seasonal variation in plasma levels of vitamin B₁₂ in Co supplemented and non-supplemented groups of lambs were observed to follow the same pattern but at different plasma concentrations (Gruner et al., 2004a). In other words, rate of production of the vitamin appeared to vary with factors other than rumen Co concentration, rather than showing evidence for a constant production of vitamin B₁₂ in supplemented animals, see Figure 2.9 (Gruner et al., 2004a). If vitamin B₁₂ production was consistent from a consistent release of Co, it would be expected that plasma vitamin B₁₂ in Co supplemented animals would reach a plateau at which Co supply would determine production and/or absorption and therefore serum concentration.
Ulvund & Pestalozzi (1990a) has suggested water-soluble carbohydrates (WSC), particularly fructan in pasture grass, as a possible predisposing factor to development of OWLD. Perennial ryegrass is rich in WSC, and in particular fructan, which accumulates in the early leafy stage of plant growth. Sub-clinical vitamin B₁₂ deficiency has been observed in lambs grazing perennial ryegrass with high WSC concentrations, but not on ‘healthy’ pastures which did not contain perennial ryegrass and had lower WSC contents. The coincidence of this observation and the period of lush grass growth supports a hypothesis that a high intake of WSC could be a predisposing factor in OWLD (Ulvund & Pestalozzi, 1990b) and is investigated within the present study.
2.10 Summary

The traditional factorial approach to estimation of mineral requirement has not been possible due to the complex nature of vitamin B₁₂ metabolism. Currently there is limited information on the amount of vitamin B₁₂ absorbed from the gastro-intestinal tract of ruminants due to the historical difficulties of measuring true vitamin B₁₂. Generally accepted rates of absorption are derived from trials performed in the 1970’s with crude and indirect experimental estimates. Information gained from obtaining more accurate information on amounts of vitamin B₁₂ absorption and metabolism may allow a better ability to interpret plasma vitamin B₁₂ reference ranges. To improve our ability to correct vitamin B₁₂ deficiency, an estimate of how much vitamin B₁₂ needed to maintain safe serum vitamin B₁₂ concentration is needed.

A similar situation was the case in respect to requirement for copper (Cu), but in that case due to analytical errors inherent in measuring low coefficients of absorption in conventional balance studies. This was resolved by studying the repletion rate of Cu in the plasma of previously depleted animals in response to change in dietary Cu intake relative to the amount of Cu needed to be infused directly into the bloodstream to achieve the same change in plasma Cu concentration (Suttle, 1974). Groups of ewes previously depleted in serum Cu were repleted with a diet supplemented with additional Cu providing 2.7 up to 8.7 mg total Cu/kg for 33 d. In a second trial ewes were depleted in serum Cu and repleted via intravenous infusion of Cu to by-pass the absorption from the gastrointestinal tract. In other words, the animals were calibrated in terms of rate of Cu absorption required to promote change in plasma Cu concentration. Calibrated responses were related to responses when additional Cu was supplied via the diet to estimated true availability of Cu.
In addition, further investigation is needed examining the effect of rumen conditions on synthesis of vitamin B$_{12}$ and on how non-Co dietary components could affect vitamin B$_{12}$ production and requirement. An extra step (i.e., production efficiency of true vitamin B$_{12}$ in the rumen) is needed to measure the rate of production and then absorption. With a model developed to measure true vitamin B$_{12}$ flows within the sheep, animals could be supplemented with varying amounts of Co to investigate how production and absorption efficiencies change with change in dietary Co intake and other possible dietary components. Measurements of the amount of the vitamin flowing from the rumen are necessary in order to measure net rate of production and a measurement of rate of entry into bloodstream to allow estimation of rate of absorption. The aim of the present study was to determine whether the repletion technique developed by Suttle 30 years ago for Cu metabolism, could be adapted and applied to vitamin B$_{12}$ metabolism. The method of development and results are discussed in the following chapters.
CHAPTER 3: General Materials and Methods

3.1 Animals
Initially, 18-month-old ewes with low vitamin B₁₂ status were obtained from a cobalt-deficient farm near Montalto, Canterbury, New Zealand. Young animals were used in the expectation of a longer service life than would be the case with the use of mature ewes and it would allow rapid establishment of the programme with ‘already depleted’ animals. All animals were drenched with 10 ml combination oral drench for sheep (37.5 g/l levamisole and 23.8 g/l albendazole, Arrest, Ancare New Zealand Ltd, Auckland, New Zealand), brought into indoor pens and offered a basal diet (see section 3.4) prior to the start of the experiment to further deplete plasma vitamin B₁₂ levels. They readily adapted to indoor feeding and were fitted with rumen and abomasal cannulae. However, after cannulation, these animals lost condition and had to be euthanased on welfare grounds. Our observations suggested that the stress of surgery coupled with their low vitamin B₁₂ status and physical immaturity were critical factors in their poor performance. It was decided to replace them with mature ewes which may be better able to handle the combined stress of deficiency and surgery. This proved to be the case, but in addition, as a precaution the Co deficient diet was improved by the addition of a small quantity of crushed barley. The experimental programme was, however, set back by nine months.

Consequently, 10 two-and-a-half year old half-bred ewes were obtained from the same property and treated as described in the previous paragraph. Eight cannulated ewes with plasma vitamin B₁₂ concentrations at least below 500 pmol/l were used in experiments described in Chapters 4, 5 and 6. During the
experiment detailed in Chapter 6, one ewe did not adapt to the high sugar diet given and was diagnosed with acidosis after rumen pH fell below 4.8 and rumen epithelium sloughed off easily as evident from abomasal fluid samples. She was removed from the trial on welfare grounds and removed from any statistical analysis due to her abnormal metabolism. A replacement ewe, one of the 10 surgically prepared sheep, was used for the remainder of the experiment but was ultimately removed from statistical analysis due to initial plasma vitamin B₁₂ values being much higher than those of the rest of the treatment groups.

3.2 Cannulation
The cannulation procedures used were an adaptation of a previously published method (Hecker, 1974). Two separate surgical procedures were performed to insert rumen and abomasal cannulae. Animals were starved for 24 h and removed from water for 12 h prior to surgery. Anaesthetic used was an intravenous combination of 0.75 mg/kg bodyweight diazepam (5 mg/ml, Pamlin, Parnell Lab NZ, Auckland) and 15 mg/kg bodyweight ketamine HCl (100 mg/ml, Phoenix Ketamine Injection, Phoenix Pharm Distributors Ltd, New Zealand).

3.2.1 Rumen Cannulation
A vertical incision was made between the last rib and hipbone on the left-hand side, about 10 cm from the lumbar vertebrae, slightly longer than the diameter of the cannula neck. The wall of the rumen was held up with Allis forceps while another slightly smaller incision was made into the rumen. The rumen mucosa was sutured to the abdominal wall with polyglycolic acid suture material (coated Vicryl 0 (3.5 metric), Johnson and Johnson Medical Pty Ltd), to create a fistula. The cannula flange, (see Plate 3.1) was folded into the neck of the
cannula and placed in the rumen, where the flange was unfolded. Permanent sutures were placed above and below the incision to close the wound completely. Two buttons were placed at the end of the permanent sutures to distribute the pressure creating tension sutures. The rubber external retaining flange was positioned and held in position by needles. The flange was firm but not tight against the abdominal wall as swelling due to oedema tightens the flange against the skin. The surgical area was cleaned and dusted lightly with 2% chlortetracycline HCl powder (Aureomycin Antibiotic Powder, Bomac Lab Ltd., New Zealand). Antibiotic cover was provided by 600,000 iu procaine penicillin, 600,000 iu benzathine penicillin and 1500 mg dihydrostreptomycin (Penstrep LA, Bomac Labs Ltd, NZ) given by subcutaneous injection immediately after surgery and again 72 h later. The animals were then placed in a warm recovery room with feed and water. Animals were returned to housing 24 h after surgery. Post-operative analgesia was available but was not required as feeding and other behavioural patterns resumed within 4 – 6 h following surgery.

Plate 3.1: A) Rumen cannula assembled, B) cannula body, C) retaining ring, D) plug, E) retaining ring needle, F) retaining flange.
3.2.2 Abomasal Cannulation

Anaesthesia was induced as for rumen cannulation. A vertical incision was then made between the last rib and hipbone on the right-hand side of the animal, about 15 cm from the lumbar vertebrae. The abdominal muscle wall was separated using blunt dissection. The abomasum was located and held up with Allis forceps while a purse string suture of polyglycolic acid suture material (coated Vicryl 2-0 with atraumatic needle, Johnson & Johnson Medical Pty Ltd), was placed around the area where the cannula was to be inserted. An incision was then made into the abomasal mucosa slightly bigger than the neck of the cannula (see Plate 3.2) and a cannula with an internal retaining flange inserted. The purse string suture was then tightened and tied off. A stab incision between the last two ribs, the approximate size of the diameter of the cannula neck was made. The cannula was returned to the abdominal cavity and exteriorised out of the incision between the last two ribs. This site was used to avoid occurrence of herniation at the fistulation site. An external retaining flange was positioned over the cannula and held in place by a rubber retaining ring positioned by elastrator applicators. The original incision was closed using polyglycolic acid suture material (Vicryl 0, Johnson & Johnson Medical Pty Ltd). The surgical area was then cleaned and dusted lightly with 2% chlortetracycline HCl powder (Aureomycin Antibiotic Powder, Bomac Lab Ltd., New Zealand). Antibiotic cover was provided as for rumen surgery. The animals were then placed in a warm recovery room with feed and water. Animals were returned to housing 24 h after surgery. Post-operative analgesia was available but was not required as feeding and other behavioural patterns resumed within 4 – 6 h following surgery.
3.3 Liver samples

To reduce the trauma of liver sampling, it was decided to collect liver samples by aspiration liver biopsy while the animal was anaesthetised during abomasal cannulation. The method used was an adaptation of the method described for use in deer by (Familton, 1985) for use in sheep. Biopsy was attempted once the abdominal muscle wall was separated and before the abomasum was located. The biopsy cannula (length 200 mm, diameter: o.d. 4.05 mm, i.d. 3.20 mm) was inserted and gently pushed towards the liver on a 30° angle from the horizontal plane.

Liver was felt and identified by the high rigidity and sighted if needed. The biopsy cannula was carefully driven into the liver 2 – 3 cm by rotation. A 10 ml luer syringe was attached to the external end of the cannula and syringe plunger withdrawn approximately 5 ml and cannula and syringe withdrawn from the animal. Any liver sample was placed on gauze swab to drain blood. The sample was then immediately placed in aluminium foil-covered tubes to protect from light.
While this method has been used successfully in a wide range of animals, it was unsuccessful in obtaining liver samples within the time constraints of an anaesthetised animal for abomasal cannulation. It proved very difficult to aspirate a sample of liver of sufficient size, since the liver of these already vitamin B\textsubscript{12} deficient animals was very friable.

As a follow up, one of the initial 18-month-old ewes obtained for experimentation (see animals) was euthanased with 10 ml sodium pentabarbitone (300 mg/ml) (NZ Veterinary Supplies) to allow practice of the technique and extraction of liver samples. Again, extraction of a core liver sample proved extremely difficult and unsuccessful. Upon removal, penetration of the liver was seen to be successful but samples were so friable that none stayed whole to allow clear separation of blood and tissue and the sample was considered unsatisfactory for liver vitamin B\textsubscript{12} analysis. This reinforced the decision not to take liver biopsies from abomasally cannulated animals due to this problem, and the additional constraint of the reduced area for surgical access with the abomasum adhered to the right flank of the sheep. It was decided that liver sampling of these valuable vitamin B\textsubscript{12} deficient animals was too dangerous for routine components of the work, consequently the possibility was abandoned.

### 3.4 Diets, water and housing

The diet consisted of low quality meadow hay made from mature pasture and crushed barley sourced from a cobalt-deficient farm near Montalto, Canterbury, New Zealand. The quality of the hay was enhanced with the addition of urea to increase nitrogen content. All animals were offered and ate approximately 400 g of hay, 250 g of crushed barley and 9 g of Nitrogen (N)
per day as urea. The hay contained 0.04 mg Co/kg DM and an estimated 7.5 mega-Joules metabolisable energy (MJME)/kg dry matter (DM). The barley contained 0.01 mg Co/kg DM and an estimated 12 MJME/kg DM. The 9 g N/d was provided in 50 ml of urea solution containing 0.2 g N/ml and was poured over the diet. The urea solution was made up by dissolving 2.2 kg urea (Ravensdown Urea 46% N) providing 1 kg N, in 5 l. Ewes were fed at approximately 0900 h each day. All water supplied was deionised. Storage drums, hosing and troughs were plastic to ensure that additional cobalt was not administered. All pens were lined with untreated downgrade plywood and had wooden slat flooring. During active infusion periods pens were modified using downgrade 3-plywood in order to temporarily halve the original size preventing the sheep from being able to turn around and interfere with infusion equipment. An example is given in Plate 3.3. There was no access to metal railing.

**Plate 3.3:** Example of wood-lined pens temporarily halved during the active infusion period used in Chapter 6. Note the automatic feeders administering barley during the 4 h after feeding of hay.
3.5 Feed analysis

3.5.1 Dry matter percentage (DM%)
DM % was determined after storing a daily representative sample of the feed offered in a clean polythene bag. Weekly sub-samples were dried to constant weight at 95°C.

3.5.2 Soluble carbohydrates
Soluble carbohydrates were extracted in water and determined spectrophotometrically, conducted at Lincoln University, Canterbury NZ as detailed in Appendix A. The technique was described by Ministry of Agriculture, (1987).

3.5.3 In-vitro digestibility
The cellulase assay used for prediction of digestibility was described by McLeod & Minson, (1978) and described in Appendix A.

3.5.4 Metabolisable energy (M/D)
Metabolisable energy was calculated using predicted % in-vitro digestibility on organic matter multiplied by 0.16 as described by Barber et al., (1984).

3.5.5 Detergent fibre analysis
Neutral Detergent Fibre (NDF) was determined on hay samples while amylase Neutral Detergent Fibre (aNDF) was determined on barley samples due to the high starch content. Acid Detergent Fibre (ADF) was determined on both hay and barley samples. Respective reagents were prepared as described in Van Soest (1963), Van Soest & Wine (1967) with updates described in Van Soest et al., (1991). Specific methodology is detailed in Appendix A.

3.5.6 Crude protein
The Kjeldahl procedure was followed to obtain nitrogen content. An accurately weighed sample (approximately 0.5 g) of the ground feed was placed in
digestion tubes with 7 g potassium sulphate (K$_2$SO$_4$) and a selenium (Se) catalyst in a 1000:1 ratio K$_2$SO$_4$:Se. Concentrated sulphuric acid (H$_2$SO$_4$), 12 ml was added and samples digested for 30 min at 420°C. Samples were cooled and analysed on a 1035 Analyser, (Tecator, Sweden) which automatically added an excess of 40% sodium hydroxide (NaOH), distilled off the ammonia and titrated it against 0.2-molar hydrochloric acid (HCl) to give nitrogen content. Indicators used were bromocresol green and methyl red. The N content was multiplied by 6.25 to give crude protein content. Samples were corrected for dry matter.

3.5.7 Cobalt content
Hay and barley samples were sent to Hill Laboratories, Hamilton, New Zealand for Co analysis during the experiments and treated as described in Appendix A.

3.6 Tissue & digesta sample preparation and analysis

3.6.1 Vitamin B$_{12}$ in abomasal digesta
Abomasal samples were collected in 45 ml centrifuge tubes and covered with aluminium foil until centrifuged at 28 000 g for 30 min at 4°C. The supernatant was decanted off into clean 30 ml conical tubes and a 2 ml sub-sample placed into a labelled 5 ml polyethylene tube. Samples were stored at -20°C until required for analysis when the sub-sample was sent to Gribbles Alpha Scientific (Hamilton, New Zealand) for vitamin B$_{12}$ analysis by chemiluminescence. See Appendix A for further detail. The resulting supernatant was also used for digesta marker analysis.

