Vitamin B_{12} Requirement of Early Weaned Pigs

By

Chancie Fletcher

A Thesis

Submitted to the Faculty of Graduate Studies

In Partial Fulfillment of the

Requirements for the Degree of

Master of Science

Department of Animal Science

The University of Manitoba

Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION PAGE

VITAMIN B_{12} REQUIREMENT OF EARLY WEANED PIGS

BY

Chancie Fletcher

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

ABSTRACT

Fletcher, Chancie M. T. M.Sc., The University of Manitoba, May 2002. Vitamin B_{12} Requirement of Early Weaned Pigs. Major Professor; Dr. James D. House.

Four experiments were conducted in early weaned pigs from 17 to 32 days of age to: 1) determine their vitamin B_{12} requirement, and 2) examine the effects of dietary antibiotics on their vitamin B_{12} status. In the first experiment, 60 pigs were *ad libitum* fed a pelleted corn-soyabean meal-lactose based diet (devoid of vitamin B_{12}) supplemented with 0, 35, 70, 105, or 140μg of vitamin B_{12} kg^{-1} of diet. Average daily gain, average daily feed intake, feed conversion efficiency, packed cell volume, and plasma homocysteine for the pigs were not influenced by the vitamin B_{12} content of the diet (overall means = 200 g, 212 g, 0.90, 33 %, and 25 μmoles L^{-1}, respectively; $P>0.05$). However, plasma vitamin B_{12} concentrations were significantly decreased (50 pg mL^{-1} versus 164, 168, 165, 178 pg mL^{-1}, respectively; $P<0.05$) in pigs given the vitamin B_{12}-unsupplemented diet. Furthermore, plasma concentrations of methylmalonic acid and cystathionine were markedly decreased in pigs supplemented with 140 μg of vitamin B_{12} kg^{-1} of diet (432 and 1096 nmoles L^{-1}, respectively; $P<0.05$) versus pigs given the vitamin B_{12}-unsupplemented diet (1481 and 1646 nmoles L^{-1}, respectively). In the second experiment, 48 pigs were *ad libitum* fed the vitamin B_{12}-unsupplemented diet with or without 10 g of ASP250 (an antibiotic) kg^{-1} of diet. Average daily gain, average daily feed intake, feed conversion efficiency, packed cell volume, plasma vitamin B_{12}, and plasma homocysteine were similar for both the ASP250-unsupplemented and ASP250-supplemented pigs. In the third experiment, 60 pigs were *ad libitum* fed either 0, 10, 20, 30, or 40 μg of vitamin B_{12} kg^{-1} of diet. Average daily gain, average daily feed intake were not different among

the dietary treatment. Overall feed conversion efficiency was significantly decreased in pigs fed the dietary vitamin B_{12} supplementation of 10 μg kg^{-1} of diet than for pigs fed 0, 20, 30 or 40 μg of vitamin B_{12} kg^{-1} of diet (81 versus 92,93, 89, and 88, respectively; $P<0.05$). Plasma analysis revealed significantly decreased vitamin B_{12} (188 and 216 pg mL^{-1}, respectively; $P<0.05$) and increased homocysteine (31 and 27 μmoles L^{-1}, respectively; $P<0.05$) concentrations in pigs fed 0 and 10 μg of vitamin B_{12} kg^{-1} of diet. In experiment 4, which was conducted to validate the findings of experiment 1, 60 pigs were *ad libitum* fed 0, 50, 100, 150, or 200 μg of vitamin B_{12} kg^{-1} of diet. Average daily gain, average daily feed intake, feed conversion efficiency, packed cell volume, and plasma homcysteine for the pigs were not influenced by the vitamin B_{12} content of the diet (overall means = 120 g, 167 g, 0.65, 35 %, 23 umoles L^{-1}, respectively; $P<0.05$). Analogous to experiment 1, vitamin B_{12} was the only plasma metabolite to show a response to the various levels of vitamin B_{12} supplementation. Pigs given the vitamin B_{12}-unsupplemented diet had significantly lower concentrations of plasma vitamin B_{12} than pigs fed 50, 100, 150, and 200 μg of vitamin B_{12} kg^{-1} of diet. Taken together, the results of the 4 experiments indicate that the dietary vitamin B_{12} for early weaned pigs from 17 to 32 days of age is 20 μg of vitamin B_{12} kg^{-1} of diet. Furthermore, ASP 250 does not alter the vitamin B_{12} of the early weaned pig.

ACKNOWLEDGMENTS

I am honoured to dedicate this thesis to my Mom and Dad for teaching me

the courage to envision, the confidence to pursue,

and the patience to achieve.

I appreciate and thank Drs. James House, Gary Crow, Loyd Campbell, and Carla Taylor for their contributions towards this thesis. I am indebted to Dr. James House for his supervision, enthusiastic interest, and constructive comments, which greatly facilitated accomplishment of this work. I should also like to acknowledge my debt to the animal caretakers of the Animal Science Research Unit. I thank Ricky Araneda and Peter Mills for their wisdom and invaluable technical assistance.

I am grateful for the monetary and product support from the University of Manitoba, Natural Sciences Engineering Research Council, FeedRite Ltd., and Parrheim Foods (a division of Parrish & Heimbecker Ltd.).

To Ricky Araneda, the meaning of true friendship is found in a friend like you. Our fruitful discussions on love and life were a precious gift! To my husband Danny, thank you for lightened my load with your understanding, support, and humour when things got difficult. I am grateful to have a hugger on call 24 hours a day!

♥ Chancie

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADFI	average daily feed intake
ADG	average daily gain
BHMT	betaine-homocysteine methyltransferase
CoA	coenzyme A
FCE	feed conversion efficiency ratio
IF-B_{12}	intrinsic factor-vitamin B_{12} complex
NRC	National Research Council
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
TC II	transcobalamin II
TC II-B_{12}	transcobalamin II-vitamin B_{12} complex

1.0 INTRODUCTION

There has been a continuing trend in the swine industry over the past 40 years to wean pigs at a younger age (Cline 1991). By removing the pig from one of its primary sources of infection (the sow), early weaning allows dramatic improvements in the health and performance of growing pigs (Thacker 1999). Consequently, the majority of swine producers wean pigs from the sow at 18 days of age or less. Weaning at this age requires feeding programs designed for pigs weighing less than 5 kg. For many nutrients, including vitamin B_{12}, virtually no studies have been conducted with pigs of this weight range (Thacker 1999). Lack of knowledge of the nutrient requirements for early weaned pigs makes it difficult for nutritionists to formulate starter pig phase 1 diets that will maximize growth.

Current vitamin B_{12} requirements of young pigs are based on extrapolations from studies with heavier (older) pigs conducted 40 to 50 years ago. The current vitamin B_{12} requirement for 3 to 20 kg pigs ranges from 15 to 20 μg of vitamin B_{12} kg^{-1} of dry matter (NRC 1998). Problems are encountered in establishing vitamin B_{12} requirements for young pigs based upon data collected from heavier pigs. As weight and age of the pig increases, the dietary vitamin B_{12} requirement will decrease. This occurs because body composition changes, more fat and less protein towards weight gain, as the pig matures (Baker 1986). In addition, body stores of vitamin B_{12} tend to rise throughout the life of the pig. Thus, tissue storage, primarily in the liver, can delay the onset of vitamin B_{12} deficiency symptoms after a vitamin B_{12} deficient diet is fed (NRC 1998). It follows that the body stores of heavier (older) pigs could exert profound effects on the estimated level of vitamin B_{12} needed by the young pig.

Modern genetic strains of pigs have an 80 % greater capacity for lean tissue accretion, than pigs 40 to 50 years ago (Stahly 1995). Thus, they potentially have greater vitamin B_{12} needs. According to Stahly (1995), pigs with a high capacity for lean tissue accretion may demand 4 to 8 times the daily B vitamin needs currently defined by the NRC. A lack of vitamin B_{12} will lead to depressed rates of lean tissue accretion in young, growing pigs. Vitamin B_{12} is a key element in lean tissue accretion because of its role in protein and energy metabolism.

2.0 REVIEW OF THE LITERATURE

2.1 HISTORY OF VITAMIN B_{12}

Vitamin B_{12}, or cobalamin, was the last known vitamin to be discovered. However, the history behind its discovery began in the early 1800s. The discovery of vitamin B_{12} stemmed from studies investigating pernicious anemia, a disease characterized by large immature red blood cells and damage to the nervous system (Sizer and Whitney 1997).

In 1824, Combe first associated pernicious anemia with "some disorder of the digestive and assimilative organs" (Herbert 1996). Addison later detailed symptoms accompanying pernicious anemia in 1855 (Cook and Easter 1991). The Nobel Prize winning discovery by Minot and Murphy in 1926 proved feeding large quantities of liver could cure pernicious anemia (Herbert 1996). Shortly thereafter, Castle postulated that the anti-pernicious anemia principle in liver required prior binding to an "intrinsic factor" secreted by the stomach for proper intestinal absorption (Cook and Easter 1991).

The anti-pernicious anemia principle was isolated almost simultaneously in 1948 by Rickes of the Merck and Company Laboratories in the United States and Smith of the Glaxo Laboratories in England (Herbert 1996). Although isolated by the two laboratories in 1948, the chemical structure of vitamin B_{12} was not identified until 1955. Hodgkin received the Nobel Prize for ascertaining the very complex structure utilizing x-ray crystallography (Herbert 1996). That same year, Woodward's group at Harvard University synthesized vitamin B_{12} using a very complicated and expensive procedure (Ensminger *et al.* 1990). Fortunately, soon thereafter, it was learned that vitamin B_{12} could be manufactured from certain bacteria

cultures; and this remains the main method of commercial production today.

2.2 CHEMISTRY OF VITAMIN B_{12}

Vitamin B_{12} is a complex molecule containing carbon, hydrogen, oxygen, nitrogen, phosphate, and cobalt. The structural formula of one vitamin B_{12} compound, cyanocobalamin ($C_{63}H_{90}O_{14}N_{14}PCo$), is depicted in Figure 1. The structure consists of a corrin ring, a variable ligand above this ring, and a dimethylbenzimidazole ribonucleotide base below it. The corrin ring is comprised of four pyrrole nuclei coupled together, with the inner nitrogen atom of each pyrrole joined to a single cobalt atom (McDowell 2000). The term cobalamin is applied to compounds where a cobalt atom occupies the active centre of the corrin ring (McDowell 2000).

As Figure 1 illustrates, a cyanide ion is attached above the central cobalt atom, thus the name cyanocobalamin. Cyanocobalamin is the commonly used commercial form of vitamin B_{12} for animal supplementation because of its relative stability (McDonald *et al.* 1995). Cyanocobalamin acquires its stability from an ester linkage between the phosphate group of the nucleotide and the propionic acid group of the D ring of the corrin ring (McDowell 2000). Cyanocobalamin is freely soluble in water and ethanol (Lassiter and Edward, Jr. 1982) but is extremely sensitive to prolonged sunlight exposure, oxidizing, and reducing agents (McDowell 2000).

The cyanide ion can be replaced by other ions to form nitrocobalamin, bromocobalamin, chlorocobalamin, sulfatocobalamin, hydroxylcobalamin, methylcobalamin, or 5'-deoxyadenosylcobalamin. Methylcobalamin and 5'-deoxyadenosylcobalamin, also known

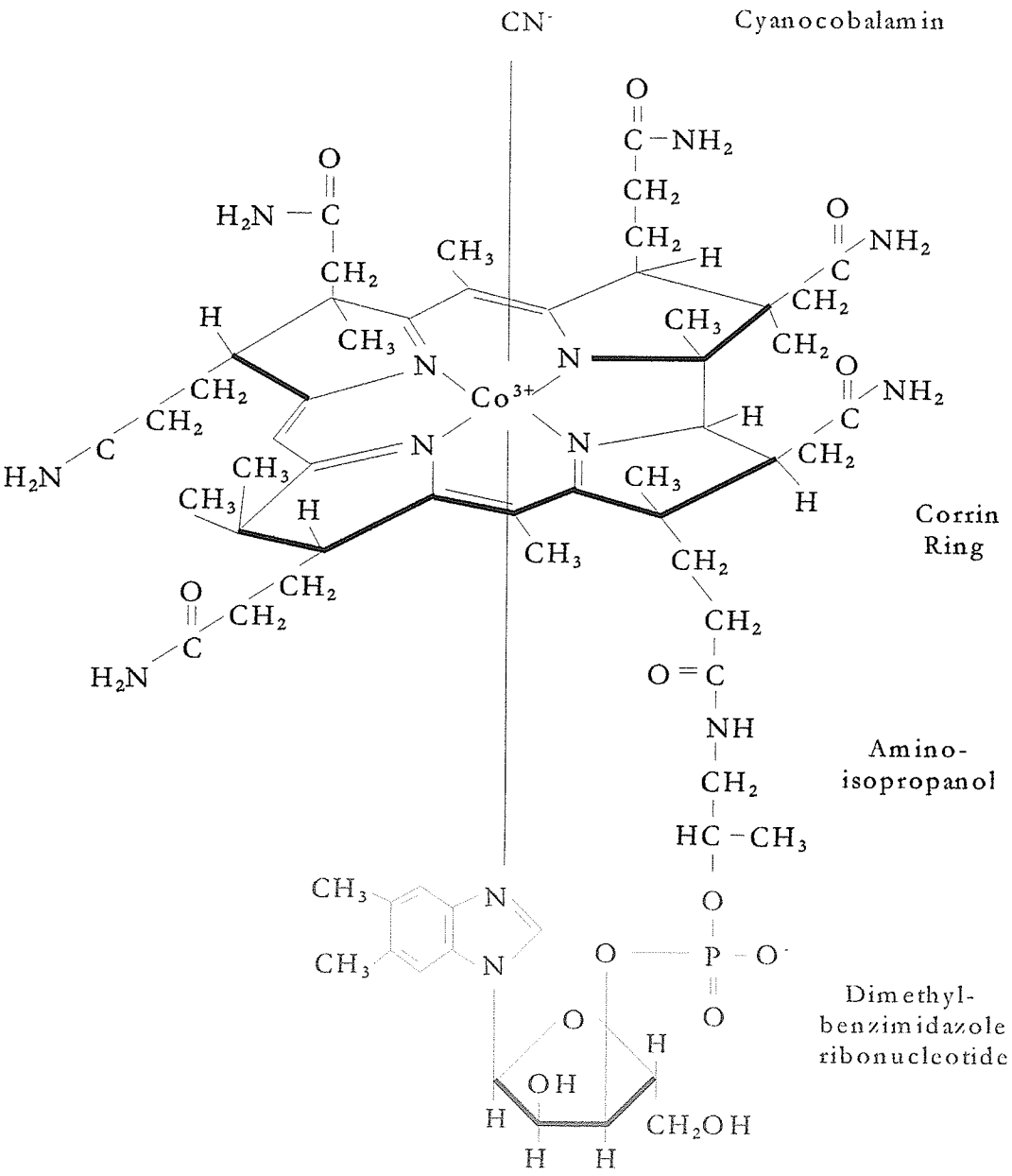


Figure 1. Structure of vitamin B_{12} (cyanocobalamin). Adapted from Lehninger *et al.* (1993).

as coenzymes B_{12}, are the two forms of vitamin B_{12} acting as coenzymes in animal metabolism (McDonald *et al.* 1995).

All the above mentioned cobalamins are biologically active (Ensminger *et al.* 1990) and are readily converted to cyanocobalamin after treatment with cyanide. Cyanocobalamin is not a naturally occurring form of the vitamin, but rather an artifact due to the presence of cyanide in the charcoal used in the extraction procedure (McDowell 2000).

2.3 SOURCES OF VITAMIN B_{12}

It is presently recognized that only microorganisms are capable of synthesizing vitamin B_{12}. Thus, all other organisms which require vitamin B_{12} are directly or indirectly dependent upon the vitamin B_{12}-producing activity of microorganisms. As a rule, commonly eaten plant products do not contain vitamin B_{12} (NRC 1998). If they do contain small amounts, this is due to external contamination with microorganisms, often of fecal origin. Fecal matter contains appreciable quantities of vitamin B_{12} that microorganisms in the lower gastrointestinal tract synthesize (Herbert 1996).

Foods of animal origin contain measurable amounts of vitamin B_{12} (Table 1) because animals store vitamin B_{12} within their tissues (NRC 1998). The occurrence of vitamin B_{12} in animal tissue is a consequence of the consumption of animal products or absorption of vitamin B_{12} produced by microorganisms in the lower gastrointestinal tract. Numberous researchers (Hendrickx et al. 1964; Keys and DeBarthe 1974; Cullen and Oace 1977; Varel 1987; Varel and Yen 1997) emphasis that the colon harbours plenty of microorganisms. These microorganisms are capable of synthesizing vitamin B_{12}.

Table 1. Vitamin B_{12} concentration ($\mu g\ kg^{-1}$) of some commonly used feed ingredients in starter pig diets.

Whey, dried	23
Blood meal, conventional	44
Blood meal, spray-dried	?
Fish meal, anchovy	280
Fish meal, herring	403
Fish meal, menhaden	143
Corn, grain	0
Soybean meal	0
Wheat, grain	0
Oat, grain	0

Source: NRC (1998)

It has also been suggested that coprophagy may satisfy the pig's requirement for vitamin B_{12}. However, the latter contribution may be of questionable nutritional significance. Although behaviours that could permit pig ingestion of feces such as rooting the floor and the walls or rubbing and biting parts of penmate's bodies have been documented, Newberry and Wood-Gush (1988) and De Passillé *et al.* (1989) reported that the deliberate ingestion of feces was not observed in pigs raised in a range situation, on slatted floors, nor on solid floors. Vitamin B_{12} absorption is discussed in the following section.

2.4 METABOLISM OF VITAMIN B_{12}

2.4.1 DIGESTION, ABSORPTION, AND TRANSPORT

The majority of the vitamin B_{12} in feed ingredients exists in a protein-bound form (NRC 1987). In the stomach, the combined effects of low gastric pH and peptic digestion are responsible for cleaving vitamin B_{12} (Toskes *et al.* 1973) from feed proteins. However, the gastric acid secretory ability is known to be limited in the early weaned pig (Easter 1988). Elevated gastric pH impairs the conversion of pepsinogen to pepsin (Easter 1988; Kidder and Manners 1978). Conceivably, this limited gastric acid secretory ability could depress vitamin B_{12} digestion in the early weaned pig .

As vitamin B_{12} is cleaved, its binds to cobalophin, a binding protein abundant in saliva and gastric secretions (Hall 1979). The cobalophins are a group of antigenically-related, relatively unspecific, corrin binding proteins, formerly known as R-proteins because of their rapid electrophoretic mobility compared with other vitamin B_{12} binding proteins (Bender 1992). As long as vitamin B_{12} is complexed with cobalophin, it is unavailable for absorption

(Herbert 1988). In the duodenum, pancreatic proteases hydrolyze cobalophin to release vitamin B_{12} to intrinsic factor (Allen *et al.* 1978). Because pancreatic protease activity in pigs is incomplete before 6-8 weeks of age (Pekas, 1991), it is highly conceivable that vitamin B_{12} digestion could also be compromised at this point. This insufficiency was later confirmed by Jensen *et al.* (1997). As documented in Table 2, the levels of trypsin and chymotrypsin activity at weaning are less than 50% the value at digestive maturity.

Intrinsic factor, an alkali-stable glycoprotein mandatory for absorption of physiological amounts of vitamin B_{12}, is synthesized and secreted by parietal cells of the gastric mucosa (McDowell 2000), which also secrete hydrochloric acid. There is evidence that a passive mechanism, presumably diffusion (not mediated by intrinsic factor), is functional when the amount of administered vitamin is large (Doscherholmen and Hogen 1957), usually in excess of the amount available from a normal diet. However, this mechanism is believed to be extremely inefficient and nonspecific.

Secretion of intrinsic factor is induced by vagus nerve stimulation, gastrin, histamine and insulin (Bender 1992). The amount of intrinsic factor secreted greatly exceeds the quantities necessary for the binding and absorption of vitamin B_{12} (Jeffries and Sleisenger 1965), which merely requires 1% of the total intrinsic factor (Bender 1992). Excess free intrinsic factor is degraded by digestive enzymes (Gräsbeck 1959) operative throughout the gastrointestinal tract.

Upon the binding of vitamin B_{12}, intrinsic factor has been shown to shrink appreciably. According to Gräsbeck (1967) and Hippe (1970), the stokes radius of free intrinsic factor decreases 4 Å (from 36.5 to 32.5 Å) on the binding of vitamin B_{12}. Theoretically, if vitamin

Table 2. The influence of age on trypsin and chymotrypsin activity (μmol substrate hydrolyzed minute^{-1}) in the pancreas of young pigs.

Age, days	Trypsin	Chymotrypsin
3	14.6	0.94
7	22.0	3.52
14	33.8	4.91
21	32.1	6.99
28 (weaning)	55.6	9.49
35	42.1	3.90
42	150	7.79
49	346	17.4
56	515	143

Source: Jensen et al. (1997)

B_{12} was attached as a bulge on the surface of intrinsic factor, the complex would be larger than the free intrinsic factor. Therefore, it is plausible that vitamin B_{12} fits into a pit on intrinsic factor (Gräsbeck 1967) with no more than a 5 Å penetration (Lien *et al.* 1974).

The pit appears to be specially designed for the dimethylbensimidazole ribonucleotide (Gräsbeck 1967). Any alteration in the ring portion of dimethylbensimidazole ribonucleotide causes nearly complete loss of binding (Lien *et al.* 1974). In contrast, the site binding vitamin B_{12} tolerates substitution of the cyanide ligand. Cyanocobalamin, aquacobalamin and 5'-deoxyadenosylcobalamin have the same binding constant to intrinsic factor, suggesting that the ligand attached to the cobalt atom does not participate in the binding process (Hippe *et al.* 1971). As illustrated in Figure 2, this information infers that the dimethylbensimidazole ribonucleotide side of vitamin B_{12} faces intrinsic factor inward, while the cyanide side faces intrinsic factor outward (Gräsbeck 1967).

The receptor for the intrinsic factor-vitamin B_{12} complex (IF-B_{12}) is located on the apical brush border of ileal enterocytes (Donaldson, Jr. *et al.* 1967; Xu and Fyfe 2000), a long way from the site of formation of the IF-B_{12}. Ellenbogen and Highley (1970) propose the IF-B_{12} protects vitamin B_{12} from bacterial utilization and/or degradation as it travels via peristalsis from the duodenum to the ileum, where absorption occurs. This proposal seems satisfactory, because the IF-B_{12} is remarkably resistant to digestion (Gräsbeck 1969).

The IF-B_{12} receptor is a 460-kDa (Birn *et al.* 1997) multiligand-binding protein. In addition to binding the IF-B_{12}, the receptor also binds albumin (Birn *et al.* 2000), high-density lipoproteins (Hammad 1999), and immunoglobulin (Batuman *et al.* 1998). The IF-B_{12} receptor was recently named cubilin in recognition of its unique domain structure: a 27 CUB domain

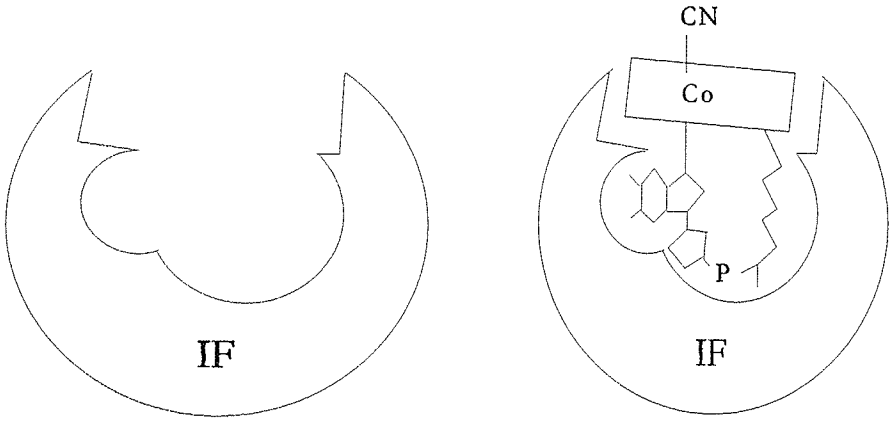


Figure 2. Binding of vitamin B_{12} to IF. Adapted from Lehninger *et al.* (1993).

cluster preceded by 8 epidermal growth factor repeats and a short N-terminal sequence (Moestrup *et al.* 1996). Published evidence shows that cubilin is co-expressed with megalin, a 600-kDa glycoprotein (Saito *et al.* 1994), in several tissues (Moestrup *et al.* 1996; Birn *et al.* 1997). Consequently, cubilin is suggested to traffic by means of megalin. Megalin is recognized as an endocytic receptor involved in the uptake of proteins (Christensen and Birn 2001). The literature on megalin is extensive and beyond the scope of this review.

The attachment of the IF-B_{12} to the receptor occurs rapidly, and is facilitated by the presence of a pH near neutrality and ionized calcium (Mackenzie and Donaldson, Jr. 1972; Hooper *et al.* 1973). This is in agreement with Sullivan *et al.* (1963) and Donaldson, Jr. *et al.* (1967) who also noted that energy is probably not required. Mackenzie and Donaldson, Jr. (1972) suggest that ionized calcium forms salt "bridges" which link anion groups on the receptor and the intrinsic factor molecule. According to Hall (1979), the attachment is specific for the IF-B_{12} but not for free intrinsic factor or vitamin B_{12}.

The capacity of the ileum to absorb vitamin B_{12} is extremely limited. Humans, for example, absorb no more than 1 to 2 μg of vitamin B_{12} from any given dose (Glass 1963). This limitation presumably results from a restricted number of receptor sites for the IF-B_{12} (Donaldson, Jr. *et al.* 1973; Hooper *et al.* 1973).

Following attachment, there is transport of vitamin B_{12} through the ileal enterocyte to the portal blood. The proposed mechanism on how the vitamin transverses the enterocyte and reaches the portal blood can be found in a recent review article (Seetharam 1999); it is illustrated in Figure 3. The proposed mechanism occurs by receptor-mediated endocytosis. After apical endocytosis of the IF-B_{12} through cubilin, there is lysosomal degradation of

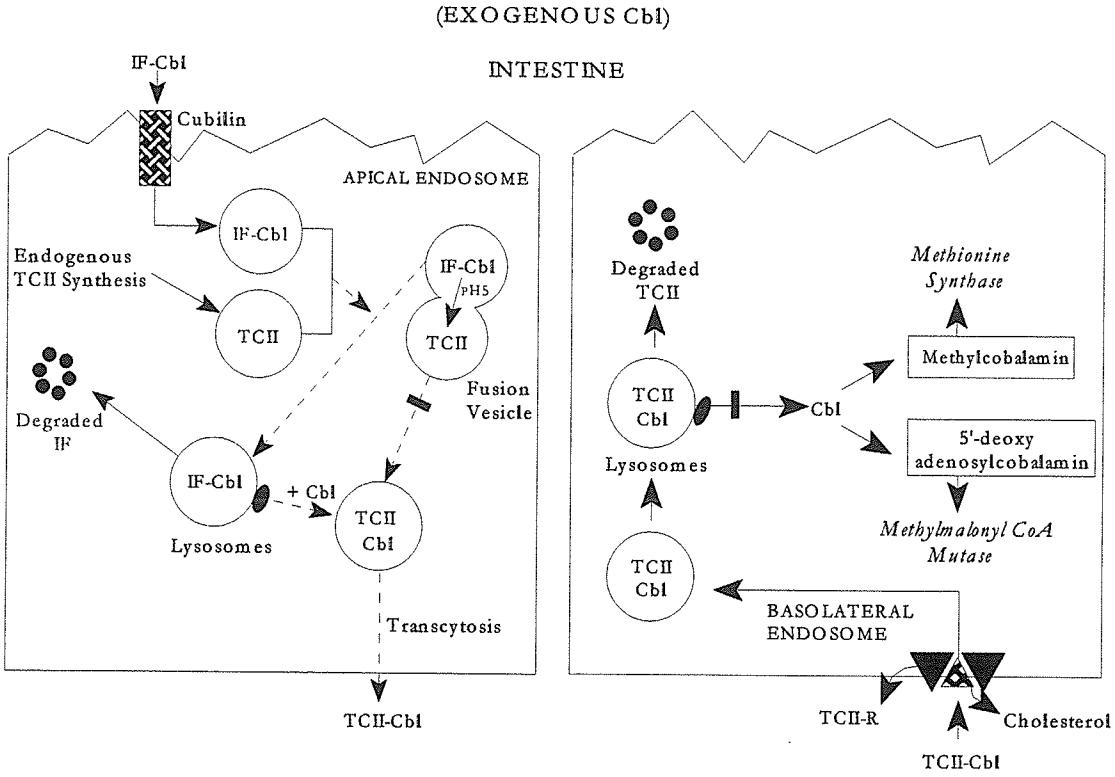


Figure 3. Receptor-mediated endocystosis. Source: Seetharam (1999).

intrinsic factor, intracellular formation of the transcobalamin II-vitamin B_{12} complex (TC II-B_{12}), and TC II-B_{12} transcytosis into the portal blood. Not clear in this scenario is how vitamin B_{12} gains access to transcobalamin II (TC II). It could occur by simple diffusion of free vitamin B_{12}, or by fluid-phase transfer during a potential fusion event involving lysosomes with the TC II-containing secretory vesicle (Seetharam *et al.* 1999). According to Hines *et al.* (1968) and Yamaguchi *et al.* (1970), intrinsic factor is not absorbed through the ileal enterocyte into the portal blood. Furthermore, Cooper and White (1968) failed to detect intrinsic factor in human portal blood, supporting the concept that lysosomes degrade intrinsic factor during the proposed receptor-mediated endocytosis mechanism. According to Jenkins *et al.* (1981) lysosomes contain powerful hydrolytic enzymes.

The appearance of vitamin B_{12} in human portal blood is delayed about 4 hours (Doscherholmen and Hagen 1957), compared to seconds for most other water-soluble vitamins. The explanation for this delay is uncertain, but it is currently postulated that the formation of TC II is the reason. Experiments using colon cancer cells have shown that the turnover of intrinsic factor degradation (Dan and Culter 1994) and TC II-B_{12} formation (Ramanujam *et al.* 1991) is about 4 hours.

It is was established early (Hall and Finkler 1965; Hall and Finkler 1966) that the function of TC II is to transport vitamin B_{12} to recipient cellular tissues. These tissues have a specific receptor, initially solubilized and purified from human placenta by Seligman and Allen (1978), for the TC II-B_{12}. The receptor is a glycoprotein with a single TC II-B_{12} binding site. The attachment of the TC II-B_{12} to the receptor is the first step in the process leading to the absorption of vitamin B_{12} into the tissue (as can also be seen in Figure 3) and is calcium

dependent (Youngdahl-Turner and Rosenberg 1978). The TC II-B_{12} enters the tissue via endocytosis and is transiently localized in lysosomes where the TC II is degraded and the vitamin B_{12} released to the cytoplasm, an observation in accordance with Youngdaul-Turner and Rosenberg (1978), Hall (1984), and Begley *et al.* (1993). Subsequently, liberated vitamin B_{12} in the cytoplasma is converted to and utilized as methylcobalamin and 5'-deoxyadenosylcobalamin.

Detailed thus far is intrinsic factor mediated absorption of vitamin B_{12} in mammals, including weaned pigs. However, the situation in the suckling pig is different, in that absorption of vitamin B_{12} successfully occurs in the absence of intrinsic factor. Ford *et al.* (1975) suggested that the vitamin B_{12}-binder in sow's milk promotes intestinal absorption of vitamin B_{12} in the suckling pig, and showed the suckling pig absorbed and retained the vitamin efficiently in the first two weeks of life, despite the absence of intrinsic factor in the gut. After the second week of life, the ability of vitamin B_{12} to be absorbed independently of intrinsic factor is no longer demonstrable while intrinsic factor mediated absorption has increased.

Studies conducted by Trugo *et al.* (1985) compliment the report of Ford *et al.* (1975) by comparing the uptake and retention of vitamin B_{12} by pigs reared either with or without the vitamin B_{12}-binder in their diet. The studies concluded that from birth to 7 days of age, suckled pigs consistently absorbed and retained a higher proportion of a single oral dose of vitamin B_{12} than did non-suckling pigs receiving a diet containing no vitamin B_{12}-binder.

2.4.2 Tissue Distribution and Storage

Vitamin B_{12} is effectively stored within the body (NRC 1998). Estimates of the average total body vitamin B_{12} pool in human adults are as high as 3.9 mg (Gräsbeck *et al.*

1958). The liver is the principle site of storage for vitamin B_{12} (Ford *et al.* 1975; Trugo *et al.* 1985) in mammals. Hendrickx *et al.* (1964) reported a total retention of 20 to 23 % of an oral dose of vitamin B_{12} in pig organs, 13 to 15 % of which abided in the liver. Aside from the liver, smaller quantities of vitamin B_{12} are maintained in the kidneys, heart, lungs, spleen, pancreas, and brain (Ford *et al.* 1975; Trugo *et al.* 1985).

Brief mention has been made of the conversion of vitamin B_{12} to methylcobalamin and 5'-deoxyadenosylcobalamin: a necessary step for vitamin B_{12} activation. According to McDowell (2000), this transformation occurs primarily in the liver but also the kidneys. Methylcobalamin accounts for 60 to 80 % of plasma vitamin B_{12}, whereas 5'-deoxyadenosylcobalamin is the major form of vitamin B_{12} in cellular tissues (Bender 1992).

2.4.3 Enterohepatic circulation

Vitamin B_{12} is continually secreted in the bile. El Kholty *et al.* (1991) estimated that the secretion of vitamin B_{12} into the human bile averaged 1.4 μg day^{-1}, or 0.1 % of the aboved presumed total body vitamin B_{12} pool. Other investigators (Gräsbeck *et al.* 1958; Ardeman *et al.* 1965; Green *et al.* 1981) indicate higher figures of 3 to 6 μg day^{-1} in both humans and baboons. Regardless, the greater part of this vitamin B_{12} is normally reabsorbed in the ileum by means of the intrinsic factor mediated absorption (McDowell 2000). Thus, enterohepatic circulation is important for maintanance of normal vitamin B_{12} balance.

2.4.4 Excretion

Total loss of vitamin B_{12} from the human body, via urine and feces, varies between 2 and 5 μg daily (McDowell 2000). Minuscule amounts, 0 to 250 ng day^{-1}, of vitamin B_{12} are excreted in urine (Ellenbogen and Cooper 1991). The main excretory route is feces. Sources

of fecal vitamin B_{12} include unabsorbed vitamin B_{12} from food or bile, gastric and intestinal secretions, and vitamin B_{12} synthesized by microorganisms in the colon.

2.5 Metabolic Functions of Vitamin B_{12}

There are two confirmed vitamin B_{12}-dependent reactions in mammals. These involve the synthesis of methionine and the conversion of L-methylmalonyl-coenzyme A (CoA) to succinyl-CoA.