Repeatability of determination of vitamin B$_{12}$ in vitamin deficient abomasal samples (n=10) gave a mean of 735 pmol/l with a Standard Deviation (SD) of 30 pmol/l and Coefficient Variation (CV) of 4.12%. Abomasal samples (n=10)
from vitamin B\textsubscript{12} sufficient sheep gave a mean of 8950 pmol/l, SD of 372 pmol/l and CV\% 4.16\%. Recovery of vitamin B\textsubscript{12} in abomasal fluid spiked with vitamin B\textsubscript{12} (cyano-cobalamin V-2878, Sigma, USA) to increase concentration by 2000, 10 000 and 20 000 pmol/l was 95\%, the linear relationship of observed vitamin B\textsubscript{12} in pmol/l = 0.9454 x the concentration of added vitamin B\textsubscript{12} in pmol/l – 255 with correlation coefficient of 0.9997 (pers. Comm. Julie Furlong, Lincoln University, New Zealand).

3.6.2 Vitamin B\textsubscript{12} in plasma samples
Ten 10 ml vacutainer tubes containing sodium heparin (143 IU, Becton Dickinson, USA) were collected at the outset from a sheep with low plasma vitamin B\textsubscript{12} concentration housed in the animal laboratory. A standard plasma sample was sent with each batch of samples for use as an internal reference of analytical accuracy. Experimental blood samples were also collected in 10 ml heparinised vacutainers. Ethylenediaminetetra Acetic acid (EDTA) is considered to have significant effect on the analysis of vitamin B\textsubscript{12} and therefore, was avoided as an anticoagulant (Diagnostic-Products-Corporation(DPC), 1999). Samples were covered from light during transport and spun at 3000 rpm at 4\(^\circ\)C for 10 min. Plasma was pipetted off using disposable pipettes and stored in clean plastic vials at -20\(^\circ\)C until required for vitamin B\textsubscript{12} analysis. Samples were analysed at Gribbles Alpha Scientific, Hamilton, New Zealand by chemiluminescence (Babson, 1991). See Appendix A for further detail.

3.6.3 Digesta marker concentration
Polyethylene glycol (PEG) was used as the liquid phase digesta marker. Animals detailed in Chapter 5 received 2 g PEG/animal/d and those detailed in Chapter 6 received 10 g PEG/animal/d as described in the respective Chapters.
Approximately, 40 ml of abomasal digesta was collected and covered with aluminium foil until centrifuged at 28 000 g for 30 min at 4°C and the supernatant was then stored at -20°C until required for analysis of PEG concentration. Polyethylene glycol in abomasal supernatant was determined by a modification of the method described by Ulyatt, (1964), which was based on the turbidimetric method of Hyden, (1956). Details are described in Appendix A.

3.6.4 Fatty acid analysis
Rumen liquid (30-40 ml) was collected for fatty acid analysis during the trial described in chapter 6. Collection was via a 50 ml syringe connected to flexible plastic tubing with 2 mm holes drilled in the sampling end. Once collected, 20 ml rumen liquid was dispensed into 30 ml plastic conical tubes, pH measured and then placed on ice. The last 10-20 ml of rumen liquid was placed in a second 30 ml conical tube and acidified immediately for ammonia analysis with 5 drops of 2M sulphuric acid to bring the pH below 3, and then placed on ice. Samples were stored on ice during sampling and subsequently stored at −20°C until required. Fatty acid concentration was determined by Gas Chromatography. Details are described in Appendix A.

3.7 Animal Ethics
All experimentation was conducted under Lincoln University Animal Ethics Committee guidelines. Those described in Chapter 4 were performed under application number 959, protocol number LU 21/02 and those in Chapters 5 and 6 under application number 76.
CHAPTER 4: Calibration of Plasma Response to Vitamin $B_{12}$ Entry Rate

4.1 Introduction

The conventional balance techniques to determine nutrient requirement are not suitable to estimate vitamin $B_{12}$ requirements (Grace, 1994) because of the complexity of its metabolism. Production of the vitamin occurs in the rumen and relies on a regular intake of cobalt for its synthesis by rumen bacteria. Without information on this rate of synthesis it is impossible to determine the coefficient of absorption of the vitamin in the small intestine. Moreover, since the biliary route is a route of excretion and the vitamin can be synthesised by bacteria in the hindgut, conventional balance approaches are not helpful, quite apart from problems of estimation of the vitamin in digesta.

The present chapter describes an experiment designed to evaluate the depletion-repletion approach used by Suttle (1974) to estimate rate of copper absorption for the determination of vitamin $B_{12}$ absorption. Vitamin $B_{12}$-depleted sheep were used to determine whether a change in plasma vitamin $B_{12}$ initially reflects a change in the rate of entry of the vitamin into the bloodstream and to quantify the relationship. If successful, this would open up the possibility of predicting rates of absorption of the vitamin for use in other studies of factors affecting rates of synthesis of the vitamin in the rumen on the assumption that infused vitamin $B_{12}$ behaves biologically similarly to endogenous vitamin $B_{12}$. 
4.2 Materials and methods

4.2.1 Experimental design

Eight rumen and abomasally cannulated vitamin B₁₂-depleted ewes, as described in Chapter 3, were offered approximately 400 g of hay and 250 g of crushed barley to continue to deplete plasma vitamin B₁₂ levels. The average composition of 7 samples of the diet is given in Table 4.1. The ewes were placed in a paired 4x4 Latin square design. Each treatment period of the Latin square was run for a total of 21 d, which included a 96 h infusion and a 17 d recovery period. Intra-jugular vitamin B₁₂ infusions were set at four levels of treatment, namely 0 (control), 7.5, 50 and 200 nmols vitamin B₁₂/day. These were based on previous experience of vitamin B₁₂ infusions (Ludemann, 2001) in which rates ranging from 0 - 2500 nmol vitamin B₁₂/d for 48 h were used and in which the highest dose rate increased plasma vitamin B₁₂ concentration to over 100 000 pmol/l. Based on these data the highest rate of infusion was established as likely to produce concentrations in plasma at the upper end of the range seen in practice, namely below 5000 pmol/l.

Table 4.1: Mean composition of the components of the diet offered to sheep during an infusion of vitamin B₁₂ into the jugular vein.

<table>
<thead>
<tr>
<th>Feed</th>
<th>Cobalt Content (mg/kg DM)</th>
<th>Dry Matter %</th>
<th>% Organic Matter in DM</th>
<th>% Crude Protein in DM</th>
<th>Metabolisable Energy (MJ ME/kg DM)</th>
<th>% Digestible Organic Matter in Dry Matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meadow Hay</td>
<td>0.061</td>
<td>90.6</td>
<td>92.8</td>
<td>9.9</td>
<td>8.3</td>
<td>51.9</td>
</tr>
<tr>
<td>Crushed Barley</td>
<td>0.01</td>
<td>86.8</td>
<td>97.8</td>
<td>10.4</td>
<td>14.1</td>
<td>88.1</td>
</tr>
<tr>
<td>Urea</td>
<td>0</td>
<td>95</td>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Diet average</td>
<td>0.041</td>
<td>89.1</td>
<td>94.8</td>
<td>10.1</td>
<td>10.6</td>
<td>66.0</td>
</tr>
</tbody>
</table>
4.2.2 Preparation of infusates

The vitamin was provided as Prolaject (Bomac Lab Ltd, NZ, 1000 µg-hydroxycobalamin vitamin B₁₂/ml) diluted in isotonic sterile saline solution. Infusion bags used were Viaflex® 1000 ml 0.9% sodium chloride (Baxter HealthCare Pty Ltd, N.S.W, Australia). A concentrated preparation bag (4428 nmol hydroxycobalamin /l) was produced by adding 6 ml of Prolaject to the bag after an equivalent volume of saline had been removed. The higher concentration was used to produce the lower infusion concentrations by dilution. Basically 100, 25 and 3.75 ml of solution from the preparation bag was injected into new recipient saline infusion bags after the equivalent volume of saline had been removed. The dilution of 1:10, 1:40 and 1:267 from the preparation bag made up infusion rates of 200, 50 and 7.5 nmol vitamin B₁₂/d, respectively. Four bags of each concentration were made (one bag per 2 d for each sheep on each infusion rate). Infusion rate was regulated at 0.32 ml/minute, equivalent to 461 ml/day. Infusion bags were kept in a darkroom next door to the experimental sheep, an example of which can be seen in Chapter 6 Plate 6.1. All infusion lines were sterilised by flushing with alcohol, rinsed with sterile saline solution and the outside of the lines spray-painted black to minimise any destabilisation of the vitamin B₁₂ solution by light and the lines primed with solution prior to the start of experiments. Vialon™ Insyte™ 16 gauge 1.7 x 45 mm IV catheters (Becton Dickinson, USA) were stitched into the left jugular vein. Infusion lines were connected once all catheters were in place. Bags were weighed prior to and after infusion and actual amounts given calculated by difference. All infusion lines were pumped by a ChemLab (England) peristaltic pump - model CPP30.
4.2.3 Blood sampling
Blood collection was done via the jugular catheter during infusions, discarding the first 5 ml collected and via the jugular vein during the recovery period into 10 ml vacutainers. Samples were taken directly prior to attachment of infusion lines, time 0 h, and 4, 8, 12, 24, 48, 72, 96, 100, 104 and 108 h and at 5, 6, 8, 11, 14, 18 and 21 d after the start of the infusion and analysed as described in Chapter 3.

4.2.4 Statistical Analysis
Plasma vitamin B\textsubscript{12} concentrations were analysed using repeated measures ANOVA to determine significance of differences and any possible interactions. Due to the large scale of the responses, raw data were log\textsubscript{10} transformed. Exponential curves were fitted to the appearance plasma vitamin B\textsubscript{12} response (up to time 96 h) using Sigmaplot 8 (Version 14, SPSS Inc, 1999). Due to the fact that plasma responses did not reach a plateau in all cases, particularly in lower treatment groups, the appearance curve was used to obtain maximum peak values for plasma vitamin B\textsubscript{12} where the curve reached the upper asymptote. Genstat 8 (Release 8.2, Lawes Agricultural Trust, Rothamstead Experimental Station, 1997) was used for statistical analysis and graphs plotted using Sigmaplot 8.
4.3 Results

4.3.1 Feed intake
Animals generally consumed all the feed offered which produced an intake of 0.03 mg Co/d and 7 MJME/d. The starting and finishing live-weight of the experimental animals averaged 46 ± 1.14 and 44.9 ± 0.66 kg, respectively.

4.3.2 Plasma vitamin B$_{12}$ response to intravenous infusion of vitamin B$_{12}$
The mean plasma vitamin B$_{12}$ response to infusion is given in Figure 4.1. Infusion of saline maintained relatively stable base line plasma vitamin B$_{12}$ levels, values fluctuating between 300 - 400 pmol/L. Pre-infusion plasma vitamin B$_{12}$ levels were similar for all groups but rose sharply within 4 h after the start of infusion of vitamin B$_{12}$ and achieved maximum values after 96 h. Peak values were proportional to infusion rate and highly significantly different between treatments. Plasma levels declined once vitamin B$_{12}$ infusions stopped and had generally returned to pre-treated levels by the end of each equilibration period, 17 d later. Due to the very high plasma levels obtained in sheep infused with 200 nmol vitamin B$_{12}$ /d, some carry-over effect was observed but this had no detectable effect on response to the following treatment. There were highly significant time and treatment effects and time x treatment interactions (P<0.001 in all cases).
**Figure 4.1:** Mean response of log₁₀ plasma vitamin B₁₂ to intravenous infusion of vitamin B₁₂ (hydroxycobalamin) at rates of 0 (●), 7.21 (∇), 48.3 (▲) and 193 (◊) nmol B₁₂ /d while offered a diet containing 0.03 mg Co/d. ▭ denotes period of infusion. Log₁₀ SEM = 0.091.

Fitted maximum responses appearing in the plasma up to 96 h are presented in Figure 4.2. To compensate for the fact that plateau values may not have been reached at the lower rates of infusion of vitamin B₁₂, exponential curves were used to determine maximum values using Sigmaplot 8 (version 14 SPSS Inc, 1999). These appearance asymptote values were subsequently used for calculations of vitamin B₁₂ production and presented on Figure 4.2.
Figure 4.2: Fitted maximum appearance response of vitamin B₁₂ in the plasma during 96 h intravenous infusion of vitamin B₁₂ (hydroxycobalamin) at rates of 0 (●), 7.21 (□), 48.3 (▲) and 193 (◊) nmol B₁₂/d while offered a diet containing 0.03 mg Co/d.
4.4 Discussion
Infusions of 200 nmol/d of vitamin B_{12} resulted in peak plasma appearance levels of vitamin B_{12} of around 10 000 pmol/l. These were well above generally observed clinical values which are usually below 5000 pmol/l when measured by Gribbles Alpha Scientific (Hamilton, New Zealand) though values of >5000 pmol/l have been observed after supplementation of sheep on pasture with more than one Co bullet (Gruner et al., 2004b). The infusion rate of 200 nmol/d was used in the absence of reliable data in the literature to ensure data would encompass results obtained from subsequent Co infusions into the rumen. It was not known, at the outset, what the response to infusion would be and what the uniformity of response between animals would be. The aim, however, was to ensure the entire natural response curve was covered so that a region of linearity and high sensitivity could be determined.

The major assumption in the use of this approach to estimate rate of absorption of vitamin B_{12} is that the infused vitamin behaves similarly to, and is transported by the same route as endogenous vitamin B_{12} and the vitamin entering the circulation through normal channels of absorption. The disappearance rate of vitamin B_{12} from the plasma was influenced by treatment, namely the lowest treatment rate (i.e. 7.5 nmol/d) was cleared from the plasma the fastest. There was no difference in disappearance rate between 50 and 200 nmol/d treatment rates. This result is surprising as it might have been expected that all plasma vitamin B_{12} would be cleared at the same rate, or at least the highest treatment level would be cleared the fastest. One could have anticipated greater saturation of transport proteins or even the possibility of unbound vitamin in the circulation. There is insufficient quantitative data on
the metabolism of transport proteins in sheep to assess whether they are present in excess normally and/or how quickly proteins are synthesised in response to vitamin B_{12} loading of the plasma.

There is one very important contrast of the current technique to that of the Cu depletion/repletion technique of which it is based on. The current model only infused vitamin B_{12} for 4 days which was arguably only marginally long enough to obtain steady state plasma concentration. Suttle (1974) infused Cu for 17 days and the rate of plasma increase reflected synthesis of the transport protein caeruloplasmin, as is the case with oral Cu repletion. In future studies, vitamin B_{12} infusions could be restricted to rates at and below 30-nmol vitamin B_{12}/d and continued for 14 – 21 days to provide stable plasma concentrations reflected by synthesis of transport proteins.

However, the fact that the plasma response to increasing infusion rates of vitamin B_{12} even at 200 nmol/l was tending to exponential (Figure 4.3) may suggest that transport proteins had not been saturated or that unbound vitamin B_{12} is not instantaneously excreted. Were that the case, one might have anticipated a plateau of plasma concentration if unbound vitamin is more susceptible to excretion probably via the kidney. Excretion via the kidney only appears to happen when total binding capacity of the plasma is exceeded (Cooksley et al., 1974). However, while the assumption of uniform behaviour has been made, given the distribution of data points, a linear or sigmoid relationship in Figure 4.3 can not be discarded due to the large gap in infusion rates, particularly between 50 and 200 nmol/d. A design which used lower rates of infusion would have been more informative.
Figure 4.3: Mean maximum plasma vitamin B₁₂ response to intra-jugular infusions of vitamin B₁₂ (as hydroxycobalamin) at rates of 0 (●), 7.21 (□), 48.3 (▲) and 193 (◊) nmol B₁₂/d while offered a diet containing 0.03 mg Co/d.