2.5.1 Methionine Synthesis

Figure 4 illustrates the current concepts of methionine metabolism. The first product in the metabolism of methionine is S-adenosylmethionine (SAM). SAM is synthesized from methionine and ATP through the enzyme SAM synthetase. SAM serves as a universal methyl donor to a variety of acceptor molecules (Selhub 1999). Also, after undergoing decarboxylation, it is capable of donating an aminopropyl group in polyamine synthesis (Green and Jabobsen 1995). The transfer of SAM's methyl group to an acceptor molecule always produces S-adenosylhomocysteine (SAH). SAH is subsequently hydrolyzed by the enzyme SAH hydrolase to yield adenosine and homocysteine, a non-protein sulfur amino acid (Selhub 1999). This hydrolysis is a reversible reaction that strongly favours SAH formation (De La Haba and Cantoni 1959). However, the rapid metabolism of adenosine and homocysteine results in an equilibrium shift toward SAH hydrolysis (Green and Jacobsen 1995). This is an important regulatory measure since SAH is an inhibitor of most methyltransferase reactions (Green and Jacobsen 1995). At this point adenosine is converted to inosine, while homocysteine is remethylated to methionine or catabolized to cysteine.

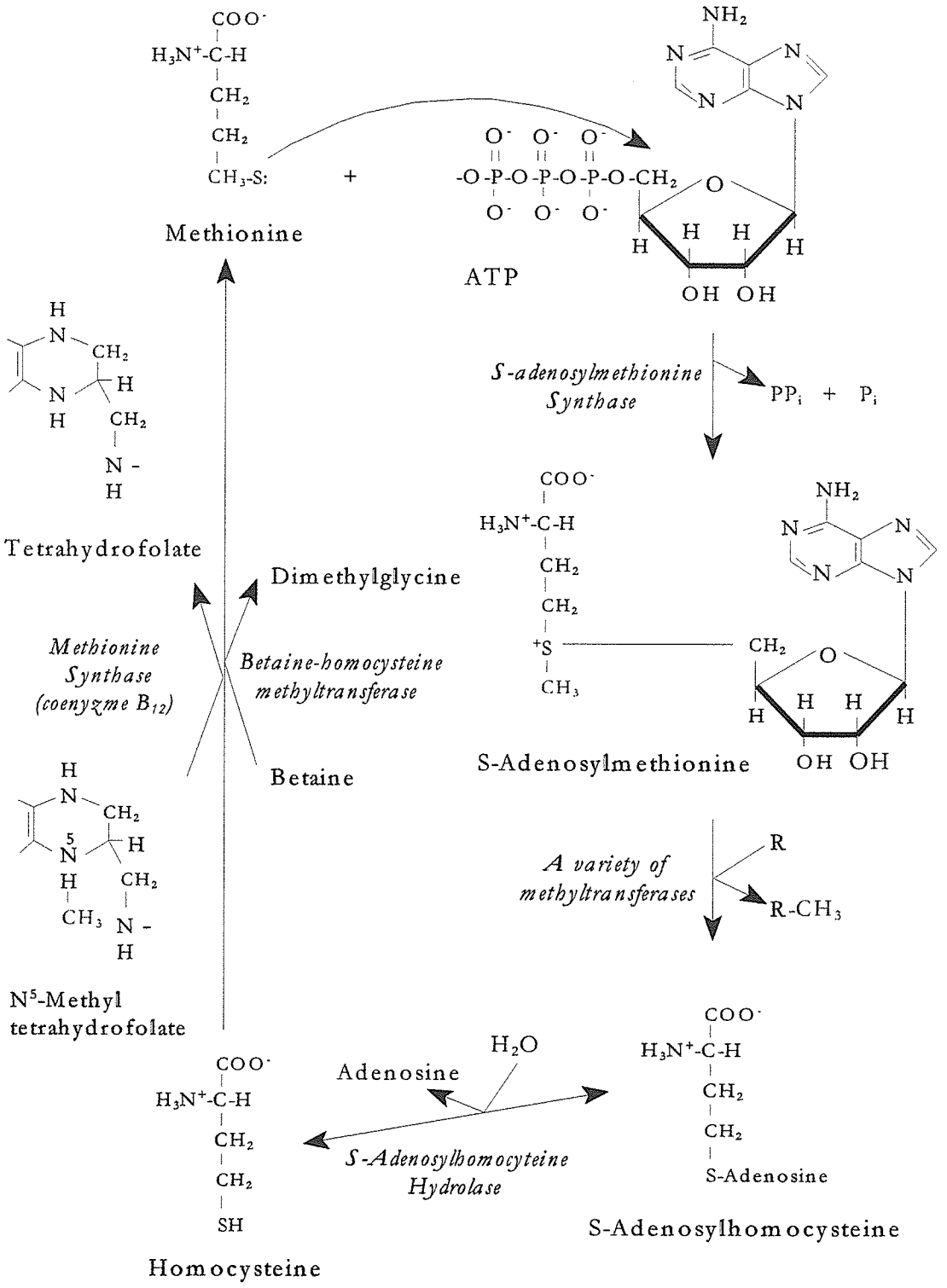


Figure 4. Synthesis of methionine. Adapted from Lehninger *et al.* (1993).

In vitro data reveals that approximately half (53 %) of the homocysteine is remethylated back to methionine, while the other half is irreversibly committed to cysteine formation (Finkelstein and Martin 1984). Homocysteine is not a normal dietary ingredient, the dietary precursor of homocysteine is methionine. Foods contain only trace amounts of homocysteine since tissue levels are generally low in plants and animals (Green and Jacobsen 1995).

2.5.1.1 REMETHYLATION

Homocysteine remethylated back to methionine is catalyzed by means of either the betaine-homocysteine methyltransferase (BHMT) or methionine synthase, the latter, not the former requiring the vitamin B_{12} cofactor, methylcobalamin. The liver and kidney are the primary sites of BHMT expression in monogastric species (Finkelstein *et al.* 1971; McKeever *et al.* 1991; Sunden *et al.* 1997; Delgado-Reyes *et al.* 2000). In contrast to BHMT, methionine synthase is detectable in all tissues except the small intestine (Finkelstein *et al.* 1971). BHMT facilitates the transfer of a methyl group from betaine, an intermediate in the catabolism of choline, to homocysteine forming dimethylglycine and methionine. BHMT is the only means for the degradation of betaine (Finkelstein 1990). Similarly, methionine synthase is the only enzyme which removes the methyl group from N^5-methlytetrahydrofolate (methyl THF) (Finkelstein 1990), thereby generating tetrahydrofolate (THF) and methionine.

Either a genetic defect in one of the enzymes of remethylation or a nutritional deficiency of vitamin B_{12} or folate, in the forms of methlycobalamin and methyl THF respectively, that participate in remethylation can lead to depressed methionine synthesis and hyperhomocysteinemia. Hyperhomocysteinemia is defined as a sustained elevation above

normal of total homocysteine in plasma and serum (Green and Jacobsen 1995). On the basis of work carried out by many researchers, the normal total plasma concentration of homocysteine for adults is 5 to 15 μmoles L^{-1} (Ueland *et al.* 1993). The normal total plasma concentration of homocysteine for children (Vilaseca *et al.* 1997) and neonates (Minet *et al.* 2000) is 3.3 to 11.3 and 4.7 to 10.9 μmoles L^{-1}, respectively. This data is consistent with that recently published by Monsen *et al.* (2001). But data for pigs is sparse and differs considerably from humans. Stangl *et al.* (2000) reported the normal plasma concentration of homocysteine in pigs to be 18 to 22 μmoles L^{-1}.

Perhaps due to its limited tissue distribution, BHMT appears to be incapable of handling excess homocysteine accumulation when the vitamin B_{12}-folate remethylation pathway is impaired (Green and Jacobsen 1995). In vitro data unveiled that merely 27 % of homocysteine can be remethylated back to methionine via BHMT (Finkelstein and Martin 1984).

In the absence of vitamin B_{12}, there is a blockade in getting from methyl THF to THF causing methyl THF to become trapped which leads to impaired folate metabolism. This "methylfolate trap" hypothesis explains why signs of folate deficiency generally accompany vitamin B_{12} deficiency. Vitamin B_{12} and folate deficiency signs are reviewed in Section 2.7.

2.5.1.2 TRANSSULFURATION

In a pathway referred to as transsulfuration (Figure 5), homocysteine condenses with serine to form cystathionine in an irreversible reaction catabolized by the vitamin B_6-dependent enzyme, cystathionine-β-synthase. The irreversibility of the cystathionine-β-synthase reaction explains the inability of cysteine to serve as a precursor of methionine. In

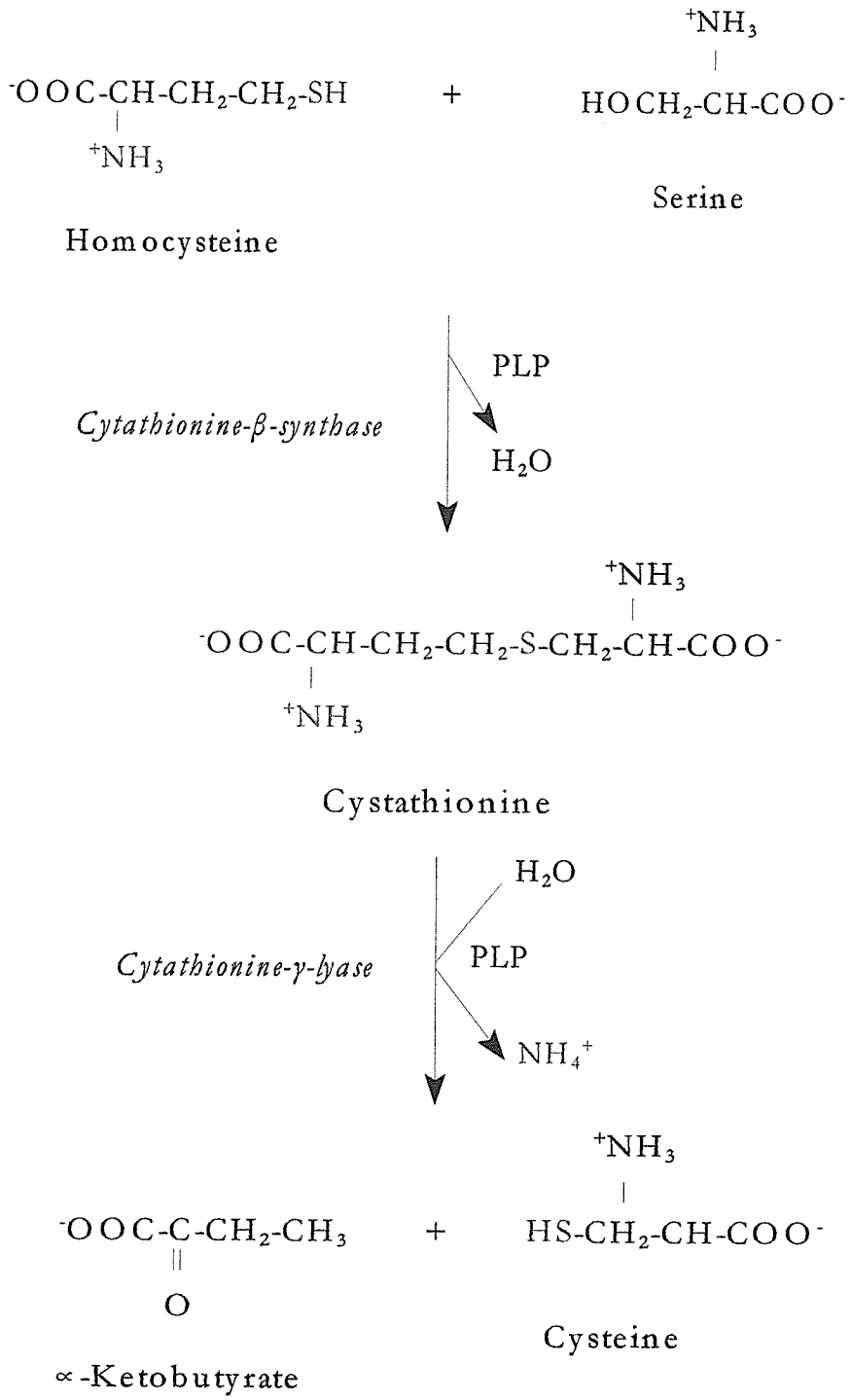


Figure 5. Transsulfuration pathway; synthesis of cysteine from homocysteine and serine. Adapted from Lehninger *et al.* (1993).

the final reaction cystathionine-γ-lyase, another vitamin B_6-dependent enzyme, catalyzes the removal of ammonium (NH_4^+) and cleavage of cystathionine to yield free cysteine and ∝-ketobutyrate. Cysteine is the precursor of metabolites including glutathione and taurine. The literature on cysteine metabolism is extensive and beyond the scope of this review.

Because both cystathionine-β-synthase and cystathionine-γ-lyase are vitamin B_6-dependent, it is highly conceivable that a vitamin B_6 deficiency, whatever the cause, would decrease homocysteine conversion through this pathway, with a resulting increase in homocysteine concentrations. Interestingly, this only occurs in the fed not fasted state. House *et al.* (1999) observed significant increases in the plasma homocysteine of rats fed a vitamin B_6 devoid diet; however the direct cause of such an increase remains to be elucidated.

Contrary to the fed state, a vitamin B_6 deficiency is associated with increased basal cystathionine, but not homocysteine in the fasting state (Miller *et al.* 1992; Miller *et al.* 1994; Ubbink *et al.* 1996; Stabler *et al.* 1997). This may be explained by studies demonstrating that liver cystathionine-γ-lyase activity in vitamin B_6 deficient rats is decreased considerably as compared with cystathionine-β-synthase, which is either normal or only slightly decreased (Swendseid *et al.* 1964; Sturman *et al.* 1969; Finkelstein and Chalmers 1970). It is therefore apparent that, at least in the fasting state, homocysteine can still be converted to cystathionine. Also, Finkelstein and Martin (1984) claim that cystathionine does not inhibit cystathionine-β-synthase activity.

2.5.2 Conversion of L-methylmalonyl-CoA to Succinyl-CoA

The conversion of L-methylmalonyl-CoA to succinyl-CoA is illustrated in Figure 6. Propionyl-CoA, derived from ∝-ketobutyrate produced during transsulfuration, is carboxylated

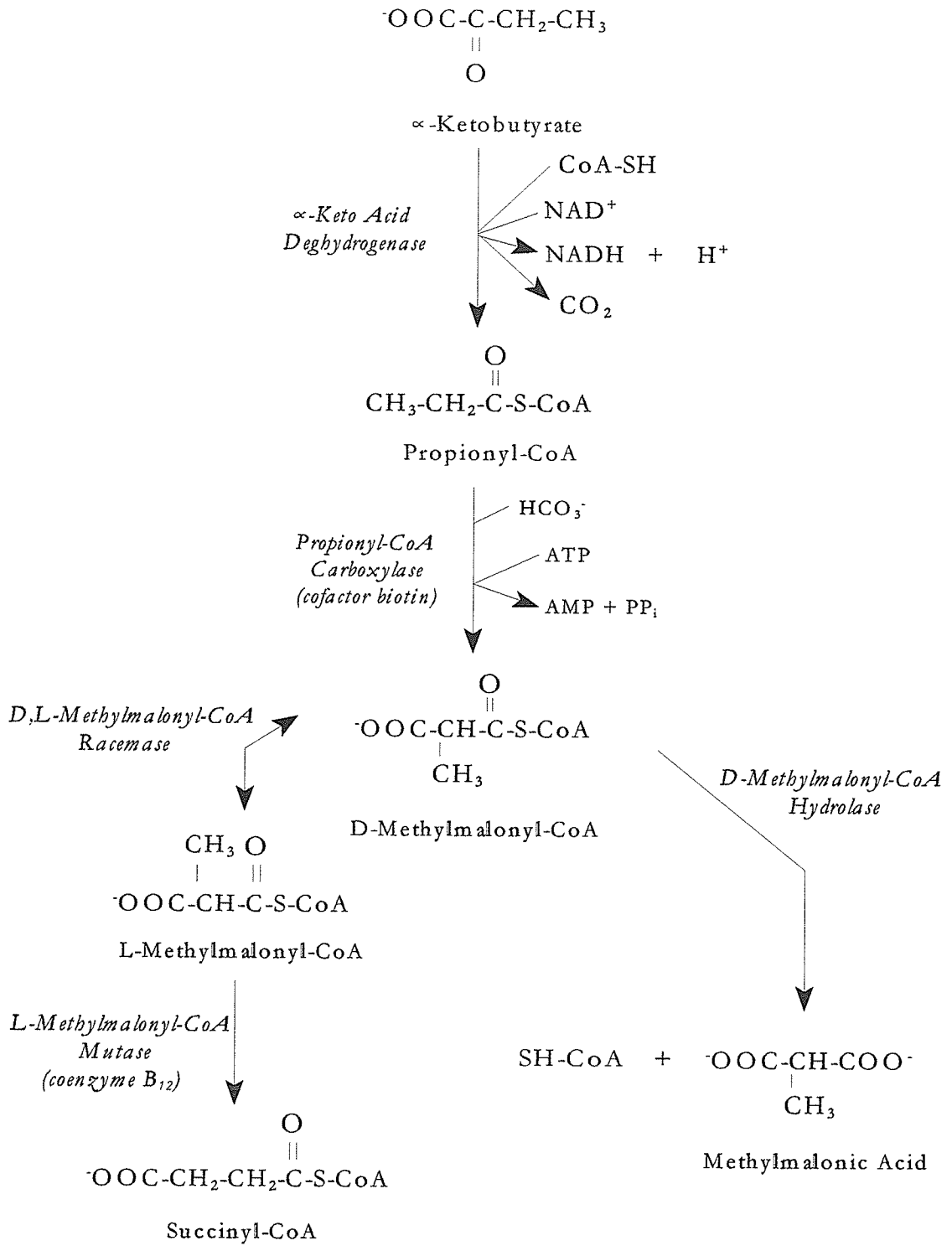


Figure 6. Conversion of propionyl-CoA to succinyl-CoA. Adapted from Lehninger *et al.* (1993).

to form the D stereoisomer of methylmalonyl-CoA by propionyl-CoA carboxylase, which contains the cofactor biotin (Lehninger *et al.* 1993). The D-methylmalonyl-CoA formed is enzymatically epimerized to its L stereoisomer by the action of D,L-methylmalonyl-CoA racemase. The L-methylmalonyl-CoA undergoes an intramolecular rearrangement to form succinyl-CoA, which enters the Krebs cycle. This rearrangement is catalyzed by L-methylmalonyl-CoA mutase, which requires vitamin B_{12} coenzyme 5'-deoxyadenosylcobalamin. In the absence of vitamin B_{12}, this conversion fails to occur and there is an accumulation of methylmalonic acid. This accumulation has been verified by numerous researchers (Stabler *et al.* 1986; Stabler *et al.* 1988; Lindenbaum *et al.* 1990; Moelby *et al.* 1990; Allen *et al.* 1993; Savage *et al.* 1994).

2.6 Dietary Vitamin B_{12} Requirement Of Pigs

Early work by Anderson and Hogan (1950), Fredrick and Brisson (1961), and Nesheim *et al.* (1950) resulted in an estimate of the vitamin B_{12} requirement to be 15 to 20 μg kg^{-1} diet when a synthetic milk diet was fed to weaned pigs (approximately 56 days of age). However, Neumann *et al.* (1950) had shown that pigs under 9 weeks of age required 50 μg of vitamin B_{12} kg^{-1} diet. The latter researchers, however, included 2 g of sulfasuxidine L^{-1} in a synthetic milk diet to inhibit microbial intestinal synthesis of vitamin B_{12}. Theoretically, pigs should have a greater dietary requirement for the vitamin when microbial intestinal synthesis is inhibited. Catron *et al.* (1952) and Richardson *et al.* (1951), using a corn-soyabean oil meal diet supplemented with a combination of antibiotics, concluded that pigs weighing 10 to 45 kg required 8.8 to 11 μg of vitamin B_{12} kg^{-1} diet, respectively. On the basis of such findings, the

NRC (1998) currently recommends a daily intake of 15 to 20 μg of vitamin B_{12} kg^{-1} dry matter for pigs weighing 3 to 20 kg.

Recent work has suggested that the NRC level of one or more of the B vitamins (vitamin B_{12}, niacin, vitamin B_6, riboflavin, and folate) are inadequate to permit maximum growth potential (Stahly and Cook 1996; Stahly *et al.* 1995). Between 9 and 28 kg, pigs obtained greater rates of gain and feed efficiency as the supplementation levels rose from 70 % to 470 % of the NRC requirements for all 5 B vitamins. Subsequent work by this group (Lutz and Stahly 1997) has since eliminated folate as the source of improvement. In addition, Woodworth *et al.* (1997) observed an improved weight gain in pigs weighing 6 to 12 kg fed a corn-soyabean meal based diet supplemented with 2 to 3 mg of vitamin B_6 kg^{-1} of diet. This level exceeds the current recommended NRC (1998) level of 1 to 2 mg of vitamin B_6 kg^{-1} diet for pigs weighing 2 to 10 kg.

2.7 Vitamin B_{12} Deficiency Symptoms

Neumann and Johnson (1950) noted a reduction in growth rate, enlarged tongue and liver, posterior incoordination, and an unsteady gait in vitamin B_{12} deficient pigs. Similar symptoms were observed by Neumann *et al.* (1950), who also noted hyper-irritability, voice failure, and pain in the rear quarters of vitamin B_{12} deficient pigs. Gross observation in older pigs revealed dermatitis, rough hair coats, and exudate around the eyes (Richardson *et al.* 1951; Catron *et al.* 1952). Examination of blood samples taken from deficient pigs have shown normocytic anemia and high neutrophil with concomitantly low lymphocyte counts (Neumann and Johnson 1950).

Signs of folate deficiency generally accompany vitamin B_{12} deficiency (NRC 1998) and include a reduction in growth rate, fading hair colour, macrocytic and normocytic anemia, leukopenia, thrombopenia, reduced packed cell volume, and bone marrow hyperplasia. The "methylfolate trap" hypothesis was introduced earlier. In the absence of vitamin B_{12}, there is a blockade in getting from methyl THF to THF which causes methyl THF to become trapped and leads to impaired folate metabolism. An obvious consequence of impaired folate metabolism is depressed methionine synthesis. Less obvious is abnormal DNA synthesis and cessation of cell proliferation. Folate participates in nucleic acid synthesis including purines and pyrimidines. These metabolic roles of folate explain the common clinical symptoms, reduced growth rate and anemia, seen in folate and vitamin B_{12} deficiencies.

Nervous disorders described by Neuman and Johnson (1950), including posterior incoordination and an unsteady gait, have also been linked in the literature to the "methylfolate trap" hypothesis. Briefly, with decreased synthesis of methionine there is subsequent decreased synthesis of SAM (refer to Figure 4). Central nervous system cells depend heavily upon SAM to provide methyl groups (Scott *et al.* 1994). In response to falling SAM levels in neural tissue, additional methionine is taken up in a concentration-dependent manner (Scott *et al.* 1994). This methionine temporarily serves as a methyl donor and produces SAH. As detailed earlier, SAH is broken down to homocysteine. In the occurrance of a "methylfolate trap", homocysteine is not remethylated. This in turn leads to the build-up of SAH, since the hydrolysis of SAH to homocysteine is a reversible reaction that strongly favours SAH formation. SAH accumulation has a detrimental effect on neural tissue by inhibiting the activity of all methyltransferases (Scott *et al.* 1994).

2.8 Assessment of Vitamin B_{12} Status

A search of the literature revealed numerous laboratory tests that could be used to evaluate vitamin B_{12} status. In this review, only packed cell volume, plasma vitamin B_{12}, plasma homocysteine, and plasma methylmalonic acid will be discussed.

2.8.1 Packed Cell Volume

The "methylfolate trap" hypothesis proposes that in a vitamin B_{12} deficiency, production of methyl THF is impaired. This cause a defect in DNA synthesis and cessation of cell proliferation (megaloblastic anemia) in the marrow. The result is the production of large, immature red blood cells. With megaloblastic anemia, a decrease in packed cell volume is anticipated.

However, Savage *et al.* (1994) claims that both homocysteine and methylmalonic acid are elevated in >89 % of the episodes in which the packed cell volume was normal. This was not an unexpected finding. Earlier researchers (Newmann *et al.* 1950; Stabler *et al.* 1986; Stabler *et al.* 1988; Lindenbaum *et al.* 1990; Stabler *et al.* 1990; Savage *et al.* 1994) had discovered that vitamin B_{12} deficient subjects have normal values for packed cell volume. An explanation for this finding can not be provided.

2.8.2 Plasma Vitamin B_{12}

Among laboratory tests to evaluate vitamin B_{12} status, determination of serum (or plasma) vitamin B_{12} has been most commonly used. Low serum concentrations are associated with low body content of the vitamin (McDowell 2000); the concentration of serum vitamin B_{12} reflects both intake and stores. As vitamin B_{12} intake decreases, serum values may be maintained at the expense of vitamin B_{12} stores in the tissues. A microbiological assay or

radioisotope dilution assay may be used to measure serum levels. The latter assay measures the extent to which vitamin B_{12}, after first being liberated from bound materials, competes with radioactively labeled cyanocobalamin for binding sites on the intrinsic factor (Ellenbogen and Cooper 1991). However, limitations in the sensitivity and specificity in diagnosing a vitamin B_{12} deficiency have been published (Lindenbaum *et al.* 1990; Moelby *et al.* 1990; Hølleland *et al.* 1999). These researchers found normal serum vitamin B_{12} levels in humans with elevated homocysteine and methylmalonic acid. Contrary to serum vitamin B_{12}, homocysteine and methylmalonic acid are recognized as sensitive and specific in diagnosing a vitamin B_{12} deficiency (Stabler *et al.* 1986; Stabler *et al.* 1988; Lindenbaum *et al.* 1990; Moelby *et al.* 1990; Allen *et al.* 1993; Savage *et al.* 1994; Møller *et al.* 1999; Bolann *et al.* 2000). The normal serum (or plasma) vitamin B_{12} concentration for humans is 200 to 900 pg mL^{-1} (McDowell 2000).

2.8.3 Plasma Homocysteine

As previously mentioned, the normal total plasma concentration of homocysteine for adults is 5 to 15 μmoles L^{-1} (Ueland *et al.* 1993), and is inversely correlated to vitamin B_{12} status. In urine the level of homocysteine is the same range (Stabler *et al.* 1988). The normal total plasma concentration of homocysteine for children (Vilaseca *et al.* 1997) and neonates (Minet *et al.* 2000) is 3.3 to 11.3 and 4.7 to 10.9 μmoles L^{-1}, respectively. This data is consistent with that recently published by Monsen *et al.* (2001). But data for pigs is sparse and differs considerably from homocysteine concentrations found in human plasma. Stangl *et al.* (2000) reported the normal plasma concentration of homocysteine in pigs to be 18 to 22 μmoles L^{-1}. Plasma homocysteine is measured via high performance liquid chromatography

after being released from S-S bonds it forms with protein (Ellenbogen and Cooper 1991).

2.8.4 Plasma Methylmalonic Acid

Stabler *et al.* (1986) did pioneering work to establish the use of methylmalonic acid concentrations in evaluating vitamin B_{12} status. They showed that serum methylmalonic acid accumulates when a vitamin B_{12} deficiency impairs the conversion of L-methylmalonyl-CoA to succinyl-CoA. Allen *et al.* (1993) found the normal serum concentration of methylmalonic acid in pigs to be 215 nmoles L^{-1}; and may vary up to 20 % from day to day in a given animal. Plasma methylmalonic acid values are comparable to serum methylmalonic acid values (Pheiffer *et al.* 1999). Presently, gas chromatography-mass spectroscopy is used to measure methylmalonic acid.

2.9 Toxicity of Vitamin B_{12}

No reports are known to suggest a maximum dietary tolerable level of vitamin B_{12} for pigs (NRC 1987). However, levels as high as 1600 mg kg^{-1} body weight (several hundred times the requirement) have been administered to albino mice with no ill effects noted (Winter and Mushett 1950). Since vitamin B_{12} absorption is limited, as mentioned previous, consuming megadoses of this vitamin would not be expected to increase the amount absorbed.

3.0 HYPOTHESIS AND OBJECTIVES

Previous research (Stahly *et al.* 1995) invites a reassessment of the B vitamin requirements for the weaned pig in light of the industry's push for rapid lean growth. The Stahly *et al.* (1995) examined five B vitamins (riboflavin, niacin, pantothenic acid, folic acid, and vitamin B_{12}) collectively, fed at various percentages (70, 170, 270, 370, and 470) of the NRC requirements (1988). Between 9 and 28 kg, pigs obtained greater rates of gain and feed efficiency as the supplementation level rose to 470 % of the NRC requirements for the five B vitamins collectively. The experimental design makes it impossible to determine how much each vitamin affected the results, although, subsequent research by Lutz and Stahly (1997) have since eliminated folic acid as the source of improvement.

The current vitamin B_{12} requirement for 3 to 20 kg pigs ranges from 15 to 20 μg of vitamin B_{12} kg^{-1} of dry matter (NRC 1998). Based upon on the above statements, it is hypothesized that this current NRC recommendation is not optimal to maximize the growth potential of modern genetic strains of (early weaned) pigs. It is also hypothesized that the addition of an antibiotic to the diet adversely affect the microbial synthesis of vitamin B_{12} in the colon. Therefore, early weaned pigs consuming a diet containing an antibiotic would have a greater vitamin B_{12} requirement than pigs consuming a diet not containing an antibiotic. Finally, it is hypothesized that measures of plasma metabolite levels are a better means of diagnosing a vitamin B_{12} deficiency and establishing a vitamin B_{12} requirement in the early weaned from 17 to 32 days of age compared to growth performance parameter.

The present thesis extends and complements the research of Stahly *et al.* (1995) by

determining the vitamin B_{12} requirement of the early weaned pig. Presented in this thesis are the description and results of four experiments designed to:

- examine the effects of graded levels of vitamin B_{12} in a corn-soyabean meal-lactose based diet on the development of the early weaned pig
- examine the effects of dietary antibiotics on the vitamin B_{12} status of the early weaned pig

Also offered was the opportunity to compare the sensitivities of growth, packed cell volume, plasma vitamin B_{12}, plasma homocysteine, plasma methylmalonic acid, and plasma cystathionine concentrations in detecting an early depletion of the vitamin.

4.0 MATERIALS AND METHODS

4.1 GENERAL METHODOLOGY

The experimental protocol was reviewed and approved by the University of Manitoba Protocol Management Review Committee. All animals in the experiments were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993), as enforced by the University of Manitoba Protocol Management Review Committee.

4.2 EXPERIMENT 1

4.2.1 ANIMALS

A total of 60 Cotswold pigs were used in the 15-day experiment. At approximately 17 days of age, pigs were weaned from the sow at the site of origin (Glenlea Swine Research Unit). Pigs were then transported to the Animal Science Research Unit at the University of Manitoba. Pigs were equalized for sex, and randomly allocated to one of five treatment groups each represented by three replicate pens of four pigs. For 3 days post-entry, all pigs were administered an intramuscular injection of tylosin (200 mg ml^{-1}, Elanco, Guelph, ON, Canada) to combat diarrhea.

4.2.2 HOUSING AND SANITATION

The pigs were group housed in an environmentally-regulated room. Temperature was initially set at 30°C, and then gradually reduced according to normal heating requirements. The room was continuously lit throughout the experiment. The pens were 5'9" x 3'1" and equipped with raised tenderfoot floors. Each pen had one self-feeder and two nipple waterers

to allow *ad libitum* intake of feed and water. The pens were thoroughly washed every second day to prevent coprophagy.

4.2.3 DIETARY TREATMENTS

Pigs were *ad libitum* fed a pelleted corn-soyabean meal-lactose basal diet, the composition of which is given in Table 3. Omission of vitamin B_{12} from the basal diet, via the exclusion of animal products, was necessary to assess the dietary essentiality of vitamin B_{12}. To this basal diet were added 0, 35, 70, 105, or 140 μg vitamin B_{12} kg^{-1} diet (providing 0, 200, 400, 600, or 800 % of the NRC (1998) recommendation for pigs weighing 3 to 20 kg). Vitamin B_{12}, in the form of cyanocobalamin, had a concentration of 1 g kg^{-1} (FeedRite Ltd., Winnipeg, Manitoba, Canada). Inclusion of these levels were based upon the results of Stahly and Cook (1996) and Stahly et al. (1995). Except for vitamin B_{12}, the basal diet was formulated to meet or exceed the NRC (1998) recommendation for all nutrients. All other vitamins were added to provide at least 150 % of the NRC (1998) recommendation. Ingredients of low concentrations were hand-mixed prior to processing the complete feed. Feed offered and wastage were recorded daily.

4.3 EXPERIMENT 2

4.3.1 ANIMALS

A total of 48 Cotswold pigs were used in the 15-day experiment. At approximately 17 days of age, pigs were weaned from the sow at the site of origin (Glenlea Swine Research Unit). Pigs were then transported to the Animal Science Research Unit at the University of Manitoba. Pigs were equalized for sex, and randomly allocated to one of two dietary treatment

Table 3. Basal diet composition for experiments 1, 2, and 3 (90 % dry basis).

Ingredient [1]	Amount, %
Corn	33.46
Soybean meal, dehulled	23.99
Lactose	15.54
Wheat	15.00
Propulse 975 pea protein [2]	3.91
Canola oil	2.50
Dicalcium phosphate	1.88
Calcium carbonate	0.67
Salt, deiodized	0.66
Trace mineral and vitamin mix [3]	1.00
L-Lysine	0.78
L-Threonine	0.28
DL-Methionine	0.22
ASP250 [4]	0.10
Total	100.00

[1] Ingredients were chosen to obtain a vitamin B_{12} devoid diet.
[2] Generously supplied by Parrheim Foods (a division of Parrish & Heimbecker Limited), Portage La Prairie, MB, Canada, through the courtesy of Mr. Ferdi van Dogen.
[3] Generously supplied by FeedRite Ltd., Winnipeg, MB, Canada, through the courtesy of Mr. Bruce Mollison. Provided per kg of diet: vitamin A, 3299 IU; vitamin D, 330 IU; vitamin E, 16 IU; vitamin K, 0.75 mg; niacin, 0.79 mg; calcium pantothenate, 7.52 mg; riboflavin, 3.91 mg; folic acid, 55.78 μg; Fe, 12.33 mg; Cu, 2.06 mg; I, 0.16 mg. Wheat middlings acted as the carrier.
[4] Super Chlor-250 Provided per kg of diet: chlortetracycline hydrochloride, 1.10 g; sulfamethazine, 0.44g; penicillin from procaine penicillin, 0.55 g

groups each represented by six replicate pens of four pigs. Some pigs developed brown encrustations on their skin (diagnosed as greasy pig syndrome) during the experiment. Therefore, an intramuscular injection of tylosin (200 mg ml^{-1}, Elanco, Guelph, ON, Canada) and/or excenel (50 mg mL^{-1}, Pharmacia & Upjohn Co., Orangeville, ON, Canada) was given to those animals. In addition, a combination of mineral oil and pirsue (50 mg 10 mL^{-1}, pirlimycin hydrochloride, Pharmacia & Upjohn Co., Orangeville, ON, Canada) was applied topically. Pig deaths occurring during the experiment were recorded.

4.3.2 HOUSING AND SANITATION

Pigs were group housed as described in Section 4.2.2, except the room was lit daily from 7:00 a.m. till 5:30 p.m..

4.3.3 DIETARY TREATMENTS

The same basal diet was used in this experiment as in the previous experiment (Table 3) except that 10 g ASP 250 kg^{-1} diet was or was not added to the diet. The 10 g of ASP 250 was substituted with 10 g of wheat middlings. As in the previous experiment, feed offered and wastage were recorded daily.