The magnitude of response of plasma vitamin B₁₂ concentration from the specific vitamin B₁₂ infusions highlights important response issues that need to be explained. The plasma vitamin B₁₂ response divided by the rate of infusion reveals increases of 44.4, 21.3 and 54.2 pmol plasma B₁₂/nmol B₁₂ infused for infusion rates of 7.5, 50 and 200 nmol B₁₂/d respectively. The plasma response at the 50 nmol/d rate of infusion was approximately half that at 7.5 and 200 nmol/d. This raises the possibility that the infused amount was actually closer to 25 nmol/d than 50 nmol/d. However after careful review of dilutions and procedures this was shown not to be the case. Had the infusion concentration been 25 nmol/d, the plasma response to infused vitamin B₁₂ would have been linear over the range of infusates used instead of the curvilinear response shown in Figure 4.3. The length of time allowed for plasma response to reach plateau was arguably too short, particularly at the lower infusion rates and
could, theoretically, have been responsible. From responses presented in Figure 4.2, it could be argued plasma concentrations were still responding to infusion when it was stopped and hence the plateau fitted was too low. It is difficult to explain the responses presented if they are a true reflection of what occurs when vitamin B₁₂ is naturally absorbed. It is possible that the response up to 7.5 nmol/d reflects the uptake of the infused vitamin B₁₂ on transport proteins. At the rate of infusion of 50 nmol/d, it is possible that the transport proteins could have become saturated and that the rate of excretion of unbound vitamin determines the plasma response up to the rate which equates with maximum rate of excretion, degradation or transfer to the tissues. However, if there is a maximum rate of removal from plasma, it would be expected that plasma response at 200 nmol/d would not plateau as in Figures 4.1 and 4.2. Clearly to fully understand the plasma response to infused vitamin B₁₂, the dynamic of transport proteins need to be measured but is beyond the scope of this experiment.

The disappearance curves of vitamin B₁₂ from plasma following intravenous infusion of vitamin B₁₂ (Figure 4.1) appeared to have two components with different half-lives. The initial clearance was very rapid, within hours of the cessation of infusion. The second and slower component cleared the bloodstream over a number of days. The rapid initial clearance may represent the early release of vitamin B₁₂ bound to transcobalamin (TC) II and the slower second component may represent release from TC I. The TC II-cbl complex is believed to have a half-life between 5 (Chanarin et al., 1978) and 90 min (Schneider et al., 1976) in rabbits and humans (Hom, 1967). Rapid clearance of TC II-cbl from the plasma probably occurs via the liver, the functional site of
vitamin B₁₂. Free cbl released from the liver is considered to be able to re-enter the bloodstream bound to TC II for transport to other tissues or bound to TC I which has a longer ½ life of approximately 10 d in non-human cells (Seetharam & Alpers, 1994) and 9-10 d in humans (Hom, 1967) and appears to act as a plasma ‘buffering’ pool. A proposed relationship between TC I and TC II is presented in Figure 4.4. After initial rapid absorption by TC II, there is an equilibrium with TC I. Such a dynamic between TC I and II could help explain the rapid clearance of B₁₂ from plasma after infusion of B₁₂ (within hours) and then a lag (over days) until plasma B₁₂ level return to normal as seen in Figure 4.1. When measuring plasma vitamin B₁₂, no account is taken as to which binding protein the vitamin is bound to due to the protein denaturing boiling step involved in the methodology.

![Figure 4.4: Simplified schematic diagram of a proposed relationship between transport proteins transcobalamin I and II.](image)

Estimation of extra-cellular fluid (ECF) volume of 10.5 l has been made on the assumption that it comprises 245 ml/kg live weight (Coghlan et al., 1977) for ewes in low body condition and of the live-weight (43 kg) of the present sheep. The total vitamin B₁₂ retained in the ECF can be calculated using peak plasma vitamin B₁₂ concentrations (Figure 4.2) less the baseline values at the 0 nmol/d infusion rate (naturally occurring vitamin B₁₂ in the bloodstream from native
Co present in the diet). When presented as a percentage of the total amount of vitamin B₁₂ infused over 4 days at the time of plateau ECF response, ECF vitamin B₁₂ represented 12.2%, 6.8% and 12.8% of infused vitamin B₁₂ at the infusion rates of 7.5, 50 and 200 nmol/d, respectively. With such a low proportion of infused vitamin appearing in the ECF, clearly a large proportion is involved in the dynamic between excretion and possible uptake into the tissues. For liver uptake to have accounted for the remaining infused vitamin B₁₂, based on liver mass of 15 g/kg live weight (Sykes et al., 1980), treated ewes would have needed to retain 9.77, 33.8 and 260 nmol vitamin B₁₂/kg fresh weight of liver each day. Considering that the normal concentration of vitamin B₁₂ in liver in sheep is above 220 nmol/kg liver fresh weight (Grace, 1994), it seems unlikely that accumulation of an additional 260 nmol vitamin B₁₂/kg liver fresh weight per day would have occurred. Clearly, at this point, liver biopsy samples would have been helpful, but due to the problems and potential dangers detailed in Chapter 3, changes in vitamin B₁₂ concentrations in liver are not available. After initial problems with the original trial animals, these sheep were considered too valuable to risk the death of them during a difficult liver biopsy. The livers were abnormally friable presumably due to prolonged exposure to vitamin B₁₂ deficiency from their property of origin. Urinary vitamin B₁₂ may also have helped explain the rapid clearance of vitamin B₁₂ from the ECF but was not measured. The need to house the animals in sizable wood-lined pens in a woolshed prevented the collection of urine at the time of planning the experiment. Smith & Marston (1970) showed only 2.5% of an oral dose 1 mg Co containing 0.2 mCi ⁶⁰Co appeared in urine after 14 days but did not measure how much Co was actually absorbed.
With only 7-13% of infused vitamin present in the ECF, the other 87-93% must either have been taken up by cells or excreted from the body. Urinary excretion is considered negligible and faecal excretion via the biliary route is very difficult to measure and no satisfactory estimate has been made. The problem in attempting to estimate excretion from the plasma into faeces is that only a small proportion of the vitamin produced in the rumen is absorbed (see Chapter 5), so that a large amount appears in the faeces relative to endogenous excretion. Also, compounding this is the probable additional production of vitamin B$_{12}$ in the lower gut by bacteria. Therefore, of the vitamin appearing in the faeces it would be impossible to differentiate the origin of the vitamin and particularly the amount coming from biliary excretion.

If there is a dynamic interplay between plasma and tissues, it is possible that apparent movement out of plasma could be lower when tissue levels are replete. Gruner et al., (2004b) showed lambs from cobalt bullet supplemented ewes which were subsequently supplemented with an intramuscular injection of vitamin B$_{12}$, showed a bigger plasma vitamin B$_{12}$ response (104 to 1500 pmol/l) than lambs suckling unsupplemented ewes (95 to 750 pmol/l) even though, at the time of injection, plasma vitamin B$_{12}$ concentrations were identical. The dynamic relationship between tissues and plasma could be bidirectional, for example, movement of vitamin B$_{12}$ out of plasma into depleted tissues could dampen the increases in plasma concentration at high vitamin B$_{12}$ intakes, and conversely, at high tissue concentrations movement of the vitamin out of tissue into plasma limits reduction in plasma concentration if vitamin B$_{12}$ absorption is reduced. In the absence of tissue concentrations, results from the current research suggest that in short-term supplementation, plasma
concentration is determined by the rate of entry of the vitamin. This is supported by a relatively constant 7-13% of infused vitamin appearing in the ECF pool irrespective of infusion rate.

If the data from outside the plasma normal range in Figure 4.3, i.e. the response to 200 nmol B\textsubscript{12}/d are omitted, the infusion rate of vitamin B\textsubscript{12} required to obtain a particular plasma vitamin B\textsubscript{12} concentration can be calculated and are given in Figure 4.5.

![Graph showing the relationship between plasma vitamin B\textsubscript{12} concentration and vitamin B\textsubscript{12} infusion rate. The equation Y = 238 + 102 x\textsuperscript{0.74} and R\textsuperscript{2} = 0.945 are displayed.]

**Figure 4.5:** Working range of maximum individual plasma vitamin B\textsubscript{12} response to intra-jugular infusions of vitamin B\textsubscript{12} (as hydroxycobalamin) at rates of 0 (●), 7.21 (□) and 48.3 (▲) nmol B\textsubscript{12} /d while offered a diet containing 0.03 mg Co/d.

There was considerable individual variation to response of infused vitamin B\textsubscript{12} the source of which is not known. It may be attributable to variation in tissue concentrations as discussed earlier but is difficult to know without this
information. One possible source of variation in tissue concentration could be a previously high rate of infusion in the Latin square, but there was no evidence for such carryover effects. Similarly, blocking by animal showed there was no significant effect (P=0.100) of animal within each treatment which could indicate inherent genetic variation. Suttle, (1974) found repletion of copper deficient sheep with intravenous copper for 17-30 d reduced the variation in response when comparing individual ‘slow’ and ‘fast’ responding sheep to an oral dose of copper. His conclusion was that individual variation was due to differences in absorption rather than in post absorptive metabolism of the mineral. In future work, it would be advantageous to determine whether tissue (predominantly liver), vitamin B\textsubscript{12} concentration affect plasma vitamin B\textsubscript{12} concentration and hence response to intravenous infusion of vitamin B\textsubscript{12}. Increasing the duration of infusion period to 8 - 10 d may reduce individual variation observed as it would have allowed more time for response to reach a maximum asymptote and fitted responses would not have been required.

Despite these reservations, the rate of entry of the vitamin into the bloodstream needed to achieve a particular plasma vitamin B\textsubscript{12} response when absorbed naturally can be predicted from Figures 4.2 and 4.6. These data suggests that the plasma reference range used in New Zealand \textit{viz}, 336 to 500 pmol vitamin B\textsubscript{12}/l (Clark \textit{et al.}, 1989) will be maintained by rates of vitamin B\textsubscript{12} entry into the bloodstream of between 0.15 to 3.3 nmol/d (from Figure 4.6) above absorption of vitamin B\textsubscript{12} on a diet supplying 0.03 mg Co/d. Once total vitamin B\textsubscript{12} flow past the abomasum is obtained, absorption coefficient can be calculated. The Australian plasma vitamin B\textsubscript{12} range is lower than that used in New Zealand, namely 200 to 400 pmol/l vitamin B\textsubscript{12}/l (Judson \textit{et al.}, 1987).
The bottom of this range is lower than that of control animals maintained on 0.03 mg Co/d, being 316 pmol/l. The top of the Australian plasma reference range (400 pmol/l) would be maintained by an infusion of 1.1 nmol vitamin B₁₂/d above natural absorption of vitamin B₁₂ from a diet containing 0.03 mg Co/d.

![Graph showing vitamin B₁₂ infusion rate vs. fitted plasma vitamin B₁₂ response](image.png)

**Figure 4.6:** Working range of mean fitted plasma vitamin B₁₂ response to intra-jugular infusions of vitamin B₁₂ (as hydroxycobalamin) at rates of 0 (●), 7.21 (□) and 48.3 (▲) nmol B₁₂/d while offered a diet containing 0.03 mg Co/d.

Historically, the generally accepted requirement for vitamin B₁₂ came from the studies of Smith and Marston (1970). These authors estimated vitamin B₁₂ requirement for ‘normal’ metabolism to be 11 ug/d, made up from 5 ug/d absorbed from ruminal synthesis and 6 ug/d that needed to be injected to maintain constant serum vitamin B₁₂ without increasing liver concentration on a diet containing 0.03 mg Co/d. The diets used by Smith and Marston (1970)
and the current data both contained 0.03 mg Co/d, therefore the 6 ug/d needed to be injected is the equivalent of infusing 4.4 nmol vitamin B\textsubscript{12}/d. Infusing 4.4 nmol/d would maintain a plasma concentration of 542 pmol/l, slightly higher than the New Zealand reference range of marginal vitamin B\textsubscript{12} status. Assuming the reference range used in New Zealand maintains normal metabolism of vitamin B\textsubscript{12} and doesn’t increase liver concentration, it implies the net requirement for vitamin B\textsubscript{12} is lower than previous estimates of 11 ug/d (Smith & Marston, 1970).

The results from this chapter have calibrated the plasma vitamin B\textsubscript{12} response to known amounts of vitamin B\textsubscript{12} infused and found a response seen in practice. While individual variation is evident, this information will allow a unit change in plasma vitamin B\textsubscript{12} concentration to be used to estimate changes in rates of absorption of the vitamin as a result of dietary change. Future modifications to the infusion approach for quantifying vitamin B\textsubscript{12} rate of entry include;

- Measurement of liver vitamin B\textsubscript{12} concentration before and after infusion of vitamin B\textsubscript{12}. Alternative experimental animals and/or surgical equipment could provide a solution.
- Measurement of urinary vitamin B\textsubscript{12} concentration. While urinary excretion is considered negligible in Co deficient sheep, confirmation of this would have helped in the interpretation of the experiment.
- Intravenous infusion rates of vitamin B\textsubscript{12} could have been held at and below 30 nmol/d to obtain plasma responses closer to the normal range seen in practice.
- Infusions rates of vitamin B$\text{_{12}}$ could have been continued for 14 – 21 days. This may allow more time for plasma vitamin B$\text{_{12}}$ to reach a true equilibrium with infused vitamin B$\text{_{12}}$.

- Measurement of plasma transport proteins TC I and TC II to help better understand plasma vitamin B$\text{_{12}}$ pool responses to increasing supplementation of vitamin B$\text{_{12}}$ or increasing Co intakes.
CHAPTER 5: Intra-ruminal Cobalt Infusions

5.1 Introduction
One problem with the use of plasma concentrations to diagnose vitamin B\textsubscript{12} deficiency is our lack of understanding of the quantitative relationship between these and the rate of absorption or transport of the vitamin into the body. The fact that the recommended reference range for interpretation of vitamin B\textsubscript{12} status are so broad, is probably, in part at least, a reflection of this lack of knowledge. The previous chapter quantified plasma response in terms of the amount of directly infused vitamin B\textsubscript{12} required to achieve a specific change in plasma concentration. With this information the dietary Co supply required to achieve a particular rate of vitamin B\textsubscript{12} absorption can be determined.

The work described in the current chapter was an attempt to measure the range of dietary Co over which the plasma vitamin B\textsubscript{12} response curve in previously vitamin B\textsubscript{12}-depleted sheep is sensitive to change in rate of vitamin B\textsubscript{12} production in the rumen.

If the flow of vitamin at the abomasum is also measured this would provide data on the rate of net production of the vitamin. Also with the plasma response data determined in the previous Chapter, estimates of the coefficient of absorption of the vitamin can be calculated, on the assumption that any vitamin synthesised in the hindgut is not absorbed.
5.2 Materials and methods

5.2.1 Experimental design & sampling procedure
The eight rumen and abomasally cannulated ewes, described in Chapter 3, were used in a paired 4 x 4 Latin square design. All sheep were offered approximately 400 g of hay and 250 g of crushed barley as described in Chapter 3, and were infused with one of four rates of Co directly into the rumen for 4 d (96 h) followed by a 19 d recovery period. There were few data available on which to calculate rate of infusion. The rates were therefore below the lowest single-shot dose of 1 mg Co used by Suttle et al., (1989), with the aim of finding the most responsive part of the response curve of vitamin B\textsubscript{12} in plasma from Co dosing in the rumen. Greater differentiation between treatment groups was expected since single-shot treatments varying from 1 – 32 mg Co showed the same response in plasma vitamin B\textsubscript{12} concentration (Suttle et al., 1989). From previous experience from single shot intra-ruminal cobalt injections of between 0 - 1.0 mg Co/d (Ludemann et al., 2005) showed maximum response of 550 pmol/l 48 h after injection of the highest rate which returned to original levels after 8 – 10 d. While these responses were within the responsive range to treatment with vitamin B\textsubscript{12}, the infusion of the Co solution during 4 d was anticipated to provide a greater range of response. Infusions were therefore set at 0 (control), 0.20, 0.60 and 1.0 mg Co/d during 4 d.

Digesta flow at the abomasum was measured with a liquid phase marker infused via a separate infusion line commencing 5 d prior to the start of Co infusions to allow time to equilibrate. Polyethylene glycol (PEG) 4000 was used as a liquid phase marker of digesta instead of the more common, chromium ethylenediaminetetra acetic acid (Cr-EDTA) which interferes with
vitamin B₁₂ analysis by chemiluminescence. Abomasal samples for the estimation of digesta flow were taken after feeding and at 6 h intervals on the day before and after, as well as on the 4 days of Co infusion. Samples were pooled and measured for each day.

Blood samples were taken on days 0, 5 to 12, 14, 16, 19, 23 and 28 d of each treatment period. Samples were prepared and analysed for vitamin B₁₂ as described in Chapter 3. The sampling timetable is given in Table 5.1.