4.4 EXPERIMENT 3

4.4.1 ANIMALS

A total of 60 Cotswold pigs were used in the 15-day experiment. At approximately 17 days of age, pigs were weaned from the sow at the site of origin (Glenlea Swine Research Unit). Pigs were then transported to the Animal Science Research Unit at the University of Manitoba. Pigs were randomly allocated to one of five dietary treatment groups each

represented by three replicate pens of four pigs. Some pigs had received tylosin (200 mg ml^{-1}, Elanco, Guelph, ON, Canada) the day prior to weaning for incidence of diarrhea. Their treatment was continued for 2 days post-weaning. As a precaution, the remaining pigs were administered tylosin for 3 days post-entry.

4.4.2 HOUSING AND SANITATION

The pigs were group housed in an environmentally-regulated room. Temperature was initially set at 30°C, and then gradually reduced according to normal heating requirements. The room was lit daily from 7:00 am till 5:30 pm. The pens were 4'10" x 3'8" and equipped with raised tenderfoot floors. Each pen had one self-feeder and one nipple waterer to allow *ad libitum* intake of feed and water. The pens were thoroughly washed every second day to prevent coprophagy.

4.4.3 DIETARY TREATMENTS

The basal diet in the present experiment was identical to that used in experiment 1 (Table 3). The experimental treatments were formed by adding vitamin B_{12} at 0, 10, 20, 30, or 40 $\mu g\ kg^{-1}$ of diet. As in experiment 1, feed offered and wastage were recorded daily.

4.5 EXPERIMENT 4

4.5.1 ANIMALS

A total of 60 Cotswold pigs were used in the 15-day experiment. At approximately 17 days of age, pigs were weaned from the sow at the site of origin (Glenlea Swine Research Unit). Pigs were then transported to the Animal Science Research Unit at the University of Manitoba. Pigs were randomly allocated to one of five dietary treatment groups each

represented by three replicate pens of four pigs. During the experiment, some pigs were administered tylosin (200 mg mL^{-1}, Elanco, Guelph, ON, Canada) for intermittent attacks of diarrhea.

4.5.2 Housing and Sanitation

Section 4.4.2 contains a complete description of the housing facilities and the sanitation practices used in this experiment.

4.5.3 Dietary Treatments

The basal diet in the present experiment was similar to that used in the latter experiments, except the ASP250 was removed and replaced with corn and soyabean meal (Table 4). The experimental treatments were formed by adding vitamin B_{12} at 0, 50, 100, 150, or 200 μg kg^{-1} of diet. For this experiment, the vitamin B_{12} was manufactured by the primary investigator using 99 % pure vitamin B_{12}, in the form of cyanocobalamin (Sigma Chemical Co., St. Louis, MO, USA), and sucrose (Sigma Chemical Co., St. Louis, MO, USA). To obtain a final concentration of 1 g kg^{-1}, 100 mg of vitamin B_{12} was added to 99.9 g of sucrose. As in the previous experiments, feed offered and wastage was recorded daily.

4.6 Data Collection

4.6.1 Measurement of Body Weight and Feed Intake

Individual body weights and feed intake on a pen basis were measured at pen entry, days 5, 10, and 15. Measurements of individual body weights and feed intake allowed for the calculation of average daily gain (ADG), average daily feed intake (ADFI), and feed conversion efficiency (FCE).

Table 4. Basal diet composition for experiment 4 (90 % dry basis).

Ingredient [1]	Amount, %
Corn	33.52
Soybean meal, dehulled	24.04
Lactose	15.54
Wheat	15.00
Propulse 975 pea protein [2]	3.91
Canola oil	2.50
Dicalcium phosphate	1.88
Calcium carbonate	0.67
Salt, deiodized	0.66
Trace mineral and vitamin mix [3]	1.00
L-Lysine	0.78
L-Threonine	0.28
DL-Methionine	0.22
Total	100.00

[1] Ingredients were chosen to obtain a vitamin B_{12} devoid diet.
[2] Generously supplied by Parrheim Foods (a division of Parrish & Heimbecker Limited), Portage La Prairie, MB, Canada, through the courtesy of Mr. Ferdi van Dogen.
[3] Generously supplied by FeedRite Ltd., Winnipeg, MB, Canada, through the courtesy of Mr. Bruce Mollison. Provided per kg of diet: vitamin A, 3299 IU; vitamin D, 330 IU; vitamin E, 16 IU; vitamin K, 0.75 mg; niacin, 0.79 mg; calcium pantothenate, 7.52 mg; riboflavin, 3.91 mg; folic acid, 55.78 μg; Fe, 12.33 mg; Cu, 2.06 mg; I, 0.16 mg. Wheat middlings acted as the carrier.

4.6.2 Blood Collection

All pigs were subjected to blood collection at the conclusion of the experiments. For confirmation of basal plasma metabolite concentrations, all pigs from experiment 2 were subjected to blood collection at pen entry. Whole blood samples were collected via jugular venipuncture into 7.0 mL heparinized vacutainer tubes (Benton Dickson, Franklin Lake, NJ, USA). Samples were placed into a cooler containing ice until collection was complete. Packed cell volume was determined in triplicate immediately following collection with heparinized microhematocrit capillary tubes (Fisher Scientific, Ottawa, ON, Canada). Within 6 hours of collection, blood samples were centrifuged at 1800 x g for 15 minutes. Plasma was removed, placed into 1.5 mL polypropylene microcentrifuge tubes (Fisher Scientific, Ottawa, ON, Canada), and frozen at -20°C until analyzed.

4.7 Proximate Analysis

Representative samples of the experimental diets were mixed and ground in a Tecator cyclotec 1093 sample mill (Tecator AB, Höganäs, Sweden). The feed samples were then analyzed in duplicate for dry matter, ash, crude fat, neutral detergent fibre, crude protein, and gross energy using established standard methods of analysis (A. O. A. C. 1990). Summaries of the feed analyzes data are given in Appendices 1 to 4.

4.7.1 Dry Matter Determination

Approximately 2.0 g of sample were weighed into pre-weighed dry matter cans. Samples were placed in a vacuum oven set at 102°C and dried for 16 hours. Samples were then removed, cooled in a dessicator, and re-weighed.

4.7.2 Ash Determination

The ash component of each feed sample were determined by placing approximately 2.0 g of the sample into pre-weighed silica crucibles and burning the samples in a muffle furnace for 12 hours at 600°C. Following the 12 hours, crucibles containing the ash were cooled in a dessicator and re-weighed.

4.7.3 Crude Fat Determination

Approximately 1.0 g of pre-dried sample were weighed and placed in cellulose extraction thimbles. Next, 35.0 mL of n-hexane was added to pre-weighed beakers. The extraction thimbles and beakers were then attached to a Goldfisch fat extraction apparatus. The extraction procedure was allowed to proceed for 5 hours after the onset of boiling. Following the 5 hours, heating elements were lowered, extraction thimbles were removed and replaced with collection thimbles. Heating elements were re-position and boiling continued until only 1.0 to 0.5 mL of n-hexane remained in the beakers. The beakers were taken to complete dryness (removal of all n-hexane) in a 100°C oven overnight. The following morning, beakers were cooled in a dessicator and re-weighed to obtain a weight of the extracted fat.

4.7.4 Neutral Detergent Fibre Determination

Approximately 0.5 g of sample were weighed into pre-weighed F57Ankom filter bags (Ankom Technology Corporation, Fairport, NY, USA) and the bags sealed with a Clamco Impulse Sealer (Model 210-8E, Clamco Corporation, Cleveland, Ohio, USA). The sample bags, 4.0 mL of heat stable alpha-amylase (Ankom Technology Corporation, Fairport, NY, USA), 20.0 g of anhydrous sodium sulfite (Anachemia Canada Inc., Montreal, PQ, Canada),

and 2.0 L of NDF solution (8.6 gm sodium phosphate dibasic heptahydrate L^{-1} distilled water) were added to the digestion vessel of an Ankom 200 Fiber Analyzer (Ankom Technology Corporation, Fairport, NY, USA). Digestion, aided by heat and agitation, was allowed to proceed for 60 minutes. Following the 60 minutes, the solution was exhausted and the sample bags rinsed for 3 minutes with 2.0 L of boiling water and 4.0 mL of alpha-amylase. This step was repeated 3 times, with the exception that alpha-amylase was not added to the third rinse. The sample bags were removed from the digestion vessel, placed in a beaker, and soaked in acetone for 3 minutes. Excess acetone was squeezed from the sample bags prior to them being placed in a drying oven at 100°C overnight. The following morning, the sample bags were cooled at room temperature and re-weighed to calculate the percentage of neutral detergent fibre.

4.7.5 Crude Protein Determination

Within a digestion tube, approximately 0.5 g of sample were digested for 45 minutes with 15.0 mL of concentrated sulfuric acid and 5.0 g of Pro-pac Power No.7 (Alfie Packers Inc., Omaha, NE, USA) catalyst in a 1015 Digester (Tecator AB, Höganäs, Sweden). Following digestion, tubes were cooled at room temperature for 10 minutes and then 55.0 mL of distilled water was slowly added. The nitrogen content was then measured via a Kjeltec Auto 1030 Analyser (Tecator AB, Höganäs, Sweden). In brief, 40 % sodium hydroxide was added to the tubes, the solution steamed distilled, mixed with receiver solution (boric acid dissolved in deionized water plus bromocresol green and methyl red as indicators), and then titrated with 0.101 N hydrochloric acid. The protein content of the feed sample was estimated by multiplying the amount of nitrogen present by a factor of 6.25.

4.7.6 Gross Energy Determination

Gross energy of the feed samples was measured by a calibrated Parr adiabatic oxygen bomb calorimeter (Parr Instrument Company Inc., Moline, IL, USA). Approximately 0.7 g of sample were weighed, pelleted, and deposited in a steel crucible. The crucible containing the sample was then positioned on an electrode unit to which a 10 cm fuse wire had been attached. The electrode unit was then enclosed in a bomb. Approximately 1.0 mL of distilled water and 25 atmospheres of oxygen were added to the bomb. The bomb was then inserted into a jacket containing 2.0 L of distilled water. After burning the sample, the crucible was removed from the electrode unit within the bomb, placed in a beaker, titrated to a yellow colour with 0.0725 N sodium carbonate and 3 drops of 0.1 % w/v methyl orange in water (indicator colour), and the titer recorded. All unburned pieces of fuse wire were removed from the electrode unit and measured. After correcting for the amount of mLs of sodium carbonate used in the titration and the length of fuse wire burned, gross energy results were obtained from a pre-programmed calorimeter control unit. Digestible energy was calculated according to the equation of Noblet and Perez (1993).

4.8 Dietary Vitamin B_{12} Analysis

The microbiological assay is the method of choice for measuring feed vitamin B_{12} concentrations (Casey *et al.* 1982). The microbiological assay, performed by Maxxam Analytics Inc., revealed the vitamin B_{12}-unsupplemented diet in experiment 1 contained 36.5 μg of vitamin B_{12} kg^{-1} of diet. However, due to the cost of the microbiological assay, an attempt was made to use a commercial radioimmunoassay to measure the vitamin B_{12} concentration in the

experimental diets. The vitamin B_{12} concentration in the experimental diets and individual feedstuff ingredients (Table 10 and Appendix 5, respectively) were determined in several assays using a quantaphase II B_{12} radioimmunoassay (Bio-Rad, Hercules, CA, USA) and was validated for use with fortified and unfortified food products by Casey *et al.* (1982).

4.8.1 Sample Preparation

Representative samples of the experimental diets and individual feedstuff ingredients were mixed and ground to pass through a 1 mm mesh sieve in a tecator cyclotec 1093 sample mill (Hoganas, Sweden). A 1 to 5 g portion of sample was weighed into a 250 mL erlenmeyer flask and 50 mL of extraction solvent (13 g sodium phosphate dibasic anhydrous, 12 g citric acid monohydrate, and 10 g sodium metabisulfite diluted to 1 L with deionized water) was added. The flasks were plugged with plastic foam stoppers. Samples were manually agitated, autoclaved for 10 minutes at 121 to 123°C or incubated for 1 hour at 100°C in a boiling water bath, and cooled rapidly in an ice water bath to room temperature. Following quantitative transfer into a 100 mL volumetric flask, samples were completed to volume with phosphate buffer (9.1 g potassium phosphate monobasic anhydrous and 18.9 g sodium phosphate dibasic anhydrous diluted to 1 L with deionized water; pH 7.0) and inverting 10 times to mix well. Samples were then centrifuged at 13 700 x g for 15 minutes, the supernatant was decanted, and frozen at -20°C until analyzed.

4.8.2 Sample Analysis

The vitamin B_{12} content of the samples were estimated using a quantaphase II B_{12} radioimmunoassay (Bio-Rad, Hercules, CA, USA). The standard for the assay was vitamin B_{12} (cyanocobalamin) in HSA base with 0.1 % sodium azide as a preservative and had a

concentration range of 0 to 2000 pg mL^{-1}. The standards were reconstituted in deionized water.

For this assay, aliquots of 200 μL of standard and sample were added to all 12 x 75 mm polypropylene reaction tubes (Bio-Rad, Hercules, CA, USA) except the total binding tubes. This was followed by the addition of 1 mL of working tracer, vortexing twice for 30 seconds, and incubation for 20 minutes at 100°C in a boiling water bath. Tubes were cooled in a ice water bath for 10 minutes and then left to sit at room temperature for 15 minutes. One hundred μL of blank reagent was then added to the blank tubes, 100 μL of microbead reagent was added to all other tubes, and all tubes were vortexed twice. Tubes were incubated at room temperature for one hour. Tubes were centrifuged at 12000 x g for 10 minutes and the supernatant fraction was decanted.

Standards and samples were counted in a gamma counter (LKB Wallac 1282 Compu Gamma Universal Gamma Counter). Total counts were approximately 16901 to 17033 cpm. Maximum binding was approximately 65 %. The standard curve concentration range was 0 to 2000 pg mL^{-1}. Nonspecific binding of all assays was <5 %. Sensitivity of the assays at 95 % binding was 15 pg mL^{-1}.

4.9 Biochemical Analyses

All plasma samples were analyzed for the concentration of vitamin B_{12} and homocysteine. Only plasma samples collected from pigs fed the dietary vitamin B_{12} supplementation of 0 and 140 μg kg^{-1} diet in experiment 1 were subjected to methylmalonic acid and cystathionine analysis.

4.9.1 PLASMA VITAMIN B_{12}

Plasma vitamin B_{12} was analyzed by a quantaphase II B_{12} radioassay (Bio-Rad, Hercules, CA, USA), as outlined in Section 4.8.2. Total counts were approximately 16013 to 18402 cpm. Maximum binding ranged between 47 and 66 %. Nonspecific binding of all assays was ≤4.4 %. Sensitivity of the assays at 95 % binding averaged 16 pg mL^{-1}. The intraassay and interassay coefficients of variation were ≤18 % and 19 %, respectively. See Appendix 6 for individual assay quality control data.

4.9.2 PLASMA HOMOCYSTEINE

The procedure used to analyze homocysteine was adopted from Vester and Rasmussen (1991). For this procedure, 150 μL of standard and sample plasma was aliquoted into 1.5 mL polypropylene microcentrifuge tubes. To liberate bound homocysteine, 20 μL of 100 mg mL^{-1} tris (2-carboxyethyl)-phosphine hydrochloride (Pierce, Rockford, IL, USA) was added and the reduction allowed to proceed for 30 minutes at 4°C. Samples were then mixed with 125 μL of 0.6 mol L^{-1} perchloric acid, containing 1 mmol L^{-1} EDTA, and centrifuged at 12 000 x g for 10 minutes. One hundred μL of the supernatant fraction was removed, placed in a new microcentrifuge tube, and mixed with a 1 mg mL^{-1} solution of ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid (Sigma Chemical Co., St. Louis, Mo, USA) and 2 mol L^{-1} potassium borate, pH 9.5. This was followed by the addition of 200 μL of 2 mol L^{-1} potassium borate, pH 10.5, containing 5 mmol L^{-1} EDTA which was then incubated at 60°C in a water bath for 60 minutes. After cooling at room temperature, the samples were ready for analysis in a SCL-10A VP Shimadzu HPLC system (Man-Tech Associates Inc., Guelph, ON, Canada).

To determine the homocysteine concentrations, 250 μL flat bottom inserts (Altech Associates Inc., Deerfield, IL, USA) were filled, placed into 12 x 32 mm clear screw cap vials (Altech Associates Inc., Deerfield, IL, USA), capped with yellow polypropyl open hole caps (Altech Associates Inc., Deerfield, IL, USA), and fitted with red tfe/silicone liners (Altech Associates Inc., Deerfield, IL, USA). The samples were then injected onto a Hypersil® ODS C^{18} reverse-phase column (4.6 x 150 mm, 5 μm) (Man-Tech Associates Inc., Guelph, ON, Canada). A 0.1 mol L^{-1} acetate buffer (a HPLC eluent), pH 4.0, containing 20 mL L^{-1} methanol was prepared from 0.1 mol L^{-1} acetic acid and 0.1 mol L^{-1} sodium acetate. The buffer was filtered through a 0.22 μm MSI filter (Fisher Scientific, Ottawa, ON, Canada) and had a flow rate of 1.0 mL $minute^{-1}$. The fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm. The detection signal was recorded and the peak areas quantified with a Class VP chromatography data system. Concentration of homocysteine (μmoles L^{-1}) was derived from a calibration curve established with pure homocysteine standards (Sigma Chemical Co., St. Louis, Mo, USA).