<table>
<thead>
<tr>
<th>Day</th>
<th>Mon</th>
<th>Wed</th>
<th>Fri</th>
<th>Mon</th>
<th>Fri</th>
<th>Wed</th>
<th>Thu</th>
<th>Fri</th>
<th>Sat</th>
<th>Sun</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co Infusion</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Marker infusion</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abomasal sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Blood samples</td>
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<td></td>
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</tr>
</tbody>
</table>

5.2.2 Preparation and delivery of infusates

A ChemLab (England) peristaltic pump - model CPP30 was used to deliver all infusates and was housed in a darkroom next door to the experimental sheep. Actual volumes delivered were calculated as the difference between volume before and after infusion. An example of infusion set up can be seen in Chapter 6 Plate 6.1 (page 85).

5.2.2.1 Cobalt solution

Solutions were prepared by dissolving an accurately measured 0, 0.517, 1.550 or 2.584 g of hydrated cobalt sulphate (CoSO₄.7H₂O) in 1000 ml of nanopure water. Aliquots of 100 ml were transferred into 25 l containers and infusion solutions made up to volume with nanopure water. Final concentrations were
calculated as 0, 0.434, 1.302 and 2.169 mg Co/l, respectively. The solutions were regulated by 0.76 mm i.d. pump tube (Astoria Pacific International, Oregon, USA) delivering 461 ml/d. The solutions were stored in clean plastic containers and kept refrigerated. Samples were sent to R J Hill Laboratories Ltd, Hamilton, New Zealand for analysis of Co concentration (for methodology see Chapter 3).

5.2.2.2 Digesta flow marker
Polyethylene glycol solutions were prepared by weighing 217 g PEG 4000 into a 50-litre container, which was then made up to volume with nanopure water. The PEG infusion contained 4.34 g PEG/L (2 g PEG/animal/d) and was regulated by 0.76 mm i.d. pump tube (Astoria Pacific International, Oregon, USA) delivering 461 ml/d. Abomasal digesta flow (l/d) was calculated by the ratio of amount of marker infused each day against the concentration of marker in abomasal fluid measured as described in Chapter 3.

5.2.3 Statistical Analysis
Repeated measures ANOVA on plasma and abomasal vitamin B$_{12}$ concentrations over time were used to determine significance of differences. Exponential curves were fitted to appearance plasma vitamin B$_{12}$ concentration using Sigmaplot 8 (Version 14, SPSS Inc, 1999). Statistical analysis was done using Genstat 8 (Release 8.2, Lawes Agricultural Trust, Rothamstead Experimental Station, 1997).
5.3 **Results**

5.3.1 **Feed intake & cobalt analysis**
Animals generally consumed all the feed offered which provided 0.03 mg Co and 7 MJME/d. The average composition of 7 samples of the diet is given in Table 5.2. Actual concentration of Co in the infusates was 0, 0.505, 1.46 and 2.44 mg Co/L and provided 0, 0.23, 0.64 and 1.08 mg Co/d.

<table>
<thead>
<tr>
<th>Feed</th>
<th>Cobalt Content (mg/kg DM)</th>
<th>Dry Matter %</th>
<th>% Organic Matter in DM</th>
<th>% Crude Protein in DM</th>
<th>Metabolisable Energy (MJ ME/kg DM)</th>
<th>% Digestible Organic Matter in Dry Matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meadow Hay</td>
<td>0.061</td>
<td>90.6</td>
<td>92.8</td>
<td>9.9</td>
<td>8.3</td>
<td>51.9</td>
</tr>
<tr>
<td>Crushed Barley</td>
<td>0.01</td>
<td>86.8</td>
<td>97.8</td>
<td>10.4</td>
<td>14.1</td>
<td>88.1</td>
</tr>
<tr>
<td>Urea</td>
<td>0</td>
<td>95</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diet average</td>
<td>0.041</td>
<td>89.1</td>
<td>94.8</td>
<td>10.1</td>
<td>10.6</td>
<td>66.0</td>
</tr>
</tbody>
</table>

5.3.2 **Plasma vitamin B\(_{12}\) response to intra-ruminal infusion of Co**
The changes in mean plasma vitamin B\(_{12}\) concentrations observed during the infusion of Co into the rumen are given in Figure 5.1. There was a significance time x treatment effect (P<0.001). Plasma vitamin B\(_{12}\) concentrations of animals receiving Co infusion increased from 300 to around 750 – 800 pmol/l. Control ewes maintained a base-line plasma vitamin B\(_{12}\) level of approximately 250 pmol/l.

The pooled mean plasma vitamin B\(_{12}\) response of each treatment group over the entire treatment period is given sequentially in Figure 5.2. Plasma vitamin B\(_{12}\) levels of all treated sheep started at similar levels to those of control ewes but rose rapidly within 4 h after the start of infusion. Although treatments had a significant carry-over effect on starting plasma values they appeared to have
no effect on the magnitude of response from Co infusions. Plasma levels declined within 24 h after Co infusion stopped and returned to pre-treatment levels by the end of each treatment, 19 d later. No significant difference in peak plasma vitamin B$_{12}$ concentration was detected between 0.2, 0.6 and 1 mg Co/d infusion rates, although clearly the trend was for maximum concentration to be determined by rate of Co infusion.

Fitted maximum plasma vitamin B$_{12}$ responses during the infusion periods between are presented in Figure 5.3. Asymptote values were predicted from the fitted appearance curves and subsequently used for calculations of vitamin B$_{12}$ absorption.

![Graph showing plasma vitamin B$_{12}$ concentration over time](image)

**Figure 5.1:** Mean plasma vitamin B$_{12}$ response to intra-ruminal infusions of cobalt (as cobalt sulphate) at rates of 0 (●), 0.23 (□), 0.64 (▲) and 1.08 (◊) mg Co/d while offered a diet containing 0.03 mg Co/d. — denotes period of infusion.
Figure 5.2: Pooled mean plasma vitamin $B_{12}$ response of all animals to ruminal cobalt infusions at rates of 0 (●), 0.23 (□), 0.64 (▲) and 1.08 (◊) mg Co/d while offered a diet containing 0.03 mg Co/d. ▬ denotes period of infusion.

Figure 5.3: Fitted mean maximum vitamin $B_{12}$ plasma concentration during 96 h intraruminal infusion of Co at rates of 0 (●), 0.23 (□), 0.64 (▲) and 1.08 (◊) mg Co/d while offered a diet containing 0.03 mg Co/d. ▬ denotes period of infusion.
5.3.3 Digesta Flow
The amount of PEG infused proved insufficient to provide concentrations at the abomasum which allowed accurate analysis. As a consequence, estimated digesta flow rates were variable and unrealistically high. Since the animals were offered a fixed amount of the same feed each day and generally consumed all of it throughout the trial, irrespective of treatment, digesta flow was calculated from data from an additional sub-trial. Basically, four of the trial animals were housed, fed and watered exactly as in the original trial. The rate of PEG infusion was increased to 10 g PEG/animal/d. Abomasal samples were taken daily for 8 d and analysed for PEG concentration and rate of flow calculated from these individual samples. The data for individual sheep are given in Table 5.3. Average daily liquid flow rates were calculated at 14.6 l/d ± 0.463 (SEM); the uniformity of flow between individuals and days gave confidence in applying these flow rates to sheep in the original experiment consuming the same amounts of the same diet.

**Table 5.3:** Daily digesta flow rates (litres/d) calculated from digesta PEG concentration during retrospective sub-trial.

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep 1</td>
<td>15.0</td>
<td>11.7</td>
<td>14.4</td>
<td>13.7</td>
<td>17.4</td>
<td>12.4</td>
<td>13.2</td>
<td>9.5</td>
<td>13.4</td>
</tr>
<tr>
<td>Sheep 2</td>
<td>14.3</td>
<td>15.7</td>
<td>13.7</td>
<td>12.0</td>
<td>11.9</td>
<td>14.0</td>
<td>15.1</td>
<td>17.7</td>
<td>14.3</td>
</tr>
<tr>
<td>Sheep 3</td>
<td>15.9</td>
<td>18.8</td>
<td>18.2</td>
<td>18.0</td>
<td>16.1</td>
<td>13.7</td>
<td>12.2</td>
<td>11.8</td>
<td>15.6</td>
</tr>
<tr>
<td>Sheep 4</td>
<td>15.0</td>
<td>15.6</td>
<td>18.8</td>
<td>13.7</td>
<td>13.4</td>
<td>12.7</td>
<td>16.4</td>
<td>13.9</td>
<td>14.9</td>
</tr>
</tbody>
</table>

14.6 ±0.46
5.3.4 Concentration of vitamin B\textsubscript{12} in abomasal fluid

Mean concentrations of vitamin B\textsubscript{12} in abomasal fluid are given in Figure 5.4. Control sheep maintained a base line abomasal fluid concentration of around 2000 pmol vitamin B\textsubscript{12}/l.

There was a significant effect of time and treatment (P<0.001 in both cases) on abomasal vitamin B\textsubscript{12} concentration (pmol/l) which had risen sharply within 24 h after the start of Co infusion. The highest concentrations (approximately 10 000 pmol/l) were obtained with infusion of 1.08 mg Co/d.

**Figure 5.4:** Change in mean vitamin B\textsubscript{12} concentration in abomasal fluid in response to 96 h intra-ruminal cobalt infusions at rates of 0 (●), 0.23 (□), 0.64 (▲) and 1.08 (◊) mg Co/d while offered a diet containing 0.03 mg Co/d. ▬ denotes period of infusion. Curved lines represent fitted maximum values.
5.4 Discussion

5.4.1 Diet
The aim was to use a diet that maintained the animal in a deficient state without inducing a severe dysfunction. Due to the low quality of the meadow hay given, crushed barley and urea were added to provide fermentable metabolisable energy to enhance cellulytic activity and microbial protein synthesis from rumen degraded protein supply in order to maintain reasonable intakes. Cobalt content was still kept to a minimum to maintain deficiency status (0.03 mg Co/d) and was still at levels of deficiency seen in practice rather than the extreme deficiencies used by other authors and which can impose a dysfunctional state (Kennedy et al., 1991; Kennedy et al., 1996). In pastoral situations, diets are very rarely seen with Co concentrations as low as used by these latter authors; the present data do represent Co intakes likely to be experienced in practice, albeit at the low end of the range.

5.4.2 Digesta flow
Polyethylene glycol (PEG) was used as a liquid phase marker due to the possibility of interference of chromium EDTA in the analysis of vitamin B₁₂ by chemiluminescence (Diagnostic-Products-Corporation(DPC), 1999). Infusion rates were kept low to minimize any modification of rumen fermentation. PEG can bind with tannins in the feed and remove the protection of protein against degradation by rumen microbes. As little as 1.8 g PEG will bind 1 g of tannins (Barry & Forss, 1983). However, concentrations of PEG were unexpectedly low and difficult to measure reliably so that the resulting liquid flow calculations were unexpectedly variable. The rate of infusion subsequently adopted, viz 10 g PEG/animal/d, was comparable to rates used by van’t Klooster et al., (1969). Moreover, tannins in meadow hay diets like the one used typically contain less
than 1% tannin (pers. Comm. Barry, TN) so any modification of the protein digestion in the rumen by PEG was probably minimal. Estimated liquid flow rates were very consistent both within and between animals, and were similar quantitatively to flow rates at the abomasum observed by previous workers in sheep consuming similar diets at the present rates of intake. For example, van 't Klooster et al., (1969) found Texel sheep had a mean flow rate of 14 - 20 l/d on intakes of 970 g/d of a hay diet. Phillipson & Ash, (1965) found Clun Forest sheep had flow rates of 10 – 12 l/d when offered 700 – 1000 g of a hay diet. The calculated average flow rate of 14.6 l/d (±0.463 SEM), which was subsequently used for calculations of total vitamin B_{12} flow through the abomasum, therefore would seem to be realistic and reliable.

5.4.3 Concentration of vitamin B_{12} in plasma after intra-ruminal cobalt infusions

The positive relationship between cobalt intake and plasma vitamin B_{12} concentrations has been well understood with ruminal synthesis of vitamin B_{12} increasing with increased supply of cobalt, but with diminishing efficiency (Suttle et al., 1989). The relationship found in the present work are presented in Figure 5.5. While plasma vitamin B_{12} increased with (or in response to) increasing cobalt intake, the size of the response similarly reduced with increasing Co supply over the range of 0.03 to 1.11 mg Co/d. This curvilinear trend is similar to that observed by other authors (Gawthorne, 1970; Marston, 1970; Hedrich et al., 1973; Suttle, 1986).
5.4.4 Plasma vitamin B$_{12}$ disappearance rate

The technique of estimating the amount of vitamin B$_{12}$ absorbed using infusions of the vitamin directly into the bloodstream against the plasma response from intra-ruminal Co infusions is based on the assumption that infused vitamin B$_{12}$ behaves identically, biologically, to vitamin B$_{12}$ absorbed from the alimentary tract. The magnitude of plasma concentration of vitamin B$_{12}$ after 200 nmol/d vitamin B$_{12}$ was infused was 14 times higher than the greatest response seen after Co was infused into the rumen (11 600 pmol/l vs 805 pmol/l, respectively), therefore a difference between trials might have been expected. However, after removing the response to infusion of 200 nmol B$_{12}$/d (Chapter 4), the difference between trial rate coefficients was still significant (P<0.001). The most plausible explanation for the difference between trials may simply have been caused by a delay of the reduction of
entry of the vitamin into the circulation after withdrawal of Co supplementation into the rumen. When Co infusions were stopped, plasma vitamin B\textsubscript{12} concentrations were still elevated 24 h later as seen in Figure 5.1. This reflects a time lag between production and appearance in the plasma. However, when vitamin B\textsubscript{12} infusions were stopped, there was no time lag and plasma concentration began to decrease immediately. To validate the assumption that infused vitamin B\textsubscript{12} behaves biologically similar to endogenous vitamin B\textsubscript{12}, transport proteins need to be measured.

The broad likeness in behaviour between modes of entry of the vitamin into the bloodstream is perhaps an indication that infused and absorbed vitamin behave similarly. For vitamin B\textsubscript{12} infusions, disappearance rate coefficients were 0.053, 0.020 and 0.018 as infusion increased from 7.5, 50 and 200 nmol/d, respectively. For Co infusions, disappearance rate coefficients were 0.0140, 0.0097 and 0.0076 as infusion increased from 0.2, 0.6 and 1 mg Co/d respectively. For both methods of entry into the plasma, vitamin B\textsubscript{12} was cleared the fastest at the lowest treatment level. This may imply equilibrium between plasma and tissue which maintains plasma levels with fall in rate of entry to the circulation, with plasma levels being maintained longer if the tissues are more replete after higher rates of infusion. Clearly data on changes in tissue concentrations will be required to test this hypothesis.
5.4.5 Flow of vitamin B\textsubscript{12} in abomasal fluid and its absorption

A positive curvilinear trend was noticed between cobalt intake and vitamin B\textsubscript{12} concentration in abomasal fluid (Figure 5.6). This production relationship saw a large response to the first increment of Co and reduced responses to further increments of Co. This could be explained by a diminishing efficiency of production of vitamin B\textsubscript{12}. This implies that the rate of production of vitamin B\textsubscript{12} is optimized at relatively low dietary concentrations and that the ability of microbes to capture additional cobalt and incorporate it into vitamin B\textsubscript{12} is diminished with increasing Co intake. Clearly, at the higher rates of infusion another factor other than Co was limiting the production of vitamin B\textsubscript{12} in the rumen. Another very plausible factor could be the amount of metabolisable energy available for microbial growth. The total amount of vitamin B\textsubscript{12} available for absorption can be calculated by converting the concentrations of vitamin B\textsubscript{12} in abomasal fluid into a flow of vitamin B\textsubscript{12} per day. From daily digesta flow rates of 14.6 l/d multiplied by vitamin B\textsubscript{12} concentration in abomasal fluid, daily flow of vitamin B\textsubscript{12} can be calculated (Figure 5.7). The maximum appearance of plasma vitamin B\textsubscript{12} per unit of metabolisable energy can be calculated from abomasal fluid concentrations in Figure 5.4, an energy intake of 7 MJME and digesta flow of 14.6 l/d equates to 4.75, 15.4, 17.4 and 20.2 nmol B\textsubscript{12}/MJME at Co intakes of 0.03, 0.26, 0.67 and 1.11 mg Co/d respectively.
Figure 5.6: Relationship between total ruminal cobalt supply and peak vitamin B\textsubscript{12} concentration in abomasal fluid.

\[ Y = 9473 + 1473(\ln(x - 0.0324)) \]
\[ R^2 = 0.9985 \]

Figure 5.7: The relationship between peak plasma vitamin B\textsubscript{12} concentration and flow of vitamin B\textsubscript{12} (nmol/d) in abomasal fluid.

\[ Y = 68 + 5.4x \]
\[ R^2 = 0.9996 \]
It might be anticipated that transport of vitamin B\textsubscript{12} across the gastrointestinal tract (GIT) will saturate implying a limit to the amount of vitamin B\textsubscript{12} that can be transported per day. When plasma concentration is plotted against abomasal flow (Figure 5.7) this does not seem to have been the case. Across the present range of supply to the intestine response of plasma concentration to increase in abomasal vitamin B\textsubscript{12} flow was linear. Rate of production in the rumen and concentration of the vitamin at the abomasum seem to have been the limiting factors to plasma concentration at the present rates of production since flow rates were the same between animals.

5.4.6 Efficiency of Production of vitamin B\textsubscript{12}

When known amounts of Co are administered each day, the theoretical maximum vitamin B\textsubscript{12} production can be calculated since it is known the cobalt ion weighs 4.35\% of the total weight of vitamin B\textsubscript{12}. The abomasal vitamin B\textsubscript{12} concentration multiplied by daily liquid flow rate (see Figure 5.7) gives a total flow of vitamin B\textsubscript{12} flowing past the abomasum which is potentially available for absorption. The total flow can be converted to a total weight of vitamin B\textsubscript{12} allowing comparison of the same units as the theoretical maximum vitamin B\textsubscript{12} production. The production efficiency of vitamin B\textsubscript{12} can be calculated from the ratio of total weight of vitamin B\textsubscript{12} flowing past the abomasum against the theoretical maximum production of vitamin B\textsubscript{12} as seen in Table 5.4. An example calculation is presented in Appendix B.
### Table 5.4: Estimate of efficiency of vitamin B<sub>12</sub> production after ruminal cobalt infusions.

<table>
<thead>
<tr>
<th>Cobalt intake (mg Co/d)</th>
<th>Theoretical maximum B&lt;sub&gt;12&lt;/sub&gt; production* (mg Vitamin B&lt;sub&gt;12&lt;/sub&gt;/d)</th>
<th>Maximum abomasal conc measured. (pmol/l)</th>
<th>Total abomasal B&lt;sub&gt;12&lt;/sub&gt; flow** (mg B&lt;sub&gt;12&lt;/sub&gt;/d)</th>
<th>Production efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03 0.69 2277 0.045</td>
<td>0.69 7376 0.146 2.43 0.67 15.4 8682 0.172 0.67 15.4 8682 0.192 0.75</td>
<td>0.045 0.146 0.172 0.192</td>
<td>6.52 2.43 1.12 0.75</td>
<td></td>
</tr>
</tbody>
</table>

*Assuming 100% incorporation of Co into vitamin B<sub>12</sub> and that 100/4.35 mg vitamin B<sub>12</sub>/mg Co

**Assuming digesta liquid flow of 14.6 l/d

When production efficiency is represented graphically (Figure 5.8) it shows an exponential reduction in capture of Co in vitamin B<sub>12</sub> as Co intake increased.

![Graph showing production efficiency (%) of vitamin B<sub>12</sub> with increasing cobalt intakes](image)

**Figure 5.8:** Production efficiency (%) of vitamin B<sub>12</sub> with increasing cobalt intakes
These efficiencies are in the same range but tend to be lower than the estimates of Smith and Marston (1970) of 13 – 3% on increasing cobalt intakes between 0.03 and 1 mg Co/d, though they, similarly, found that efficiency decreased with increasing Co intake. However, Smith and Marston’s estimate was derived from data based on the concentrations of cobalt and vitamin B₁₂ in the rumen before and after withdrawal from oral drenches of 1 mg cobalt per day.

A possible reason for Smith and Marston’s production estimate being higher than the current is that their measurement of vitamin B₁₂ was by a microbial (Ochromonas) assay which presents difficulties in the detection of low concentrations of vitamin B₁₂ with accuracy. It is also possible with such analytical methods measurement of vitamin B₁₂ was artificially high due to the measurement of vitamin B₁₂–like compounds. Little reliable literature exists on the specificity of microbial assays for vitamin B₁₂. Millar & Penrose, (1980) found a high correlation (R²=0.92 at significance level 0.1) between serum vitamin B₁₂ measured by microbiological and radioassay methods. However the radioassay values were higher than microbiological unless a purified intrinsic factor was used, when values were lower than microbiological methods. Given the variation between assays, it is difficult to conclude which assay best measures true vitamin B₁₂ and excludes vitamin B₁₂-like compounds. Vitamin B₁₂ in the current research was measured by chemiluminescence, which excludes analogues due to use of purified hog Intrinsic Factor which only binds true vitamin B₁₂ (after a boiling step to denature proteins). This could be another reason for the current estimate of production efficiency being lower than previous reports of production. Nevertheless, the difference between
estimates is small and the present reinforces the earlier conclusion that
efficiency of capture of Co in vitamin B₁₂ by rumen micro-organisms is low.

Another factor which could affect vitamin B₁₂ production in the rumen is the
amount of fermentable metabolisable energy (FME) available for microbial
production. The diet used within this research supplied approximately 8.8 MJ
FME/d based on data for FME from McDonald et al., (1995). The diet used by
Smith and Marston (1970) would have supplied less FME (estimated 6.5 MJ
FME/d) due to the higher proportion of hay-chaff. However, this does not
account for their higher estimate of production.

5.4.7 Absorption of vitamin B₁₂
In the previous chapter plasma vitamin B₁₂ concentration responses were
calibrated for rate of entry of the vitamin into the plasma pool (Figure 4.6). It is
now possible to relate plasma vitamin B₁₂ responses against abomasal flows of
vitamin B₁₂ to allow an estimate of rate of vitamin B₁₂ absorption (Table 5.5)
and therefore the absorption coefficient. An example calculation is given in
Appendix C.

<table>
<thead>
<tr>
<th>Co intake (mg Co/d)</th>
<th>Abomasal flow of vitamin B₁₂ * (nmol/d)</th>
<th>Plasma response (pmol/l)</th>
<th>B₁₂ infusion rate to give equivalent plasma response (nmol/d) (Chapter 4, Fig. 4.6)</th>
<th>Estimated absorption coefficient (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>33.2</td>
<td>248</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.26</td>
<td>108</td>
<td>640</td>
<td>7.3</td>
<td>6.8</td>
</tr>
<tr>
<td>0.67</td>
<td>127</td>
<td>748</td>
<td>10.9</td>
<td>8.7</td>
</tr>
<tr>
<td>1.11</td>
<td>142</td>
<td>833</td>
<td>14.1</td>
<td>9.9</td>
</tr>
</tbody>
</table>

* Assuming digesta liquid flow of 14.6 l/d
The vitamin B\textsubscript{12} responses to infusions of vitamin B\textsubscript{12} or Co were the responses measured in addition to the endogenous vitamin B\textsubscript{12} absorbed from the basal diet containing 0.03 mg Co/d. Therefore the estimate described in Table 5.5 is based on additional vitamin B\textsubscript{12} supplied directly into the blood above vitamin B\textsubscript{12} absorbed from the basal diet. In an ideal situation, control animals would maintain the same plasma concentrations in Chapters 4 and 5. However control animals maintained a slightly higher plasma concentration, albeit a small difference, in the calibration of Chapter 4. There is a very real possibility this is caused by short-term carry-over effects from high treatment rates in Chapter 4, artificially inflating animal values when used as a control in subsequent treatments, as discussed in the present chapter.

Coefficients of absorption calculated over the range of abomasal vitamin B\textsubscript{12} flows obtained ranged from 6.8 to 9.9% and tended to be greatest at highest abomasal flow of the vitamin. This result is a little surprising as it might have been expected that as abomasal flow of vitamin B\textsubscript{12} increases, absorption coefficient might decrease. However, the plasma responses from increasing abomasal flow of vitamin B\textsubscript{12} are not significantly different. Therefore the difference in plasma response over the range of flow of 105 – 141 nmol/d of vitamin B\textsubscript{12} at the abomasum seems mainly to be due to the change in efficiency of capture of Co, namely decreasing from 2.5 to 0.75% Co incorporated into vitamin B\textsubscript{12} respectively, the effect being minimized by the trend for increasing absorption efficiency of the vitamin.

Marston’s (1970) calculation of a coefficient of absorption of 3% was estimated by the failure of 100 ug vitamin B\textsubscript{12} given \textit{per os} to be as effective as 3 ug
vitamin B₁₂ injected intramuscularly, in terms of change in mean haemoglobin concentration and body weight. This suggests oral dosing of vitamin B₁₂ is ineffective way of supplementing sheep. It may be inappropriate to compare this method of supplementation with other methods, namely oral dosing of Co as administration of vitamin B₁₂ *per os* allows the vitamin to be degraded by the rumen microbial population and hence rendered unavailable for absorption. Possible responses may be due to release of cobalt ion due to degradation, which subsequently makes the ion available for true vitamin B₁₂ production by the microbes. However, limited data exists on the extent of degradation of vitamin B₁₂ in the rumen. Smith & Marston, (1970) demonstrated a rapid decrease in supposed ‘true’ vitamin B₁₂ 10 h after oral dosing with 500 µg cyanocobalamin and an equal increase in vitamin B₁₂–like analogues.

The plasma reference range used in New Zealand *viz*, 336 to 500 pmol/l (Underwood & Suttle, 1999) was calculated, in Chapter 4, to equate to a direct intravenous rate of infusion of vitamin B₁₂ of between 0.15 to 3.3 nmol/d. Based on the present work, Figure 5.7, the plasma concentrations in the reference range equate to an abomasal flow of vitamin B₁₂ between 50 to 80 nmol/d. Converting this flow rate to a concentration of vitamin B₁₂ based on a digesta flow of 14.6 l/d gives a concentration of vitamin B₁₂ in abomasal fluid of 2527 and 4044 pmol/l respectively. At these concentrations, the relationship listed in Figure 5.6 equates to a Co intake of 0.041 and 0.057 mg Co/d. The suggested plasma reference range appears therefore to be supported by a dietary intake of 0.041 to 0.057 mg Co/d, lower than the estimated minimum dietary requirement of 0.1 mg Co/d (Grace, 1994).
While high plasma vitamin B\textsubscript{12} responses (>3000 pmol/l) are seen from direct supplementation of sheep with vitamin B\textsubscript{12}, in pastoral situations responses are generally more modest below 2000 pmol/l. In the present work, intra-ruminal infusions of 1 mg Co/d only increased plasma to a maximum plateau value of approximately 800 pmol/l. It has been clearly demonstrated that plasma is capable of carrying very high loads of vitamin B\textsubscript{12}, yet under relatively high Co intakes (1 mg Co/d) more modest plasma levels were observed during short-term trials. Results from the present Chapter showed production efficiency decreased from 6.5 to 0.75% as Co intake increased from 0.03 to 1.11 mg Co/d. This decrease in production efficiency was offset by an increase in absorption efficiency of 6.8 to 9.9% as Co intake increased from 0.26 to 1.11 mg Co/d. Modifications to the present trial design would include:

- Restrict Co infusions to the period of feeding for better simulated grazing situation.
- A longer infusion period of 17 – 30 d as it was proved in Chapter 4 that 4 days of direct infusion into the bloodstream was marginally long enough to reach an appearance asymptote.
- Use of a diet higher in ME but low in Co to maintain animals deficient of vitamin B\textsubscript{12} but prevent any potential limitations of microbial activity.
- Measurement of urinary vitamin B\textsubscript{12} concentration. While urinary excretion is considered negligible in Co deficient sheep, confirmation of this would have helped in the interpretation of the experiment.
CHAPTER 6: Effect of Carbohydrate Source on Vitamin B$_{12}$ Production and Absorption

6.1 Introduction
While it is well documented that dietary cobalt (Co) concentration is the major limiting factor in vitamin B$_{12}$ deficiency, there is evidence that other factors may influence vitamin B$_{12}$ status of the host. Gruner et al., (2004b) observed that Co pellet-supplemented animals, although showing elevated plasma vitamin B$_{12}$ levels, still showed a seasonal pattern of change with time which mimicked concentrations in un-supplemented animals with rises and falls in plasma levels occurring at the same time. If cobalt were the only limiting factor in vitamin B$_{12}$ production, it could be assumed that in the Co-supplemented animals, vitamin B$_{12}$ production would increase and plateau at a rate consistent with the rate of release of cobalt from the pellet, rather than the fluctuations seen. Other factors therefore appear to be promoting and/or restricting the production or absorption of vitamin B$_{12}$. It may simply reflect seasonal variation in metabolisable energy intake, but components of the diet could also be implicated.

OWLD appears to mainly affect lambs during rapid pasture growth on a diet lacking cobalt (Kennedy et al., 1994) and rich in readily digestible carbohydrates (Ulvund, 1990a) and was first described in New Zealand by Sutherland et al. (1979). Anecdotal evidence has associated the deficiency with high quality reseeded pastures. These would have the characteristic of high levels of soluble carbohydrate, given the dominance of grass in reseeds, and a low content of clover, which is known to maintain a higher Co concentration than grasses, reducing potential daily Co intake and increasing soluble
carbohydrate intake. Ulvund & Pestalozzi, (1990a) for example, have observed variation in plasma vitamin B$_{12}$ concentrations and susceptibility of sheep to ovine white liver disease (OWLD) between seasons on herbage with the same Co concentration. These authors speculated that variation in soluble carbohydrate levels, especially fructan levels, may be implicated. This seems entirely plausible as the rate of vitamin B$_{12}$ synthesis in the rumen seems likely to be a reflection of the growth and activity of rumen micro-organisms. The aim of the present trial was to determine whether the source of carbohydrate for rumen microbial energy influences rate of vitamin B$_{12}$ production and absorption.
6.2 Materials and methods

6.2.1 Experimental design
Eight rumen and abomasally cannulated vitamin B$_{12}$-depleted ewes were used, as described in Chapter 3, in a 2 x 4 cross-over design employing two energy sources on two groups of 4 sheep. Dietary treatments were intra-ruminal infusion of a soluble carbohydrate (sugar)-enriched diet or the continuation of the barley (starch)-enriched diet. Animals receiving the sugar-enriched diet were introduced to the diet by gradual substitution of barley for sugar during the first 7 d of a 28 d period. On day 7, intra-ruminal infusion with 0.3 mg Co/d was commenced and continued until day 13. Cobalt infusion rates were based on data from Chapter 5 in which 0 – 1.0 mg Co/d was infused intra-ruminally for 96 h. The most responsive section of the dose-response curve was at the rate of infusion of 0.3 mg Co/d, the estimated infusion rate for the optimal response of plasma vitamin B$_{12}$ and therefore subsequently used in the current trial. Days 14 – 28 were a recovery period until the next period started. Infusions of digesta marker solution was started on day 2 and continued until day 13.

6.2.2 Sampling procedure
Blood samples were taken on days 0, 7, 10, 13, 14, 18, 23 and 28 of each treatment period. Samples were prepared and analysed for vitamin B$_{12}$ as described in Chapter 3. Abomasal samples (approximately 40 ml) were taken once each day during days 7, 8 and 13 and 4 times each day (4am, 10am, 4pm and 10pm) on days 9, 10 and 11 for estimation of digesta flow. Samples were covered with aluminium foil until centrifuged at 28 000 g for 30 min at 4°C and the supernatant then stored at -20°C until analysed for vitamin B$_{12}$ and PEG concentrations as described in Chapter 3.
Rumen samples were taken for analysis of ammonia and fatty acid (FA) concentration at 0, 2, 4, 6, 8, 12 and 24 h after feeding on day 12 as described in chapter 3. The detailed sampling procedure is summarised in Table 6.1.