4.9.3 Plasma Methylmalonic Acid

Methylmalonic acid was analyzed at the University of Colorado Health Sciences Centre in the Hematology Division. The method is based on the stable isotope dilution principle using the ratio of unlabeled methylmalonic acid to labeled methylmalonic acid ([methyl-D_3]methylmalonic acid) as previously described in detail (Allen et al. 1993). Briefly, 51 μL of water containing [methyl-D_3]methylmalonic acid was added to 400 μL of pig plasma and 1 mL of water. Samples were then vortexed, applied to 0.9 x 6 cm columns containing 400 μL anion-exchange resin, washed once with 3 mL of water and three times with 3 mL 0.01

N acetic acid in methanol, and eluted with 1.1 mL 3.6 mol/L acetic acid/0.1N HCL in 10 % water/90 % methanol. Elutes were then dried by vacuum centrifugation, derivatized by adding 30 μL of a solution containing 10 μL N-methyl-N(tert-butyldimethlsilyl)trifluoroacetamide and 20 μL acetonitrile, and incubated at 90°C for 30 minutes. The samples were analyzed on a SPB-1 column (20 m x 0.25 mm internal diameter, 0.25 um film thickness) from Supelco (Bellefonte, PA) and a Hewlett Packard (Palo Alto, CA) 5890 gas chromatography-mass spectrometer equipped with a 7673A autosampler and a Hewlett Packard 5971A mass selective detector. Quanititation was based on the ratio of the area of the base peak ion 289.2 m/z for the endogenous methylmalonic acid, to the areas of the base peak ion 292.2 m/z for the [methyl-D_3]methylmalonic acid internal standard.

4.9.4 PLASMA CYSTATHIONINE

Cystathionine was analyzed, as previously described by Stabler *et al.* (1993), at the University of Colorado Health Sciences Centre in the Hemotology Division. The method is based on the stable isotope dilution principle using the ration of unlabeled cystathionine to labeled cystathionine (D,L-[2-amino-2-carboxyethyl]-homocysteine 3,3,4,4-D_4). For this assay, 51 μL of water containing 400 pmol of D,L-[2-amino-2-carboxyethyl]-homocysteine 3,3,4,4-D_4 was added to 400 μL of plasma, 1 mL of water, and 51 μL og 1 N NaOH containing 10 mg mL^{-1} of dithiothreitol in 12 x 75 mm test tubes. Samples were then mixed by vortexing, incubated at 40°C for 30 minutes, and then applied to 0.9 x 6cm columns containing 400 μL anion-exchange resin. After samples were applied, each column was washed three times with 3 mL of water and one time with 3 mL of methanol. Each column was then eluted with 1.1 mL of 0.4 N acetic acid in methanol. Elutes were taken to dryness by vacuum centrifugation

in a vacuum centrifuge, derivatized by adding 30 μL of a solution containing 10 μL of N-methyl-N (tert-butyldimethylsilyl) trifluoracetamide and 20 μL of acetonitrile, and incubated at 90°c for 30 minutes. The samples were analyzed on a SPB-1 column (20 m x 0.25 mm internal diameter, 0.25 um film thickness) from Supelco (Bellefonte, PA) and a Hewlett Packard 5890 gas chromatography-mass spectrometer equipped with a 7673A autosampler and a Hewlett Packard 5971A mass selective detector. Quanititation was based on the ratio of the area of the base peak ion 362.2 m/z for the endogenous cystathionine, to the areas of the base peak ion 366.2 m/z for the D,L-[2-amino-2-carboxyethyl]-homocysteine 3,3,4,4-D_4 internal standard.

4.10 CALCULATIONS

$$\% \text{ Dry Matter} = \frac{\text{Final Sample Weight} \times 100}{\text{Initial Sample Weight}}$$

$$\% \text{ Ash} = \frac{(\text{Final Crucible Weight} + \text{Ash}) - (\text{Initial Crucible Weight})}{\text{Initial Sample Weight}} \times 100$$

$$\% \text{ Crude Fat} = \frac{(\text{Final Vessel Weight} + \text{Fat}) - (\text{Initial Vessel Weight})}{\text{Initial Sample Weight}} \times 100$$

$$\% \text{ Neutral Detergent Fibre} = \frac{\text{Neutral Detergent Fibre Residue Weight}}{\text{Sample Weight}} \times 100$$

$$\% \text{ Crude Protein} = \frac{[(\text{Volume of HCL (L)} \times \text{Normality of HCL (moles } L^{-1}))]}{\text{Weight of Sample}} \times 14.01 \times 6.25$$

$$\text{ADG} = \frac{\text{Final Pig Weight} - \text{Initial Pig Weight}}{\text{Total Number Days}}$$

$$\text{ADFI} = \frac{\text{Total Feed Intake}}{\text{Total Number Days}}$$

$$FCE = \frac{\text{Total Gain}}{\text{Total Feed Intake}}$$

4.11 STATISTICAL ANALYSIS

The experimental data collected on an individual pig basis (packed cell volume, plasma vitamin B_{12}, plasma homocysteine, plasma methylmalonic acid, and plasma cystathionine) were analyzed as a completely randomized design with a 2 x 5 factorial arrangement by which pigs of each sex were randomly allocated to 1 of 5 dietary treatments for experiments 1, 3, and 4. Note that experimental data collected on an individual pig basis in experiment 2 was analyzed as a completely randomized design with a 2 x 2 factorial arrangement by which pigs of each sex were randomly allocated to 1 of 2 dietary treatments . The individual pig was the experimental unit. Outliers were removed using the studentized residuals.

The model used for the completely randomized design was $y_{ijk} = \mu + s_i + t_j + \gamma_{ij} + e_{ijk}$, where y_{ijk} is a measurement on the k^{th} pig with the i^{th} sex on treatment j, μ is the population mean, s_i is the effect of sex i (i = 1 or 2), t_j is the effect of treatment j (j =1 to 5, or 1 or 2), γ_{ij} is the interaction effect of sex i and treatment j, and e_{ijk} is the error effect of the k^{th} pig with the i^{th} sex on treatment j.

However, experimental data collected on a pen basis (ADG, ADFI, and FCE) was analyzed as a split plot design with dietary treatment representing the whole plot and time representing the subplot. Pen was the experimental unit.

The model used for the split-plot design was $y_{ijk} = \mu + b_i + p_{ij} + t_k + \gamma_{ik} + e_{ijk}$, where y_{ik} is a measurement of the j^{th} pen within the i^{th} treatment over time k, μ is the population mean, b_i is the effect of treatment i (i = 1 to 5, or 1 or 2), p_{ij} is the effect of pen j (j = 1 to 3)

within treatment i, t_k is the effect of time k (k = 1 or 2), γ_{ik} is the interaction of effect of treatment i and time k, and e_{ijk} is the error effect of the j^{th} pen within the i^{th} treatment over time k.

Data for body weight, ADG, ADFI, FCE, packed cell volume, plasma vitamin B_{12}, plasma homocysteine, plasma methylmalonic acid, and plasma cystathionine were subjected to analysis of variance using General Linear Modeling in the Statistical Analysis System Institute Inc. (1988). Differences between treatment means were assessed by Student-Newman-Keuls' multiple comparison test and were considered statistically significant at $P<0.05$.

5.0 RESULTS

5.1 EXPERIMENT 1

5.1.1 PIG PERFORMANCE

The ADG, ADFI, and FCE for the pigs (Table 5) showed no significant differences ($P>0.05$) at any time between levels of dietary vitamin B_{12} supplementation of 0 to 140 μg kg^{-1} of diet. No treatment by pen interaction was detected ($P>0.05$). For the cumulative period, day 1 to 15 after weaning, the mean average daily gain of all pigs was 200 g day^{-1}.

5.1.2 BIOCHEMICAL ANALYSES

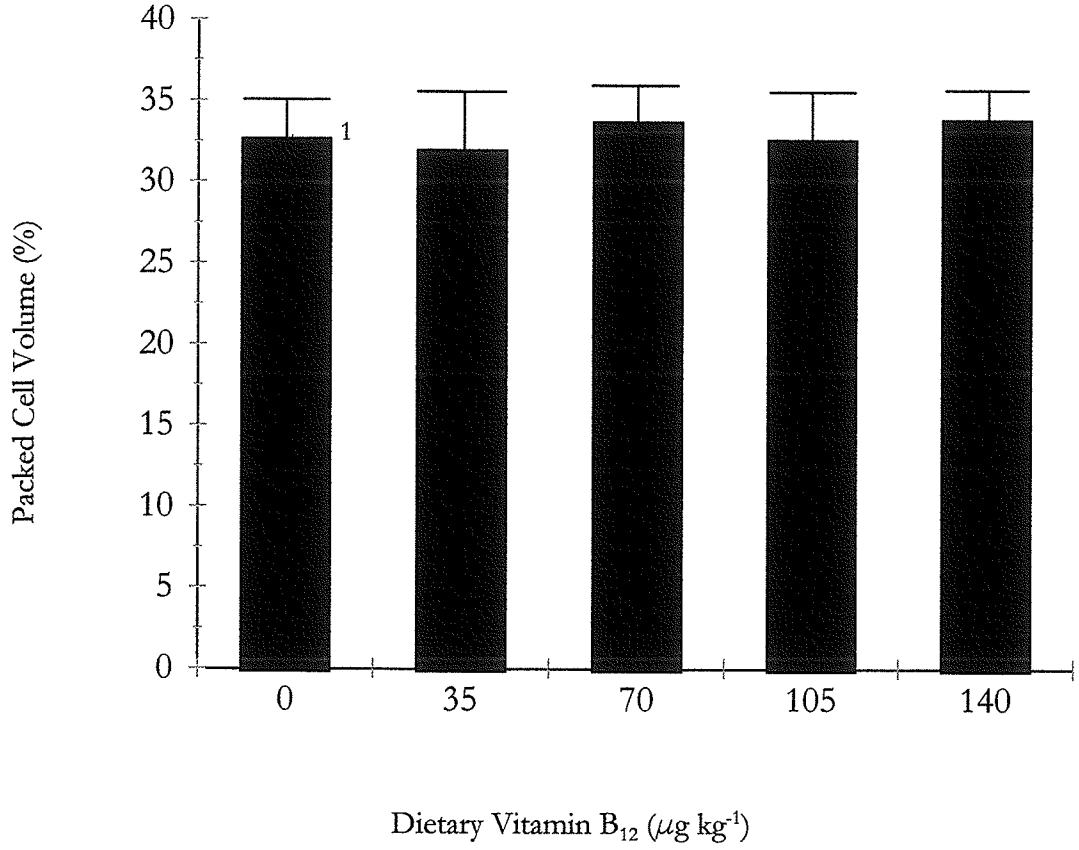
Analysis of packed cell volume (Figure 7) revealed no significant differences ($P>0.05$) between the various levels of vitamin B_{12} supplementation at the end of the 15 day experiment. This pattern also was observed for plasma homocysteine (Figure 9). However, pigs given the vitamin B_{12}-unsupplemented diet had significantly ($P<0.05$) lower concentrations of vitamin B_{12} in their plasma than pigs fed the diets supplemented with vitamin B_{12} (Figure 8). There was no further increase in the concentration of vitamin B_{12} in plasma with additional vitamin B_{12} supplementation above 35 μg vitamin B_{12} kg^{-1} diet. Furthermore, plasma concentrations of methylmalonic acid and cystathionine were markedly decreased ($P<0.05$) in pigs supplemented with 140 μg vitamin B_{12} kg^{-1} diet versus those on the 0 μg diet (Table 6). No treatment by sex interaction was detected ($P>0.05$) for any of the parameters measured.

Table 5. Performance of early weaned pigs fed diets supplemented with graded levels of vitamin B_{12} in experiment 1.[1]

	Dietary Vitamin B_{12} (μg kg^{-1})				
	0	35	70	105	140
Day 0 to 5					
ADG, g	69 ± 5[2]	85 ± 30	73 ± 36	72 ± 52	64 ± 43
ADFI, g	74 ± 5	87 ± 17	200 ± 26	84 ± 37	83 ± 20
FCE	0.93 ± 0.05	0.97 ± 0.20	0.73 ± 0.20	0.79 ± 0.28	0.71 ± 0.31
Day 5 to 10					
ADG, g	180 ± 37	181 ± 36	145 ± 43	200 ± 44	168 ± 52
ADFI, g	179 ± 18	203 ± 16	180 ± 32	212 ± 34	185 ± 29
FCE	0.99 ± 0.13	0.89 ± 0.17	0.80 ± 0.12	0.94 ± 0.08	0.89 ± 0.14
Day 10 to 15					
ADG, g	375 ± 38	356 ± 95	347 ± 65	366 ± 46	313 ± 140
ADFI, g	366 ± 33	349 ± 66	368 ± 90	364 ± 63	347 ± 80
FCE	1.03 ± 0.12	1.01 ± 0.09	0.95 ± 0.07	1.02 ± 0.17	0.88 ± 0.20
Overall					
ADG, g	208 ± 23	207 ± 37	189 ± 47	213 ± 38	181 ± 61
ADFI, g	206 ± 18	213 ± 30	215 ± 49	220 ± 42	205 ± 33
FCE	1.01 ± 0.06	0.97 ± 0.04	0.88 ± 0.03	0.97 ± 0.10	0.87 ± 0.16

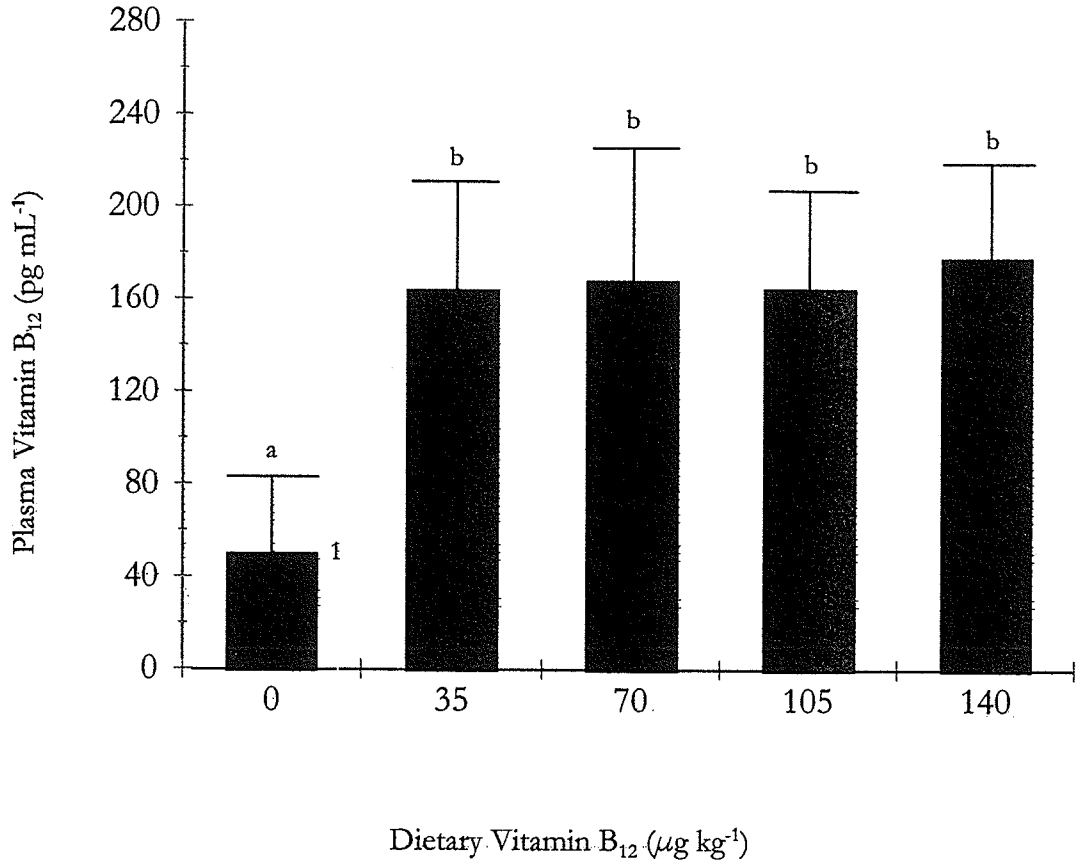
[1] Treatment by pen interaction was not significant at $P>0.05$

[2] Mean ± Standard Deviation (n=4)



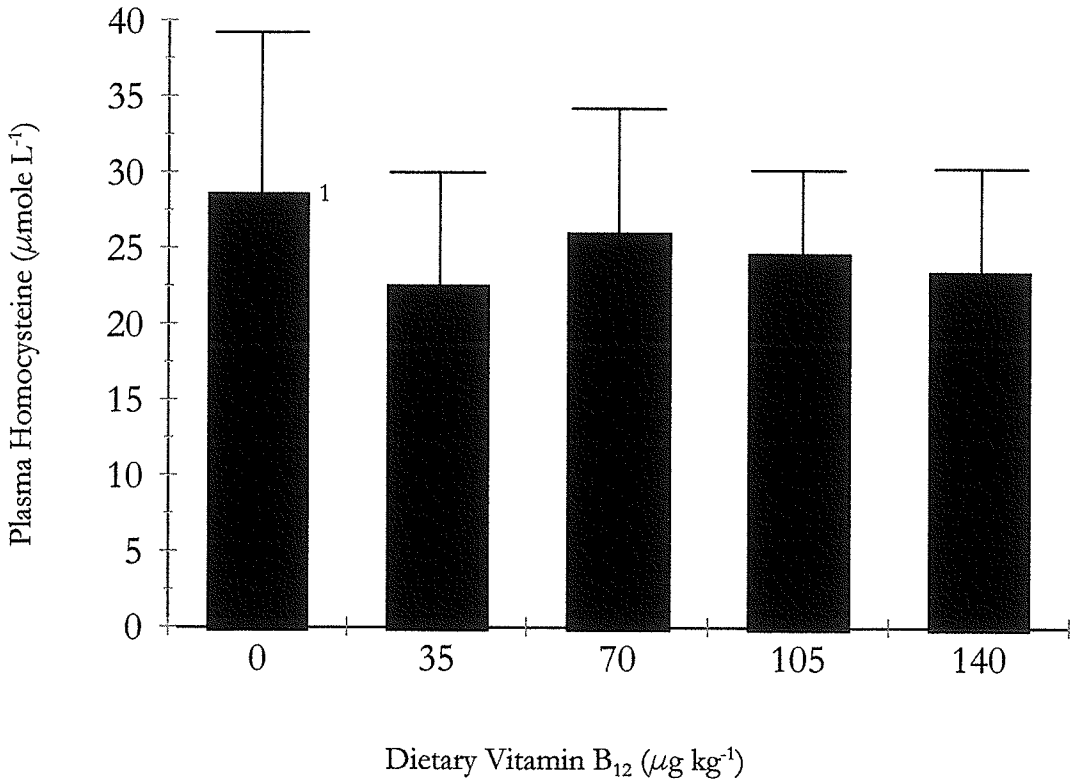
[1] Mean±Standard Deviation (n=12)

Figure 7. Impact of dietary vitamin B_{12} concentration on packed cell volume in early weaned pigs in experiment 1.