**Table 6.1:** Feeding regime, infusion times and sampling procedure for sheep during the experimental period.

<table>
<thead>
<tr>
<th>Day</th>
<th>Mon</th>
<th>Tues</th>
<th>Wed</th>
<th>Thurs</th>
<th>Fri</th>
<th>Sat</th>
<th>Sun</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar:barley substitution for applicable animals</td>
<td>150 g</td>
<td>150 g</td>
<td>200 g</td>
<td>200 g</td>
<td>250 g</td>
<td>250 g</td>
<td>250 g</td>
</tr>
<tr>
<td>PEG Infusion</td>
<td>Start</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Blood Sampling</td>
<td>1x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day</th>
<th>Mon</th>
<th>Tues</th>
<th>Wed</th>
<th>Thurs</th>
<th>Fri</th>
<th>Sat</th>
<th>Sun</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cobalt and Urea and (Sugar - if applicable) Infusions</td>
<td>Start</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Stop</td>
<td></td>
</tr>
<tr>
<td>PEG Infusion</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Stop</td>
<td></td>
</tr>
<tr>
<td>Rumen Sampling</td>
<td>6x</td>
<td>1x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abomasal Sampling</td>
<td>1x</td>
<td>1x</td>
<td>4x</td>
<td>4x</td>
<td>4x</td>
<td>1x</td>
<td></td>
</tr>
<tr>
<td>Blood Sampling</td>
<td>1x</td>
<td>1x</td>
<td>1x</td>
<td>1x</td>
<td>1x</td>
<td>1x</td>
<td></td>
</tr>
</tbody>
</table>

### 6.2.3 Diets

#### 6.2.3.1 Starch enriched diet

The diet consisted of low quality chaffed meadow hay, crushed barley and urea solution to balance rumen fermentation as described in Chapter 3. Animals were offered and consumed approximately 400 g DM of hay once a day, 250 g DM of crushed barley and 9 g DM of urea per day given over a 4 h period after the hay was offered to the sheep. During the experimental period, the barley was given via belt-driven feeder and the urea solution was supplied via intraruminal infusion line during the 4 h period from feeding of the hay to help simulate nutrient supply in normal pastoral grazing situations.
6.2.3.2 *Sugar enriched diet*

The quality of the diet was modified by the substitution of an infusion of soluble carbohydrate directly into the rumen for crushed barley. Animals were offered and consumed approximately 400 g DM of hay once a day and received, via infusion directly into the rumen, 210 g DM fructose (Raftilose, R.M.F Marketing Service, Christchurch, New Zealand; 95% oligofructose): 42 g DM sucrose (BDH Laboratory Supplies, Poole, England) per day in an 80:20 fructose:sucrose mix and a total of 21.5 g DM of urea to balance the two diets for N source. The sugar and urea were administered over the 4 h period after the hay was offered to the sheep.

Methods of estimating metabolisable energy and percent of soluble carbohydrate of each diet are described in Chapter 3.

6.2.4 *Preparation and delivery of infusates*

The Co solution was prepared by accurately measuring 0.777 g of hydrated cobalt sulphate (CoSO$_4$·7H$_2$O) dissolved in 1000 ml of nanopure water. A 100 ml aliquot was transferred and volume made up to 18 l with nanopure water. Final concentration was 0.91 mg Co/l. The solution was stored in clean plastic containers and kept refrigerated until used. The Co solution was delivered at 331 ml/d regulated by 0.64 mm inside diameter pump tube (Astoria Pacific International, Oregon, USA) supplying 0.3 mg Co/d.

The total fructosan/sucrose mix was made up by dissolving 10 kg fructooligosaccharide and 2 kg sucrose in hot water which was then made up to 18.5 l with distilled water. The daily soluble carbohydrate mix was delivered in 96
ml/h regulated by 1.65 mm inside diameter pump tube (Astoria Pacific International, Oregon, USA). Infusions of carbohydrate and urea were administered during the 4 h period from feeding of the hay to help simulate nutrient supply conditions of water soluble carbohydrate (WSC) in pastoral grazing situations.

Polyethylene Glycol (PEG) 4000 was used as a liquid marker. The PEG infusion contained 30 g PEG/l (10 g PEG/animal/d) delivered at 331 ml/d regulated by 0.64 mm i.d pump tube (Astoria Pacific International, Oregon, USA). Infusion solutions were prepared by weighing 1.2 kg PEG 4000 into a 40 l container which was then made up to volume with nanopure water.

The urea solution was delivered in a volume to balance both diets for total liquid volume administered. The urea solution for the sheep on the barley based diet was made up by dissolving 470 g urea (Ravensdown Urea 46% N) in 25 l water and delivered at the rate of 120 ml/h regulated by 1.85 mm inside diameter pump tube (Astoria Pacific International, Oregon, USA) administered during the 4 h period from feeding of the hay. The urea solution for the sheep on the sugar based diet was made up by dissolving 1.3 kg urea (Ravensdown Urea 46% N) in 6 l water and was delivered in 25 ml/h regulated by 0.89 mm i.d pump tube (Astoria Pacific International, Oregon, USA) administered during the 4 h period from feeding of the hay.

A ChemLab CPP30 (England) peristaltic pump was used to drive all infusion lines. Actual volumes delivered were calculated by difference between volume before and after infusion.
6.2.5 Sample analysis

6.2.5.1 Abomasal vitamin $B_{12}$ analysis

Vitamin $B_{12}$ in abomasal fluid was determined by chemiluminescence as previously described in Chapter 3.

6.2.5.2 Abomasal PEG analysis

Polyethylene glycol in abomasal fluid was determined by a modification of the turbidimetric method as previously described in Chapter 3.

6.2.5.3 Ammonia analysis

Acidified rumen samples were thawed, vortexed and accurately weighed (3-4 g) in duplicate into Kjeldahl tubes. Samples were inserted into Kjeltec Auto 1030 Analyser (Tecator, Sweden) which automatically adds an excess of saturated sodium tetraborate ($\text{Na}_2\text{B}_4\text{O}_7$), distils off the ammonia and titrates it against
0.03 molar hydrochloric acid (HCl). The volume titrated was converted to ammonia content (gNH₃ N/kg rumen fluid). If duplicates differed by more than 5% the analysis was repeated.

6.2.5.4 Fatty acid analysis
Rumen samples were thawed and analysed for acetic, propionic, butyric, valeric and succinic acid concentrations as described in Chapter 3.

6.2.6 Statistical analysis
Analysis of variance was performed on vitamin B₁₂ in plasma and abomasal fluid to determine significance of differences and possible interactions. All remaining measurements were analysed using repeated measures ANOVA to determine significance of difference and any possible interactions. Data were analysed using Genstat 5 (Release 4.21, Lawes Agricultural Trust, Rothamstead Experimental Station, 1997) and graphs plotted using Sigmaplot (Version 5.00, SPSS Inc, 1999).
6.3 Results

6.3.1 Diet
Analyses of the diet offered to each treatment group during infusion of sugar or continuation of the starch-enriched diet are given in Table 6.2. As an observation, animals on sugar-enriched diets generally consumed their feed at a much slower rate compared to their starch-enriched diet counterparts. One animal on the sugar-enriched diet developed acidosis and did not consume any feed. She was removed from the trial and subsequent statistical analysis.

Table 6.2: Mean composition of starch- and sugar-enriched hay diets during the experimental period.

<table>
<thead>
<tr>
<th>Hay (g)</th>
<th>Barley (g)</th>
<th>Urea (g)</th>
<th>Fruct -osan (g)</th>
<th>Sucrose (g)</th>
<th>aNDF* (g DM)</th>
<th>ADF (g DM)</th>
<th>MJ ME/ kg DM</th>
<th>% sol. CHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley enriched diet</td>
<td>400</td>
<td>250</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>296</td>
<td>190</td>
<td>10.4</td>
</tr>
<tr>
<td>Sugar enriched diet</td>
<td>400</td>
<td>0</td>
<td>21.5</td>
<td>210</td>
<td>42</td>
<td>252</td>
<td>166</td>
<td>10.5</td>
</tr>
</tbody>
</table>

*note NDF was determined for hay and aNDF was determined for the barley constituents of the diet.

6.3.2 Digesta flow
Calculated digesta liquid flow rates for each diet are given in Figure 6.1. Average digesta flow for barley and sugar enriched diets was 18.8 l/d and 14.4 l/d respectively. There was a trend for higher digesta liquid flow on the barley diet (P=0.088) but no diet x time interaction (P=0.403). The overall mean was 16.6 l/d.
6.3.3 Rumen fermentation characteristics

Mean rumen fluid acetic, propionic, butyric, valeric acid, ammonia concentration and pH during a 24 h sampling period on the starch and sugar enriched diets are given in Figure 6.2.

Rumen pH was significantly affected by time (P<0.001), values dropping to lowest pH by 8 h after feeding before returning to initial values by 24 h. It was not affected by diet (P=0.164) nor was there a diet x time interaction (P=0.614).

Valeric acid concentration was significantly affected by time (P<0.001) due to values rising to a peak after 6 – 8 h after feeding before subsequently returning.
to initial values. There was no significant effect of diet (P=0.480) or diet x time interaction (P=0.720).

Rumen ammonia concentration was affected significantly by time (P<0.001) but not by diet (P=0.231). There was a significant diet x time interaction (p<0.021), as a consequence of the sugar enriched diet causing a greater peak in rumen ammonia concentration after feeding despite having lower initial concentrations.

Acetic acid concentrations were significantly affected by time (P<0.001) but there was no significant effect of diet (P=0.111) or diet x time interaction (P=0.072).

Propionic acid concentrations were not affected by diet (P=0.230) and there was no diet x time interaction (P=0.527). A significant effect of time was identified (P<0.001) with rumen concentrations rising until 6 h after feeding before returning to initial values by 24 h after feeding.

Butyric acid concentrations were not affected by diet (P=0.286), there was an effect of time (P=0.001) with rumen concentrations rising to a peak after 6 – 8 h of feeding. There was, however, no significant diet x time interaction (P=0.089).

Rumen succinic acid concentrations were not significantly affected by diet (P=0.138) nor was there any significant diet x time interaction (P=0.080) or effect of diet (P=0.138). There was an effect of time (P=0.024) with concentrations peaking at 2 and 4 hours after feeding for animals on starch and sugar diets respectively.
6.3.4 Vitamin B$_{12}$ concentration in abomasal fluid
Vitamin B$_{12}$ concentrations in abomasal fluid during the 6-d infusion of 0.3 mg Co/d are given in Figure 6.3. There was a significant effect of time (P<0.001) on concentration but no significant effect of diet (P=0.494) or diet x time interaction (P=0.157).

6.3.5 Vitamin B$_{12}$ concentration in plasma
Mean vitamin B$_{12}$ concentrations in the plasma during the experimental period are given in Figure 6.4. Diet had no significant effect (P=0.737) on vitamin B$_{12}$ concentration whereas there was a significant effect of time (P<0.001). Vitamin B$_{12}$ concentrations in plasma peaked at day 6 and declined to initial values by day 28. There was no significant diet x time interaction (P=0.494).
Figure 6.2: Mean rumen fluid acetic, propionic, butyric, succinic, valeric acid, ammonia concentration and pH during a 24 h period following the feeding of a starch (barley - ●), or sugar (fructose/sucrose mix - ○) enriched diets.
Figure 6.3: Vitamin B₁₂ concentrations in abomasal fluid of sheep on a starch- (●), or sugar- (○) enriched diet while receiving intra-ruminal infusion of 0.3 mg Co/d during days 7-13.

Figure 6.4: Plasma vitamin B₁₂ concentrations of sheep on a starch-(●) or sugar- (○) enriched diet during a 6-d intra-ruminal infusion of 0.3 mg Co/d. — denotes period of infusion of Co solution, abomasal fluid sampling and digesta flow measurements.
6.4 Discussion

The difference in the mean WSC content of the two diets used, mean 6% and 42% WSC in the starch and sugar enriched diets respectively, encompassed the range of WSC content seen in practice, namely perennial ryegrass (*Lolium perenne*) cv Aberdart of 17% (Lovett et al., 2004) and white clover (*Trifolium repens*) at 8.4%, perennial ryegrass at 17% and cocksfoot (*Dactylis glomerata*) at 9% WSC (Mitchell, 1973). Thus, if the hypothesis of an effect of WSC on vitamin B$_{12}$ production was correct, the range of WSC utilised would be expected to have affected vitamin B$_{12}$ production.

Closer examination of the digesta flow data suggests that since flow rates on day 7 were similar before Co infusions started, Co may have been responsible for the increase in digesta flow on starch enriched diets. The real question here is whether Co stimulated greater digesta flow. Starch-enriched diets did promote a higher average digesta flow (18.8 l/d) compared to the sugar-enriched diet (14.4 l/d). While this difference was not statistically significant (P=0.088), the 30% difference in flow and trend lines does imply a difference. Some of the variation in digesta flow may be attributable to the difference in fibre content of the diets. The starch-enriched diet contained 16% more fibre which decreases digestibility and therefore could be expected to increase digesta flow (McDonald et al., 1995). However adjusting the sugar-enriched diet by 16% would only increase the digesta flow to 16.7 l/d, thus not accounting for all of the difference in digesta flow between the diets.

If diet did cause a difference in digesta flow but no difference in abomasal concentration of vitamin B$_{12}$, then diet did create a difference in vitamin B$_{12}$
production as shown in Table 6.3. The production efficiency, regardless of diet, calculated with the present work is lower than that calculated in Chapter 5 on a similar Co intake of 0.26 mg Co/d which had a production efficiency of 2.43%. The major difference between the two Chapters is the maximum vitamin B$_{12}$ concentration in abomasal fluid. In Chapter 5, vitamin B$_{12}$ concentration in abomasal fluid was double that in the present work although vitamin B$_{12}$ plasma concentration was of similar magnitude. It is difficult to explain this difference but one possible cause could have been the volume of infusates (120 ml/h) administered in Chapter 6 for the 4 hr after feeding could have added significantly to abomasal flow, diluting abomasal liquid vitamin B$_{12}$ concentration. However the sampling method of 4 times a day during days 9-11 would have accounted for any diurnal variation. Another possible cause for variation between trials is the higher concentration of PEG in digesta in the Chapter 6 (10 g/d as opposed to 2 g/d in Chapter 5) may have interfered with the measurement of vitamin B$_{12}$ by chemiluminescence. There is no literature evidence that PEG interferes with chemiluminescence but this requires investigation. As a result of the lower vitamin B$_{12}$ concentration in abomasal fluid in the present work, production efficiency is lower than expected and absorption coefficient is conversely higher as shown in Table 6.4.

Table 6.3: Estimate of efficiency of vitamin B$_{12}$ production after ruminal cobalt infusions of sheep consuming starch- or sugar- enriched diets.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Cobalt intake (mg Co/d)</th>
<th>Theoretical maximum B$<em>{12}$ production* (mg B$</em>{12}$/d)</th>
<th>Maximum abomasal conc measured. (pmol/l)</th>
<th>Total abomasal B$<em>{12}$ flow (mg B$</em>{12}$/d)</th>
<th>Production efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch**</td>
<td>0.33</td>
<td>7.6</td>
<td>3343</td>
<td>0.085</td>
<td>1.12</td>
</tr>
<tr>
<td>Sugar***</td>
<td>0.33</td>
<td>7.6</td>
<td>3214</td>
<td>0.063</td>
<td>0.83</td>
</tr>
</tbody>
</table>

*Assuming 100% incorporation of Co into vitamin B$_{12}$ and that 100/4.35 mg vitamin B$_{12}$/mg Co

**Assuming digesta liquid flow of 18.8 l/d

***Assuming digesta liquid flow of 14.4 l/d
Table 6.4: Estimation of the absorption coefficient from vitamin B₆₂ flow in the abomasum and plasma response from ruminal cobalt infusions and the equivalent plasma response from an intra-jugular vitamin B₆₂ infusion of sheep consuming starch- or sugar- enriched diets.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Co intake (mg Co/d)</th>
<th>Abomasal flow of vitamin B₁₂ (nmol/d)</th>
<th>Plasma response (pmol/l)</th>
<th>B₁₂ infusion rate to give equivalent plasma response (nmol/d) (Chapter 4, Fig. 4.6)</th>
<th>Absorption coefficient (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch*</td>
<td>0.33</td>
<td>62.8</td>
<td>631</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>Sugar**</td>
<td>0.33</td>
<td>46.3</td>
<td>703</td>
<td>9.4</td>
<td>20</td>
</tr>
</tbody>
</table>

*Assuming digesta liquid flow of 18.8 l/d
**Assuming digesta liquid flow of 14.4 l/d

While production of vitamin B₁₂ (nmol/d) appeared to be greater on the starch-enriched diet, this was not translated into plasma concentration presumably as a consequence of lower absorption of the vitamin. Overall plasma concentrations were not significantly different whether the sheep received a starch or sugar-enriched diet. Clearly, this interpretation depends on the reliability of the digesta flow data.