[1] Mean±Standard Deviation (n=12)

Figure 8. Impact of dietary vitamin B_{12} concentration on plasma vitamin B_{12} in early weaned pigs in experiment 1. Means with different letters indicate significant differences: Student-Newman-Keuls' ($P<0.05$).



[1] Mean±Standard Deviation (n=12)

Figure 9. Impact of dietary vitamin B_{12} concentration on plasma homocysteine in early weaned pigs in experiment 1.

Table 6. Levels of methylmalonic acid and cystathionine in plasma of pigs fed diets supplemented with either 0 or 140 μg vitamin B_{12} kg^{-1}. [1, 2]

	Treatments, Dietary Vitamin B_{12} (μg kg^{-1})	
	0	140
Methylmalonic Acid, nmol L^{-1}	1481 ±1126[a]	432 ± 102[b]
Cystathionine, nmol L^{-1}	1646 ± 624[a]	1096 ± 223[b]

[1] Mean ± Standard Deviation (n=12)

[2] Values in same row with different superscripts differ ($P<0.05$)

5.2 EXPERIMENT 2

5.2.1 PIG PERFORMANCE

Summaries of the growth performance data are given in Table 8. Inclusion of 10 g of ASP250 kg^{-1} of vitamin B_{12}-unsupplemented diet did not significantly ($P>0.05$) affect the ADG, ADFI, or FCE for the pigs. Over the duration of the experiment, the ADG of all pigs was 155 g day^{-1}. No treatment by pen interaction was detected.

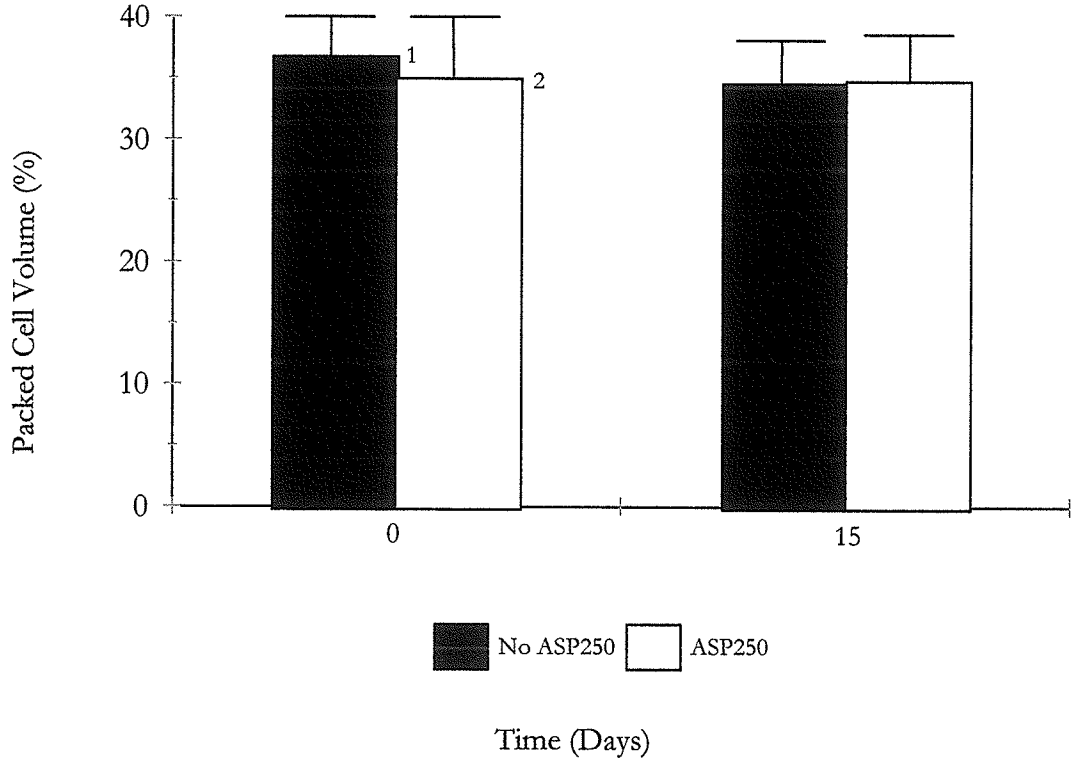
5.2.2 BIOCHEMICAL ANALYSES

No significant ($P>0.05$) differences in packed cell volume were observed for pigs fed either of the experimental treatments (Figure 10). Similarly, plasma vitamin B_{12} and homocysteine were not significantly ($P>0.05$) affected by the addition of ASP250 to the vitamin B_{12}-unsupplemented diet (Figures 11 and 12, respectively). However, concentration of plasma vitamin B_{12} was markedly decreased ($P<0.05$) in all pigs upon completion of the experiment. Concentration of plasma vitamin B_{12} was approximately 262 pg mL^{-1} at pen entry (day 0). Concentration of plasma vitamin B_{12} was approximately 50 pg mL^{-1} upon completion of the experiment (day 15). No treatment by sex interaction ($P>0.05$) was detected.

Table 7. Performance of early weaned pigs fed vitamin B_{12}-unsupplemented diets with or without ASP250.[1]

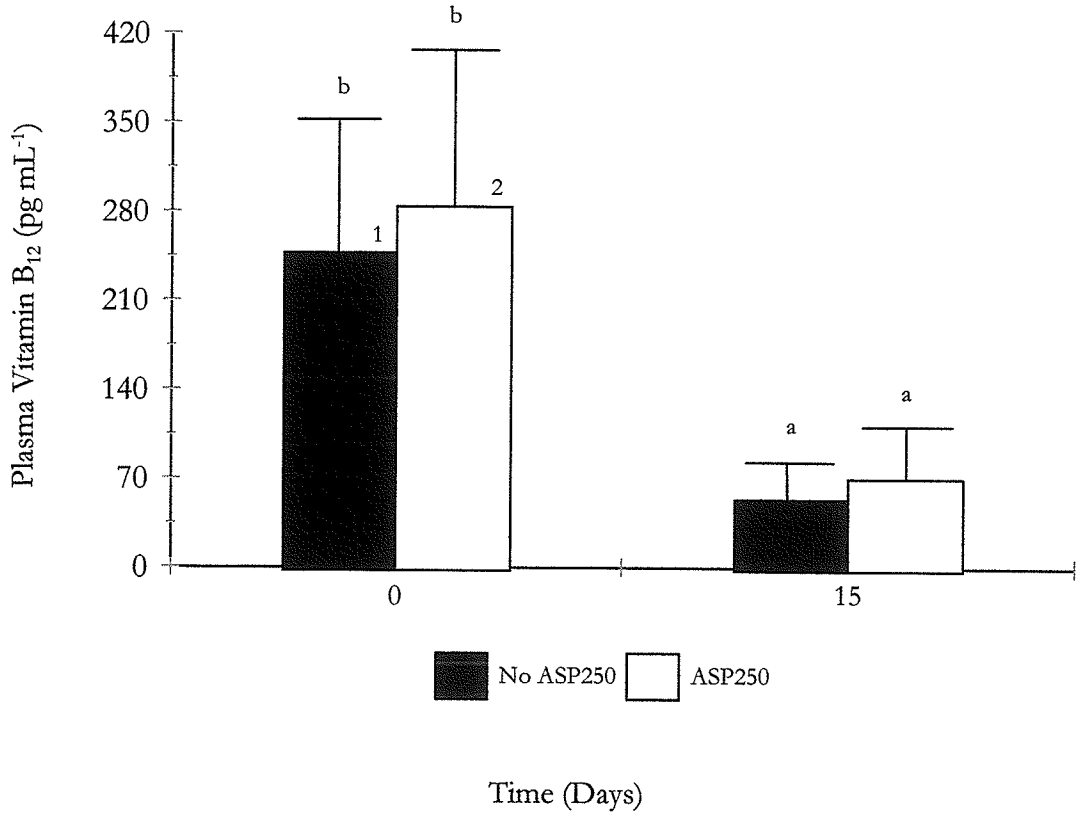
	Treatments	
Parameter	No ASP250	ASP250 (10 g kg^{-1})
Day 0 to 5		
ADG, g	120 ± 30[2]	90 ± 40[3]
ADFI, g	120 ± 10	100 ± 30
FCE	1.00 ± 0.17	0.84 ± 0.16
Day 5 to 10		
ADG, g	110 ± 40	130 ± 30
ADFI, g	170 ± 30	170 ± 30
FCE	0.61 ± 0.17	0.78 ± 0.11
Day 10 to 15		
ADG, g	240 ± 50	250 ± 80
ADFI, g	290 ± 40	300 ± 70
FCE	0.81 ± 0.11	0.81 ± 0.14
Overall		
ADG, g	150 ± 30	160 ± 40
ADFI, g	190 ± 20	190 ± 40
FCE	0.79 ± 0.08	0.81 ± 0.07

[1] Treatment by pen interaction was not significant at P>0.05
[2] Mean ± Standard Deviation (n=24)
[3] Mean ± Standard Deviation (n=22)



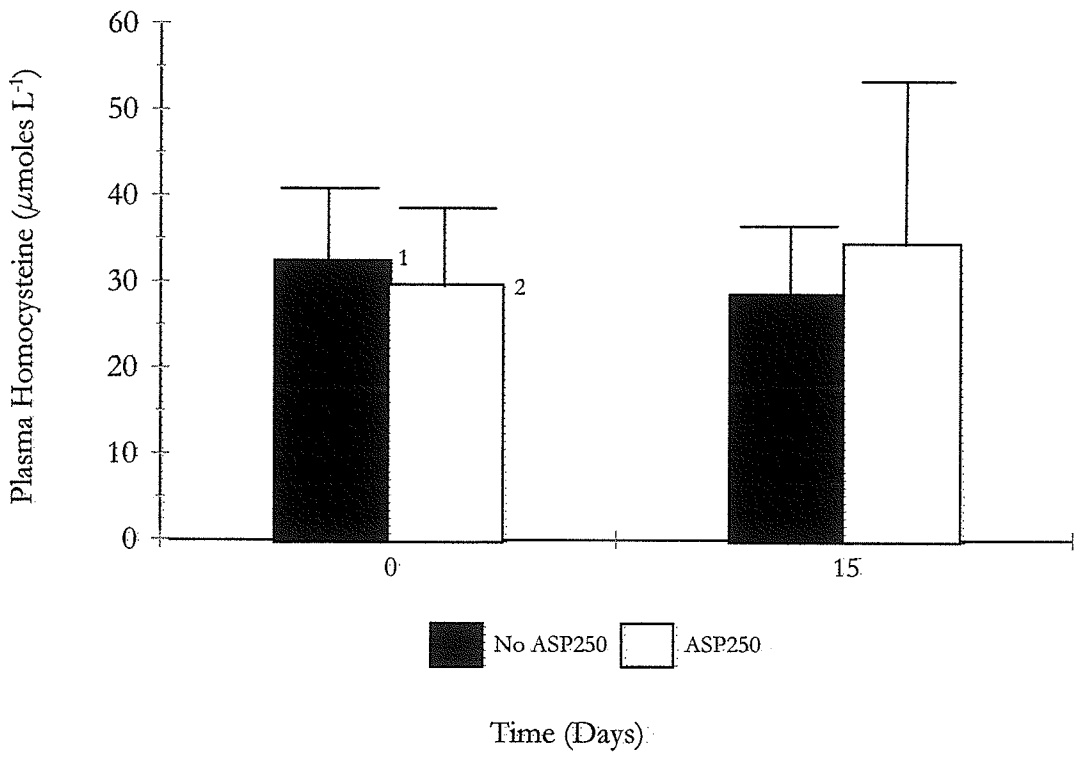
[1] Mean±Standard Deviation (n=24)
[2] Mean±Standard Deviation (n=22)

Figure 10. Influence of feeding vitamin B_{12}-unsupplemented diets with or without ASP250 on packed cell volume over time.



[1] Mean±Standard Deviation (n=24)
[2] Mean±Standard Deviation (n=22)

Figure 11. Influence of feeding vitamin B_{12}-unsupplemented diets with or without ASP250 on plasma vitamin B_{12} over time. Means with different letters indicate significant differences: Student-Newman-Keuls' ($P<0.05$).



[1] Mean±Standard Deviation (n=24)
[2] Mean±Standard Deviation (n=22)

Figure 12. Influence of feeding vitamin B_{12}-unsupplemented diets with or without ASP250 on plasma homocysteine over time.

5.3 EXPERIMENT 3

5.3.1 PIG PERFORMANCE

In this experiment, ADG and ADFI were not different ($P>0.05$) among the dietary treatments (Table 9) at any time. For the cumulative period, the mean ADG of all pigs was 248 g day^{-1}. Cumulative results also revealed a significantly poorer ($P<0.05$) FCE for pigs fed the dietary vitamin B_{12} supplementation of 10 μg kg^{-1} of diet than for pigs fed the rest of the dietary treatments. However, from day 0 to day 5 and day 5 to day 10, increasing the dietary vitamin B_{12} had no effect ($P>0.05$) on FCE's. As for the period from day 10 to day 15 after weaning, a significant difference ($P<0.05$) in FCE's were observed among dietary treatments. Pigs fed the dietary vitamin B_{12} supplementation of 10 μg kg^{-1} of diet had a decreased FCE, but pigs fed the 20 μg of vitamin B_{12} kg^{-1} of diet had a similar FCE to those fed the vitamin B_{12}-unsupplemented diet. The FCE's of pigs fed either 30 or 40 μg of vitamin B_{12} kg^{-1} of diet was comparable to the FCE's of pigs fed 0, 10, and 20 μg of vitamin B_{12} kg^{-1} of diet.

5.3.2 BIOCHEMICAL ANALYSES

Upon completion of the experiment, significant differences ($P<0.05$) in packed cell volume were observed between pigs given the vitamin B_{12}-unsupplemented diet and those on the 30 μg diet (Figure 13). In addition, the pigs fed the vitamin B_{12}-unsupplemented diet has significantly lower plasma vitamin B_{12} ($P<0.05$) than pigs fed the dietary vitamin B_{12} supplementation of 40 μg kg^{-1} of diet (Figure 14). Plasma homocysteine of pigs fed the vitamin B_{12}-unsupplemented diet were similar to pigs fed the dietary vitamin B_{12} supplementation of 10 μg, but different from pigs fed the dietary vitamin B_{12} supplementation of 20, 30, and 40 μg kg^{-1} of diet (Figure 15).