The level of Co infused was only 0.3 mg Co/d. The infusion level was kept low to ensure responsiveness to detect any different in vitamin B₁₂ production or absorption, whilst preventing repletion and carry-over effects. The level of infusion may well have been better set between 0.5 – 0.6 mg Co/d to optimise vitamin B₁₂ production (see Figures 5.5 and 5.6) which could possibly have magnified any potential difference caused by diet. On the other hand, earlier work has shown that efficiency of incorporation of Co into the vitamin decreases with increasing Co intake.

Rumen pH was not affected by diet; therefore rumen stasis was not a probable cause of any potential difference in digesta flow between the diets. While every effort was made to balance both diets for total liquid volume administered, water intake from the troughs should have also been measured in an effort to account for the difference seen in liquid digesta flow. Also, total feed intakes
should have been more accurately measured. In previous experiments using these animals the diet was completely consumed, therefore was not considered to be a factor in the current experiment. However in the current experiment, an observation was made that animals on the sugar-enriched diet did eat their diet more slowly. Also compounding factors from prolonged deficiency may have had an effect. While sampling procedure should account for difference in rate of intake, the small quantity of hay left in the corners of the troughs could have had a significant effect on digesta flow.

The degree of modification of rumen fermentation was limited. The higher succinic acid concentration of rumen fluid from sheep on the sugar-enriched diet raises the question as to whether conditions were optimal for its conversion to propionate. Conversion of succinate to propionate is a major metabolic pathway in rumen micro-organisms as shown in Figure 2.8 (page 22) a pathway which is vitamin B_{12} dependent. The lack of difference between diets in vitamin B_{12} concentration in abomasal fluid suggests a lack of the vitamin was unlikely to be responsible. The type of carbohydrate supplied was limited in the extent to which it modified rumen fermentation between the two diets. There was no significant difference in any of the rumen concentrations of fatty acids measured during the 24 h sampling period. Moreover, while modification of rumen fermentation was limited, pH was not significantly different between diets.

The infusion of highly fermentable carbohydrates meant that acidosis was a consideration. The adjustment period should perhaps have been extended from 7 days to 10-14 days to allow rumen micro-organisms more time to
adjust. It was considered 7 days would be long enough adjustment due to the animals having already been fed 250 g of crushed barley for several months. However one animal had to be withdrawn from the trial in the first treatment and the replacement animal proved not to be deficient in vitamin B₁₂ so was also withdrawn from the analysis of data.

Anecdotal evidence that dietary factors other than Co affect vitamin B₁₂ production and/or absorption originates from long term trials conducted in Norway during the 1980’s. In the trials lambs were grazed for an average of 143 days each year on ovine white liver disease (OWLD) pastures (S) and 15 km away on control (H) pasture (Ulvund & Pestalozzi, 1990a). These authors suggested a factor other than pasture Co levels may influence the development of OWLD. The pastures where the disease occurred (S) were dominated by perennial ryegrass and were high in water soluble carbohydrates (WSC), particularly fructans. Lambs grazing H pastures gained an average 10 kg more over the 5 month period and cobalt levels were actually higher on the S pasture at 0.024, 0.134 and 0.046 mg Co/kg DM at weeks 21, 26 and 32 respectively compared to H pastures which contained 0.020, 0.023 and 0.047 mg Co/kg DM, at the same time intervals. However the massive increase of Co concentration in the second pasture sample only 5 weeks later on S pastures would suggest soil contamination due to much higher levels in the soil than the pasture (Grace, 1994). Ulvund & Pestalozzi, (1990b) suggested the higher levels of fructans for the month of June on S pastures may have initiated methylmalonyl CoA mutase overload leading to hepatic lipid abnormality, growth retardation and the development of OWLD. The levels of fructans at the
start of June when lambs started to active graze the pasture were 12.9 g/kg ww on S pastures as opposed to 7.3 g/kg ww on H pastures.

Although differences were noticed between treatment groups by the latter authors, pasture species and soil acidity were different among the pastures. S pastures were dominated by *Poa* spp. (meadow grass), *Agropyron repens* (Twitch grass) and *Lolium perenne* (perennial ryegrass), while H pastures were dominated by *Poa* spp., *Festuca rubra* (red fescue) and *Agrostis tenuis* (brown top). Soils were more acidic on the H pastures which does increase the uptake of Co by plants (Grace, 1994). This may help explain the differences seen.

The results of the present chapter do add some support to the anecdotal evidence of Ulvund (1990b) that a diet high in WSC may retard production of vitamin B₁₂, or at least, prevents additional production in the presence of increased ruminal Co concentration. While the sugar enriched diet did tend to provide lower digesta flow than barley enriched diet, it was not statistically significant. However, any difference in production appears to have been masked by a possible reduction in absorption of vitamin B₁₂, as discussed in the previous Chapter. As a result, no difference was detected in plasma vitamin B₁₂ concentration between the diets. While the two diets differed highly in WSC content, rumen fermentation characteristics were not significantly modified between diets. This demonstrated the robust buffering effect of rumen micro-organisms to resist modification of fermentation. Had the diets produced significant changes in fermentation, differences in vitamin B₁₂ production and/or absorption may have been detected.
CHAPTER 7: General Discussion

The validity of the depletion – repletion technique described within the present research is dependent on the assumption that vitamin B$_{12}$ infused directly into the bloodstream behaves biologically identically to naturally absorbed vitamin. While this assumption can not be validated without measurement of transport proteins, the general trends in disappearance from the plasma were similar regardless of mode of entry into the bloodstream.

It was also observed that there was between animal variation in response to intra-venous infusion of vitamin B$_{12}$. It has been suggested in earlier discussion that tissue concentration of vitamin B$_{12}$ may have an effect in moderating the size of response in plasma vitamin B$_{12}$ to a change in vitamin B$_{12}$ absorption. Since liver samples could not be obtained as had been planned, it was not possible to test this hypothesis. Gruner, *et al.* (2004b) did, however, observe variation in plasma response to vitamin B$_{12}$ injection in lambs suckling Co supplemented and unsupplemented mother. The former had larger plasma responses to supplementation with vitamin B$_{12}$ than lambs from unsupplemented mothers even though their pre-injection plasma concentration of the vitamin was identical. This suggests that plasma responses to entry of the vitamin into the bloodstream may be dependent on tissue concentration. This suggests that rate of transfer of vitamin B$_{12}$ to tissues is inversely related to tissue concentration with the result that vitamin B$_{12}$ accumulates in plasma to a greater degree as tissues replete. This theory is depicted pictorially in Figure 7.1.
Figure 7.1: Schematic representation of the relationship between tissue and plasma pools of vitamin B\textsubscript{12}. Animals with low tissue concentration before supplementation (A), have a lower plasma response than animals with a higher tissue concentration before supplementation (B), even though initial plasma concentrations are the same.

Thus, two plausible explanations may be involved in the regulation of plasma vitamin B\textsubscript{12} concentration. Firstly, logically, for a microbially synthesised vitamin, production may be regulated by microbial activity determined by the amount of fermentable material entering the GIT. Secondly, plasma concentration response may be influenced by interplay between rates of appearance and disappearance of the vitamin into and from the plasma pool, with the latter, as tissues become more replete resulting in greater accumulation in the plasma.

The schematic representation presented in Figure 7.1 may also help explain the difference plasma response between trials. The mean plasma vitamin B\textsubscript{12}
baseline drifted from 300-400 pmol/l in Chapter 4 to 200 pmol/l in Chapter 6. Sheep would have been more Co deficient in the last than in the preceding trials and, as a result, the data may not have been directly comparable between trials.

There is also an issue of potential differences in digesta flow between Chapter 5 and 6. Overall means for each Chapter were compared in a two-sample t-test. The digesta flow used in Chapter 5 (14.6 l/d) was significantly different (P<0.001) to the digesta flow determined in Chapter 6 (16.6 l/d). This difference raises an important issue. The average flow rate used in Chapter 5 was 14.6 l/d, very similar to the rates observed in Chapter 6 on the sugar-enriched diet of 14.4 l/d but lower than 18.8 l/d on the starch-enriched diet. The starch-enriched diet was, however, the same as that used in Chapter 5 and similar flow rates would have been anticipated. However, one difference was that sheep used in earlier trial were not receiving the additional Co via infusion at the time of digesta flow measurements. It is interesting that the animals receiving the starch-enriched diet in Chapter 6 showed a trend for increase in flow rate on the commencement of infusion of Co. Therefore it may be that the addition of infused Co on a starch-enriched diet could have increased digesta flow through the abomasum or alternatively, this undermines the confidence of using the digesta flows used in Chapter 5 in calculating production and absorption efficiencies. The digesta flow rates from Chapter 6 can be applied retrospectively to Chapter 5 data (see Tables 7.1 and 7.2). Clearly in light of this information, digesta flow rates need to be calculated on sheep during infusion of additional Co to account for any such difference in digesta flow that infusions may cause. This is done in Table 7.1, which suggests a starch-
enriched diet may promote greater rates of production and therefore efficiency of production of vitamin B\textsubscript{12}.

**Table 7.1:** Estimate of efficiency of vitamin B\textsubscript{12} production after ruminal cobalt infusions used in Chapter 5, table 5.4, retrospectively using adjusted digesta flow data.

<table>
<thead>
<tr>
<th>Cobalt intake (mg Co/d)</th>
<th>Theoretical maximum B\textsubscript{12} production* (mg B\textsubscript{12}/d)</th>
<th>Maximum abomasal conc measured (pmol/l)</th>
<th>Total abomasal flow barley diet** (mg B\textsubscript{12}/d)</th>
<th>Total abomasal flow sugar diet*** (mg B\textsubscript{12}/d)</th>
<th>Production efficiency barley diet (%)</th>
<th>Production efficiency sugar diet (%)</th>
<th>Production efficiency (%) (from Table 5.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>0.69</td>
<td>2277</td>
<td>0.058</td>
<td>0.044</td>
<td>8.41</td>
<td>6.48</td>
<td>6.52</td>
</tr>
<tr>
<td>0.26</td>
<td>6.0</td>
<td>7376</td>
<td>0.188</td>
<td>0.144</td>
<td>3.13</td>
<td>2.40</td>
<td>2.43</td>
</tr>
<tr>
<td>0.67</td>
<td>15.4</td>
<td>8682</td>
<td>0.221</td>
<td>0.169</td>
<td>1.44</td>
<td>1.10</td>
<td>1.12</td>
</tr>
<tr>
<td>1.11</td>
<td>25.5</td>
<td>9706</td>
<td>0.247</td>
<td>0.189</td>
<td>0.97</td>
<td>0.74</td>
<td>0.75</td>
</tr>
</tbody>
</table>

*Assuming 100% incorporation of Co into vitamin B\textsubscript{12} and that 100/4.35 mg vitamin B\textsubscript{12}/mg Co
**Assuming digesta liquid flow of 18.8 l/d
***Assuming digesta liquid flow of 14.4 l/d

The method of estimating production efficiency relies heavily on the accuracy of the digesta flow measurement. With the data presented, production efficiency appears to be between 6.5% and 8.4% on a Co intake of 0.03 mg /d and between 0.74 and 0.97% on higher Co intake of 1.11 mg /d.

**Table 7.2:** Estimation of the absorption coefficient for vitamin B\textsubscript{12} from flow of the vitamin in the abomasum and plasma response from ruminal cobalt infusions used in Chapter 5, table 5.5 and the equivalent plasma response from an intra-jugular vitamin B\textsubscript{12} infusion.

<table>
<thead>
<tr>
<th>Co intake (mg Co/d)</th>
<th>Abomasal flow of B\textsubscript{12} barley diet * (nmol/d)</th>
<th>Abomasal flow of B\textsubscript{12} sugar diet ** (nmol/d)</th>
<th>Plasma response (pmol/l)</th>
<th>B\textsubscript{12} infusion rate to give equivalent plasma response (nmol/d) (Figure 4.6)</th>
<th>Absorption coefficient barley diet (%)</th>
<th>Absorption coefficient sugar diet (%)</th>
<th>Absorption coefficient (%) from Table 5.5***</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>42.8</td>
<td>32.8</td>
<td>248</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.26</td>
<td>139</td>
<td>106</td>
<td>640</td>
<td>7.3</td>
<td>5.3</td>
<td>6.9</td>
<td>6.8</td>
</tr>
<tr>
<td>0.67</td>
<td>163</td>
<td>125</td>
<td>748</td>
<td>10.9</td>
<td>6.7</td>
<td>8.7</td>
<td>8.7</td>
</tr>
<tr>
<td>1.11</td>
<td>182</td>
<td>140</td>
<td>833</td>
<td>14.1</td>
<td>7.7</td>
<td>10.1</td>
<td>9.9</td>
</tr>
</tbody>
</table>

* Assuming liquid digesta flow rate of 18.8 l/d
** Assuming liquid digesta flow rate of 14.4 l/d
*** Assuming liquid digesta flow rate of 14.6 l/d

Absorption of vitamin B\textsubscript{12} appears to increase with increasing Co intake, although its precise value depends on the accuracy of digesta flow measurements. Based on a digesta flow of 18.8 l/d, absorption increases from 5.3 to 7.7% as Co intake increases from 0.26 to 1.11 mg Co/d. Based on slower
digesta flow rate of 14.4 l/d, absorption is higher at 6.9-10.1% as Co intake increases from 0.26 to 1.11 mg Co/d.

While production of vitamin B₁₂ appeared to be greater on a starch-enriched diet, any difference in the plasma was eroded by a lower absorption of the vitamin. Overall plasma concentrations were not significantly different whether the sheep received a starch or sugar-enriched diet.
CHAPTER 8: General Summary

The current animal model developed within this thesis is capable of determining which factors affect vitamin B\(_{12}\) production and/or absorption. A similar approach was made to that of Suttle’s (1974) method of Cu repletion and appears quantitatively robust. Plasma responses have been calibrated with direct infusions of known amounts of vitamin B\(_{12}\) into the bloodstream. Vitamin B\(_{12}\) flows past the abomasum have been estimated to obtain measurements of total vitamin B\(_{12}\) available for absorption and related to the amount appearing in the plasma for estimates of absorption coefficient. Plasma vitamin B\(_{12}\) responses have also been measured in terms of how much dietary Co it took to obtain that response. Calibrating response from dietary Co also adds strength to the model by enabling an estimate of production efficiency of vitamin B\(_{12}\) in the rumen from daily Co intake compared to abomasal vitamin B\(_{12}\) flow.

Further information is needed on the distribution and excretion of vitamin B\(_{12}\) within the bloodstream. The amount of vitamin B\(_{12}\) appearing in the extracellular space has been estimated between 7-13\% of the infused vitamin B\(_{12}\) once response has reached a plateau. The fate of the other 87-93\% of the infused vitamin B\(_{12}\) which was absorbed into the tissues or excreted still needs to be clarified. Also more robust digesta flow data needs to be measured due to its influence on subsequent vitamin B\(_{12}\) production and absorption calculations.

Future trials which would help validate this model would investigate whether tissue status of the animal affects its plasma response to supplementation. For
example, do long-term deficient animals with low plasma and low liver vitamin 
B\textsubscript{12} levels respond differently to more recently depleted animals with low 
plasma but higher liver vitamin B\textsubscript{12} levels? Clearly, if tissue analysis of vitamin 
B\textsubscript{12} was available in the current research, a more comprehensive understanding 
of vitamin B\textsubscript{12} metabolism could have been made.

Production efficiency has been demonstrated to be low; decreasing from 8.4% 
to 0.74% as Co intake increases from 0.03 to 1.11 mg Co/d. However, there was 
evidence which suggested that as digesta flow increases, production efficiency 
may also increased. These efficiencies are lower than previous estimates 
(Smith & Marston, 1970) although similar in trend of decreasing efficiency as 
Co intake increases.

Absorption efficiency was estimated by the ratio of total abomasal vitamin B\textsubscript{12} 
flow to the quantified plasma response. Absorption efficiency was observed to 
increase from 7 to 10% as abomasal vitamin B\textsubscript{12} flow increased from 108 to 142 
nmol B\textsubscript{12}/d. These estimates are less variable than previous research (Elliot et 
al., 1971) and higher than Marston’s (1970) estimate of 3%.

The model was used to investigate the observation from the literature that the 
carbohydrate source in the diet may affect vitamin B\textsubscript{12} production and/or 
absorption. There was some evidence that when sheep were offered a high 
starch diet, via barley, and infused with additional Co, flow of the vitamin from 
the rumen may be 30% greater than in sheep on a high water soluble 
carbohydrate diet. This did not, however, translate into higher plasma 
concentrations of the vitamin.
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My thanks extend to Julie Furlong for her tireless and endless willingness to help with laboratory analysis and teach me safe practices. Also thanks to Andy Greer who answered so many of my questions throughout my research.

To James Ross, thank you for your effort, help and patience with statistical analysis of my results. It was great to have someone so approachable to help.

I would like to acknowledge the contribution Meat and Wool New Zealand have had in funding the current research. Without their support, I would not have been able to conduct the research.

Finally, I thank two dear friends, Dr Sean MW Barnes and Megan Stenswick for their sound level-headed advice, encouragement and friendship that put my thinking and approach back on level ground. Cheers guys.
Appendix A  Feed Analysis

Soluble carbohydrates
Soluble carbohydrates were extracted in water and determined spectrophotometrically as the green-blue complex formed when carbohydrates are heated with anthrone in sulphuric acid. Herbage samples were frozen at -20°C overnight and then freeze-dried and passed through a Retsch ZM100 centrifugal mill with a 1 mm sieve. Samples (0.2 g in duplicate) were placed in a plastic 250 ml wide mouth, screw cap bottle and 200 ml nanopure water added. The bottle was capped and shaken for 1 h at approximately 275 strokes/min on a Labline orbital shaker, model 4628-1. Samples were filtered through a No. 1 Whatman filter paper, the first few millilitres were rejected to eliminate any cloudy residue from the filter paper and the remainder collected. Determination was carried out within 1 h of filtration. Anthrone reagent (760 ml of nitrogen free sulphuric acid) was added slowly with constant stirring to 330 ml H₂O and cooled rapidly. Thiourea (1 g) and anthrone (1 g) was added and the mixture stirred. Glucose was used as a standard – 0.8 mg/ml D-glucose (0.4 g/500 ml). Standard aliquots were 0, 0.04, 0.08, 0.12, 0.16 and 0.20 mg glucose/ml. Standard or extract (2 g) was placed in a glass test tube (150 x 25 mm) and cooled on ice for 10 min. With the test tube held on an angle in the ice, 10 ml of anthrone reagent was added slowly so it ran down the side of the tube and formed a layer under the solution. With the tube still on ice, the contents were gently mixed and the temperature maintained below 25°C. Each tube was loosely stopped and placed in a boiling water bath for exactly 20 min. Tubes were then removed and placed on ice to reduce to ambient temperature as rapidly as possible. Absorbance was measured in a 10 mm cell at 620 nm within 30 min.
In-vitro digestibility
Sintered glass crucibles were oven dried (105°C) overnight. Duplicate 0.500 ± 0.001 g, samples of the feed, which had been ground through a 1.0 mm sieve were placed in pre-weighed crucibles. A rubber stopper was securely fitted to the base of the crucible. Pepsin solution (30 ml of 0.3% w/v in 0.125% HCl) was added to the top of the crucible and the crucible capped and incubated at 50°C for 68 h. Samples were removed and pepsin solution filtered off under low vacuum and the crucible rinsed with 2 x 10 ml aliquots of hot nanopure water, filtered and rinsed with another 10 ml aliquot of water. Cellulase solution (30 ml of 0.025 g cellulase per 0.5 g sample) was added to each crucible, the crucible recapped and the contents digested at 50°C for 48 h. Samples were removed and cellulase solution filtered off under low vacuum and rinsed with 2 x 10 ml aliquot hot nanopure water, filtered and then washed with another 10 ml aliquot of water. Crucibles were dried at 105°C overnight and weighed from a desiccator when cool. Crucibles were ashed at 500°C for 6-8 h and re-weighed from a desiccator when cool. Cellulase Organic Matter Digestibility % (% Cellulase OMD) and cellulase Dry Matter Digestibility (% Cellulose DMD) was calculated using initial and residual dry ash weights, dry weight proportion and sample weight. Predicted % in-vitro digestibility on a dry matter basis was calculated by 6.70 + (0.902 x % Cellulose DMD), and on an organic basis by 2.81 + (1.03 x % Cellulose OMD).

Detergent fibre analysis
Feed samples were freeze-dried and ground through a 1.0 mm sieve. Approximately 1 g was accurately weighed into 400 ml beakers in duplicate. Respective reagent was added (50 ml) depending on analysis and the beaker was placed on hotplate with refluxing condensers assembled over the top of the
beaker. The samples were brought to the boil and simmered for 1 h. For aNDF analysis, an additional 2 ml of standardised amylase solution was added once samples were boiling. Heating was stopped and cooled samples were filtered through pre-weighed crucibles, rinsed with boiling water under vacuum until bubbles ran clear and rinsed twice with acetone to remove water. Crucibles were then placed in an oven at 105°C overnight and re-weighed the next day once they had cooled in the desiccator. The crucibles were then place in the furnace at 500°C for 2 h, cooled and ash weight recorded. Respective detergent fibre percent was calculated by dry fibre weight divided by initial sample weight dry matter multiplied by 100.

**Cobalt content**

Samples were mixed, spread evenly in trays and divided into quarters. One quarter was placed into a drying bag and maintained at 65°C overnight which samples typically retained 5% moisture. The remainder of the sample was returned to the original bag for future analysis if needed. Dried samples were passed through a Tecator Cyclotec self-cleaning mill with a 0.5 mm screen. A 0.200 g sample of ground plant material was placed in 40 ml digestion tubes along with 3-5 glass beads. Digestion acid, 3 ml (nitric-perchloric digestion acid, 2:1 mix of nitric acid (HNO₃, MW 63, 69-70.5%) and perchloric acid (HClO₄, MW 100.5, 70%)) was added to each tube and vortexed. Tubes were placed on a 100°C pre-heated digestion block where they were heated for 2.55 h using a temperature ramp up to 205°C (Figure 3.1). Once completed, the digestion block was allowed to cool to approximately 80°C when 19.2 ml of water was added to each tube. Tubes were then placed back on the digestion block at 80°C for 10 min. While solutions were hot, each tube was vortexed thoroughly 3 times and allowed to cool for 30 min. For Co analysis, 2.5 ml
aliquots of digestes were placed into the appropriate tubes along with 2.5 ml solution of nitric acid (1% v/v) and vortexed. Samples were analysed by Inductively Coupled Plasma Mass Spectrometer (ICP-MS).

Figure 9.1: Temperature Profile for Basic Plant Digestion Analysis of Co

Tissue & digesta sample preparation and analysis

Vitamin B₁₂ in plasma samples
All samples were pre-treated to denature any carrier proteins. Plasma samples (200 µl), controls or a blank sample of 0.9% (w/v) saline solution were pipetted into 5 ml polypropylene tubes. Working solution was prepared on a daily basis (mixture of 1000 µl Borate-KCN Buffer Solution and 20 µl Dithiothreitol Solution per test) and 1000 µl added to each tube and vortex. All tubes were covered with foil and placed in a covered boiling water bath at 100°C for 20 min. Tubes were then removed and cooled in an ambient water bath for 5 min and then capped. At least 350 µl of treated sample was pipetted to a sample cup. Assay was performed by IMMULITE Chemiluminescence Immunoassay System. This system introduces samples an hog intrinsic factor into a test unit containing polystyrene beads coated with vitamin B₁₂ analogue. During an incubation period, sample vitamin B₁₂ competes for the limited number of binding sites with the vitamin B₁₂ analogue. Alkaline phosphatase-
labelled anti-hog intrinsic factor is added and binds unbound intrinsic factor, which is then removed by centrifugal wash. The IMMULITE vitamin B₁₂ procedure has a calibration range of 74 to 885 ρmol/L. Samples expected or found to be above this range were diluted with 0.9% (w/v) saline solution before assay.

**Vitamin B₁₂ in abomasal digesta**

Samples were pre-diluted with ovine albumin matrix depending on concentration to obtain measured values below 850 pmol/l. A blank ovine albumin matrix sample was also measured as a background measure and subtracted from results if needed. All samples subsequently followed the same treatment as described for vitamin B₁₂ in plasma samples, taking into account any dilution factor used.

**Digesta marker concentration**

Abomasal samples were centrifuged for 30 min at 28 000 g in a JA-20 rotor to separate particulate matter. In the work described in Chapter 4, the supernatant was clarified with ZnSO₄ and Ba(OH)₂ as described by Smith, (1959). Samples of 3 ml supernatant were pipetted into 50 ml centrifuge tubes, to which 2 ml of 4.73% (w/v) Ba(OH)₂.8H₂O was added followed by 2 ml 5% (w/v) ZnSO₄.7H₂O. The concentrations of the Ba(OH)₂.8H₂O and ZnSO₄.7H₂O solutions were measured previously to give molar solutions and neutralisation of the alkali against the zinc sulphate (Somogyi, 1945). To this, 0.5 ml of 10% (w/v) BaCl₂.2H₂O was added and mixture made up to 10 ml with nanopure water and shaken well. Samples were centrifuged at 15 000 g for 10 min.

Depending on PEG concentration, 0.5 to 5 ml of the resulting supernatant was pipetted into 15 ml test tubes and made up to 5 ml with nanopure water. PEG
turbidity was achieved by adding 5 ml of an aqueous solution of 30% (w/v) trichloroacetate acid (TCA) and 5.9% (w/v) BaCl₂·2H₂O to each test tube.

Tubes were left to stand for 60 min before the solution was transferred into disposable 4 ml curvettes, wavelength 340 – 800 nm (Biolab Ltd, Auckland, New Zealand) and transmittance read by Perkin-Elmer spectrophotometer at 525 μm. If duplicates differed in transmittance by more than 3%, the analysis was repeated. PEG standard solutions were prepared by accurately measuring 0, 0.25, 0.5, 1 and 1.5 g PEG into 100 ml volumetric flask (A) and made to volume with nanopure water. An aliquot of 1 ml was taken from respective samples and placed in a recipient 100 ml volumetric flask (B) and made to volume with nanopure water to achieve a 1:100 dilution.

The following adaptations were made for PEG analysis described in Chapter 6. Abomasal samples were centrifuged for 30 min at 28 000 g in a JA-20 rotor to separate particulate matter. Samples of 3 ml supernatant were pipetted into a 50 ml centrifuge tubes, clarified with 5 ml of 0.038 mol ZnSO₄ and 5 ml of 0.038 mol Ba(OH)₂. The concentrations of the Ba(OH)₂·8H₂O and ZnSO₄·7H₂O solutions were measured previously to give molar solutions and neutralise the alkali against the zinc sulphate (Somogyi, 1945). To this, 0.5 ml of 10% (w/v) BaCl₂·2H₂O was added and 0.5 ml H₂O and shaken well. Samples were left to stand for 10 min and then centrifuged at 15 000 g for 10 min.

The resulting supernatant (2 ml) was pipetted into 15 ml test tubes and 3 ml of 0.4 mg/ml Gum Arabic (Malawer & Powell, 1967) added. PEG turbidity was achieved by adding 5 ml of a pre-filtered (0.45 μm cellulose nitrate membrane)
aqueous solution of 30% (w/v) trichloroacetate acid (TCA) and 5.9% (w/v) BaCl₂·2H₂O into each test tube. The tubes were left to stand for 60 min before the solution was transferred into disposable 4 ml cuvettes, wavelength 340 – 800 nm (Biolab Ltd, Auckland, New Zealand) and transmittance read by Perkin-Elmer spectrophotometer at 340 nm. If duplicates differed by more than 3% transmittance, the analysis was repeated. Standard tubes were prepared using 2.7 ml blank abomasal fluid and a 300 µl spike of 6, 9, 12 and 15 mg PEG/ml to give final concentrations of 0, 0.6, 0.9, 1.2 and 1.5 mg PEG/ml of original sample.

**Fatty acid analysis**
Rumen samples for fatty acid (FA) analysis were thawed and centrifuged at 28000 g for 30 min at 4°C. A 500 µl aliquot of the supernatant was mixed with 500 µl internal standard (10 mmol of 2-methylvaleric acid in 100 ml nanopure H₂O) in a 1.5 ml micro-centrifuge tube. The final concentration of the internal standard was 5 mmol/l. Samples were vortexed then filtered through a 0.22 µm PES millipore syringe driven filter (Millex Corporation Bedford, USA) into pre-labelled autosampler tubes. Acetic, propionic, butyric, valeric and succinic acid concentrations were determined by Gas Chromatography using a Hewlett Packard (HP) 6890 GC with HP ChemStation software, version A.03.04. A 5 µl aliquot of sample was injected onto the column (HP-INNOWax polyethylene glycol 30.0 m x 250 µm x 0.25 µm capillary column) using a 20:1 split injection. The oven was kept at an initial temperature of 85°C for two min then ramped at 10°C/min to a final temperature of 200°C. Run time was 23.5 min; post run temperature was 50°C and was held for 5 min. Helium was used as the carrier gas at a pressure of 156.9 kPa to give a flow rate of 2.0 ml/min. The inlet liner (SGE, Australia) was replaced after approximately
40 samples to prevent constriction of sample delivery onto the column, in line with good laboratory practice.

At the beginning and end of each analytical run, sets of external calibration standards were run to produce linear calibration curves in the range required for quantification of FA’s present. The succinic acid standard curve was extended to cover the low concentrations found. Concentrations between 0.6 and 1.0 mmol/l were added to the final calibration curve to produce a representative linear graph. An external standard solution was prepared by mixing a FA solution containing 100 mmol of acetic and propionic, 50 mmol of butyric and succinic, 25 mmol valeric made up to volume in 100 ml nanopure H₂O. This external standard solution (500, 300, 200, 100, 50 µl) was mixed with 500 µl internal standard and made up to 1000 µl with nanopure H₂O into a 1.5 ml micro-centrifuge tube. This gave external standards at their final concentration of 50, 30, 20, 10 and 5% respectively of the original external standard solution. Standards then followed the same protocol as mixed rumen samples above.
Appendix B - Example calculation of vitamin B$_{12}$ production efficiency

Cobalt intake $\frac{0.03 \text{ mg/d}}{0.0435}$ (4.35% Co/B$_{12}$ molecule) $= 0.69 \text{ mg B}_{12}/\text{d}$ (Theoretical maximum B$_{12}$ production assuming 100% incorporation of Co into B$_{12}$).

Need to convert maximum abomasal concentration into the same units as theoretical maximum production.

Abomasal conc. $2277 \text{ pmol/d} \times 14.6 \text{ l/d}$

$= 32,444 \text{ pmol/d}$

$= 33.244 \text{ nmol/d} \times 1355 \text{ ng/nM}$ (molecular weight of B$_{12}$)

$= 45,046 \text{ ng/d}$

$= 0.045 \text{ mg B}_{12}/\text{d}$

Production efficiency $= \frac{\text{abomasal flow}}{\text{theoretical maximum}}$

$= \frac{0.045}{0.69} \times 100 = 6.52\%$
Appendix C – Example calculation of vitamin B₁₂ absorption coefficient

From Chapter 5, cobalt intake of 0.26 mg Co/d equated to abomasal vitamin B₁₂ concentration of 7376 pmol/l (Table 5.4). Multiplied by digesta flow rate of 14.6 l/d equates to an abomasal flow of vitamin B₁₂ of 108 nmol B₁₂/d.

Using the relationship described in Figure 5.7, an abomasal flow of 108 nmol B₁₂/d equates to a plasma vitamin B₁₂ responses of 640 pmol/l

Using the quantified response in Chapter 4, Figure 4.6 a plasma response of 640 pmol/l required an intra-jugular infusion of 7.3 nmol/d.

Absorption coefficient is the ratio of the quantity of B₁₂ in the bloodstream divided by the total available for absorption (i.e. abomasal flow). Therefore,

\[
\frac{7.3}{108} \times 100 = 6.8\% \text{ absorption}
\]