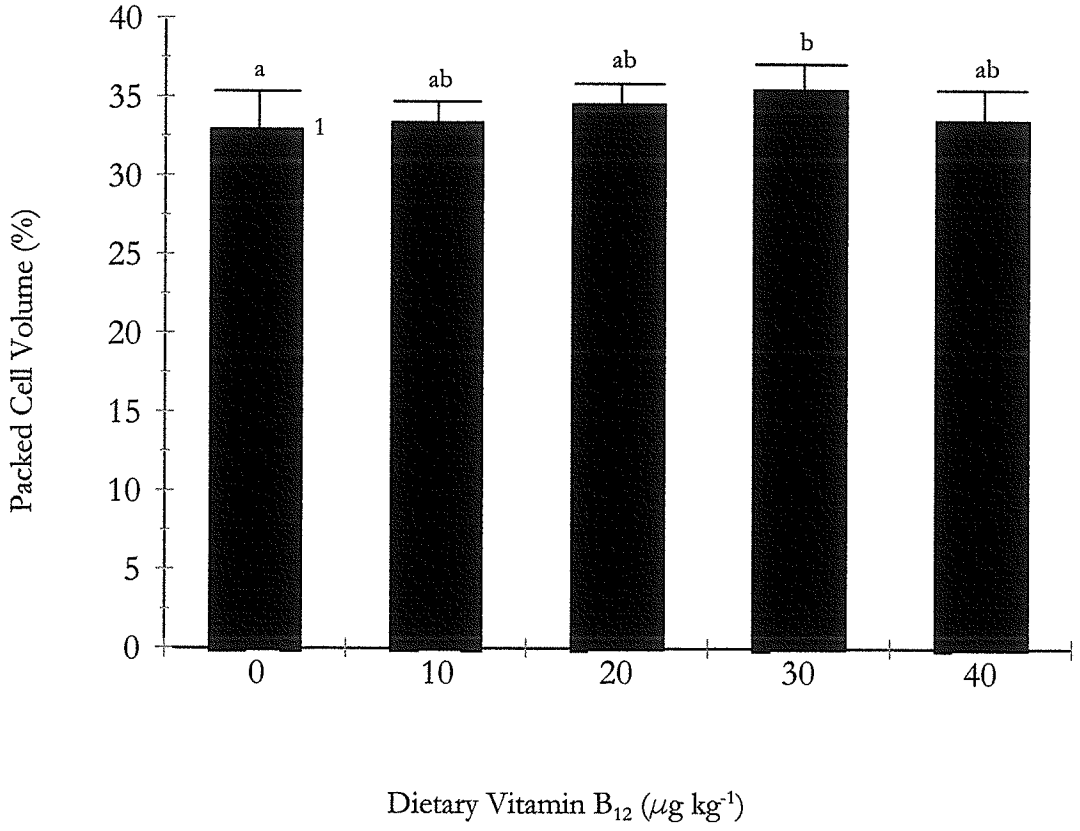
Table 8. Performance of early weaned pigs fed diets supplemented with graded levels of vitamin B_{12} in experiment 3.[1]

	Dietary Vitamin B_{12} ($\mu g\ kg^{-1}$)				
	0	10	20	30	40
Day 0 to 5					
ADG, g	135 ± 9[2]	103 ± 53	94 ± 77	106 ± 26	91 ± 7
ADFI, g	118 ± 9	110 ± 35	92 ± 26	100 ± 13	102 ± 1
FCE	1.15 ± 0.09	0.88 ± 0.24	0.92 ± 0.32	1.05 ± 0.13	0.90 ± 0.07
Day 5 to 10					
ADG, g	279 ± 55	313 ± 56	324 ± 51	361 ± 18	309 ± 26
ADFI, g	303 ± 60	346 ± 64	314 ± 26	358 ± 7	314 ± 20
FCE	0.92 ± 0.01	0.91 ± 0.09	1.03 ± 0.10	1.01 ± 0.03	0.99 ± 0.12
Day 10 to 15					
ADG, g	363 ± 59	289 ± 52	321 ± 37	303 ± 33	332 ± 44
ADFI, g	422 ± 64	417 ± 86	387 ± 42	403 ± 27	413 ± 32
FCE	0.86 ± 0.02[b]	0.70 ± 0.03[a]	0.83 ± 0.01[b]	0.75 ± 0.04[ab]	0.80 ± 0.04[ab]
Overall					
ADG, g	259 ± 35	235 ± 34	246 ± 3	257 ± 10	244 ± 21
ADFI, g	281 ± 40	291 ± 52	264 ± 5	287 ± 10	276 ± 14
FCE	0.92 ± 0.02[b]	0.81 ± 0.03[a]	0.93 ± 0.01[b]	0.89 ± 0.01[b]	0.88 ± 0.05[b]

[1] Treatment by pen interaction was not significant at $P>0.05$

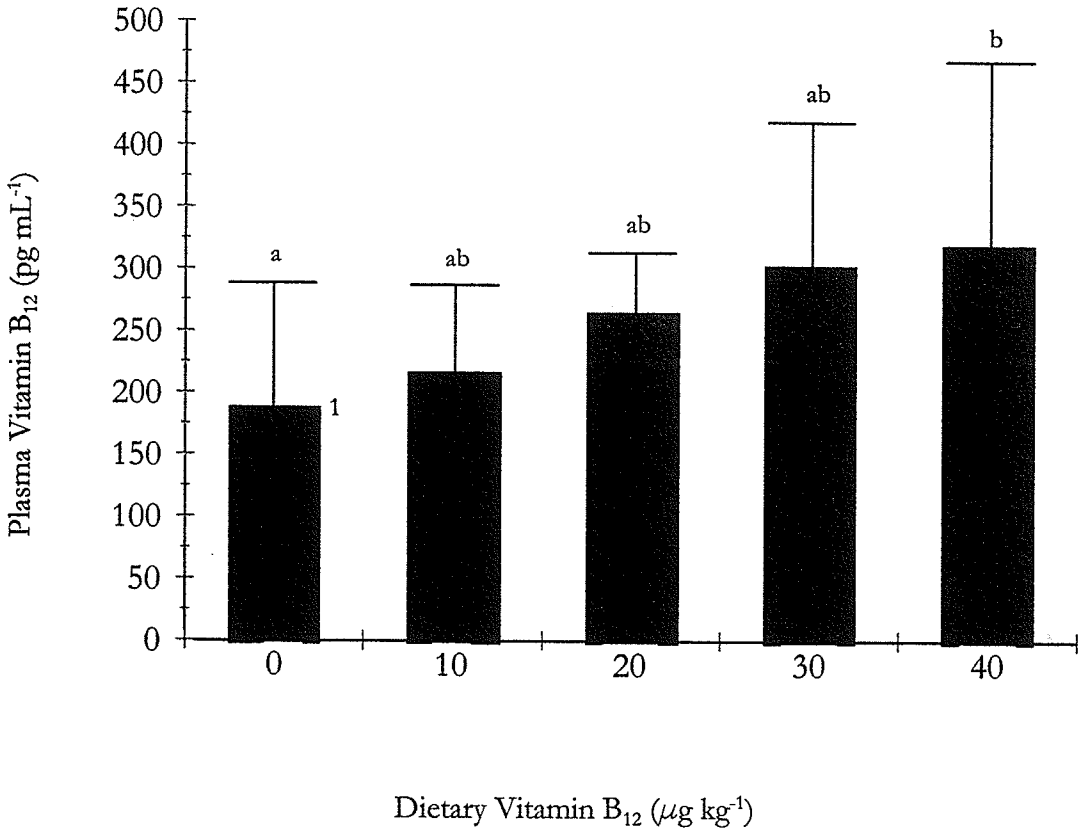
[2] Mean ± Standard Deviation (n=12)

[3] Means with different letters indicate significant difference: Student-Newman-Keul's ($P<0.05$).



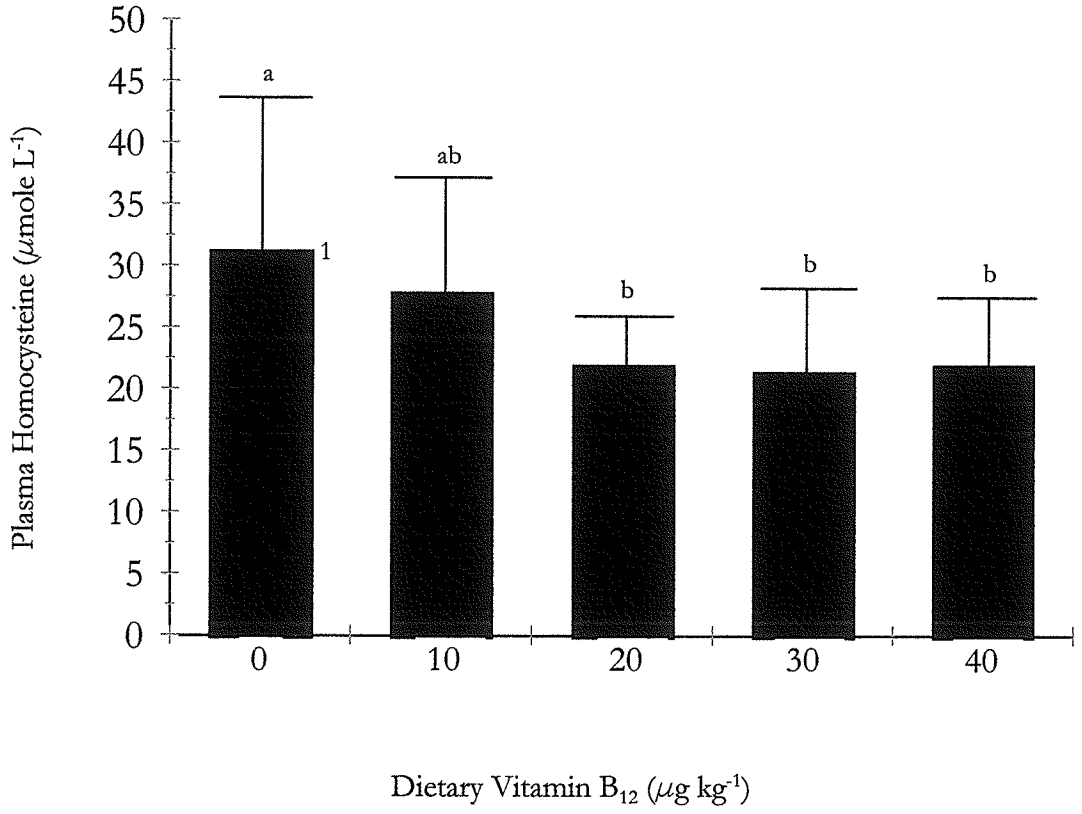
[1] Mean+Standard Deviation (n=12)

Figure 13. Impact of dietary vitamin B_{12} concentration on packed cell volume in early weaned pigs in experiment 3. Means with different letters indicate significant differences: Student-Newman-Keuls' ($P<0.05$).



[1] Mean+Standard Deviation (n=12)

Figure 14. Impact of dietary vitamin B_{12} concentration on plasma vitamin B_{12} in early weaned pigs in experiment 3. Means with different letters indicate significant differences: Student-Newman-Keuls' ($P<0.05$)



[1] Mean+Standard Deviation (n=12)

Figure 15. Impact of dietary vitamin B_{12} concentration on plasma homocysteine in early weaned pigs in experiment 3. Means with different letters indicate significant differences: Student-Newman-Keuls' ($P<0.05$).

5.4 EXPERIMENT 4

5.4.1 DIET COMPOSITION

Samples from all dietary treatments in experiment 1, 2, and 3 were collected and analyzed for concentrations of vitamin B_{12} (Table 10). Samples were analyzed following completion of all 3 experiments.

Analyzed concentration of vitamin B_{12} increased with increased supplementation. However, analyzed values showed considerable variation from calculated values. The dietary treatments were described as containing 0, 35, 70, 105, and 140 μg vitamin B_{12} kg^{-1} of diet in experiment 1, but according to radioimmunoassay, they contained 4, 179, 437, 655, and 876 μg vitamin B_{12} kg^{-1} of diet. The vitamin B_{12}-unsupplemented diet in experiment 2 actually contained 5 μg vitamin B_{12} kg^{-1} of diet. The dietary treatments in experiment 3 calculated to contain 0, 10, 20, 30, and 40 μg vitamin B_{12} kg^{-1} of diet, in fact, contained 33, 65, 260, 300, and 404 μg vitamin B_{12} kg^{-1} of diet. The vitamin B_{12} used in these 3 experiments were described as having a concentration of 1 g kg^{-1}, but unfortunately, according to radioimmunoassay, had a concentration 10 times greater. Furthermore, majority of the plant based ingredients in the basal diet were contaminated with vitamin B_{12} (Appendix 5).

Based on these findings, the dietary treatments in experiment 4 were formulated to contain 0, 50, 100, 150, and 200 μg vitamin B_{12} kg^{-1} of diet. The vitamin B_{12} used in experiment 4 was manufactured by the primary investigator using 99 % pure vitamin B_{12}, in the form of cyanocobalamin, and sucrose. See Section 5.5.3 for complete details. Analysis revealed the dietary treatments contained 15, 73, 100, 133, and 110 μg vitamin B_{12} kg^{-1} of diet.

5.4.2 Pig Performance

No significant differences ($P>0.05$) were observed in ADG, ADFI, or FCE among pigs fed the various levels of dietary vitamin B_{12} supplementation of 0 to 200 $\mu g\ kg^{-1}$ of diet (Table 11). No treatment by pen interaction was detected ($P>0.05$). For the cumulative period, day 1 to 15 after weaning, the mean ADG of all pigs was 120 g day^{-1}.

5.4.3 Biochemical Analyses

There were no apparent differences ($P>0.05$) among dietary treatments with regard to packed cell volume (Figure 16) at the end of the 15 day experiment. However, pigs given the vitamin B_{12}-unsupplemented diet had significantly ($P<0.05$) lower concentrations of vitamin B_{12} in their plasma than pigs fed the diet supplemented with 200 $\mu g\ kg^{-1}$ of diet (Figure 17). Plasma homocysteine concentrations were not significantly ($P>0.05$) different among the dietary treatments (Figure 18). No treatment by sex interaction was detected ($P>0.05$) for any of the parameters measured.

Table 9. Analyzed vitamin B_{12} concentrations for dietary treatments in experiments 1, 2, 3, and 4.

Calculated vitamin B_{12} inclusion rate ($\mu g\ kg^{-1}$ diet)	Analyzed vitamin B_{12} inclusion rate			
	n[1]	Mean ± Standard Deviation ($\mu g\ kg^{-1}$ diet)	Range ($\mu g\ kg^{-1}$ diet)	Coefficient of Variation (%)
Experiment 1				
0	4	4.1 ± 0.5	3.6 - 4.7	11
35	4	178.5 ± 21.1	160.8 - 208.0	11.8
70	3	437.3 ± 13.0	422.2 - 445.3	3.0
105	3	654.8 ± 110.7	578.5 - 781.8	16.9
140	3	875.8 ± 137.3	717.3 - 959.7	15.7
Experiment 2				
0	3	5.2 ± 1.5	3.4 - 6.2	29.6
Experiment 3				
0	2	32.9 ± 1.1	32.1 - 33.7	3.4
10	3	65.2 ± 7.9	56.9 - 72.5	12.0
20	3	260.1 ± 45.1	210.4 - 298.5	17.4
30	3	300.1 ± 129.3	210.0 - 448.2	43.1
40	3	404.0 ± 170.5	281.4 - 598.8	42.2
Experiment 4				
0	3	14.7 ± 4.4	9.6 - 17.4	30.2
50	3	72.7 ± 14.8	55.8 - 83.4	20.3
100	4	99.5 ± 48.6	49.3 - 155.4	48.8
150	4	132.8 ± 46.3	89.1 - 188.3	34.9
200	3	110.6 ± 16.6	91.8 - 123.1	15.0

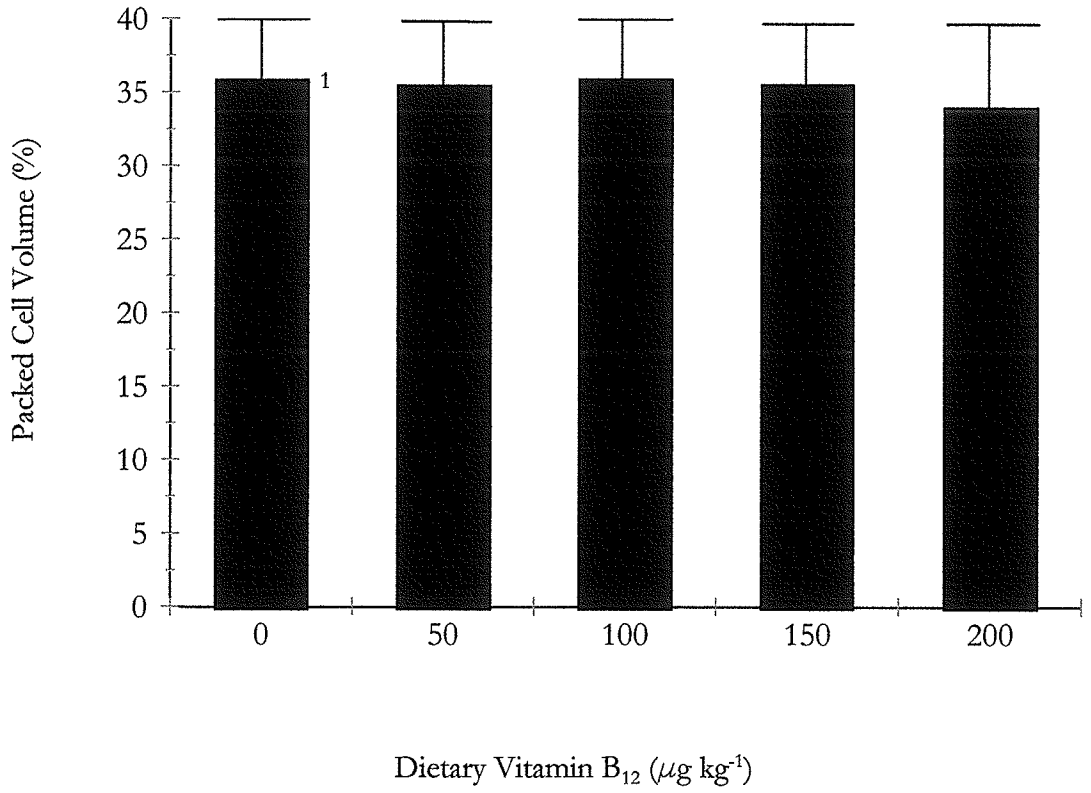
[1]n represents the number of samples analyzed per diet.

Table 10. Performance of early weaned pigs fed diets supplemented with graded levels of vitamin B_{12} in experiment 4.[1]

	Dietary Vitamin B_{12} (μg kg^{-1})				
	0	50	100	150	200
Day 0 to 5					
ADG, g	39 ± 28[2]	44 ± 38	66 ± 29	49 ± 42	59 ± 49
ADFI, g	68 ± 15	98 ± 21	104 ± 17	85 ± 21	95 ± 32
FCE	0.55 ± 0.36	0.41 ± 0.35	0.62 ± 0.23	0.52 ± 0.38	0.53 ± 0.40
Day 5 to 10					
ADG, g	76 ± 46	127 ± 24	108 ± 46	72 ± 51	93 ± 19
ADFI, g	144 ± 20	171 ± 18	152 ± 39	142 ± 30	152 ± 27
FCE	0.50 ± 0.27	0.76 ± 0.21	0.68 ± 0.16	0.47 ± 0.23	0.62 ± 0.10
Day 10 to 15					
ADG, g	198 ± 35	210 ± 103	185 ± 75	226 ± 77	251 ± 46
ADFI, g	243 ± 61	285 ± 80	240 ± 57	250 ± 22	277 ± 46
FC	0.83 ± 0.16	0.71 ± 0.23	0.75 ± 0.13	0.89 ± 0.23	0.90 ± 0.08
Overall					
ADG, g	104 ± 25	127 ± 43	120 ± 39	116 ± 38	134 ± 19
ADFI, g	152 ± 29	185 ± 33	166 ± 30	159 ± 12	175 ± 21
FCE	0.68 ± 0.08	0.68 ± 0.16	0.71 ± 0.11	0.72 ± 0.19	0.77 ± 0.05

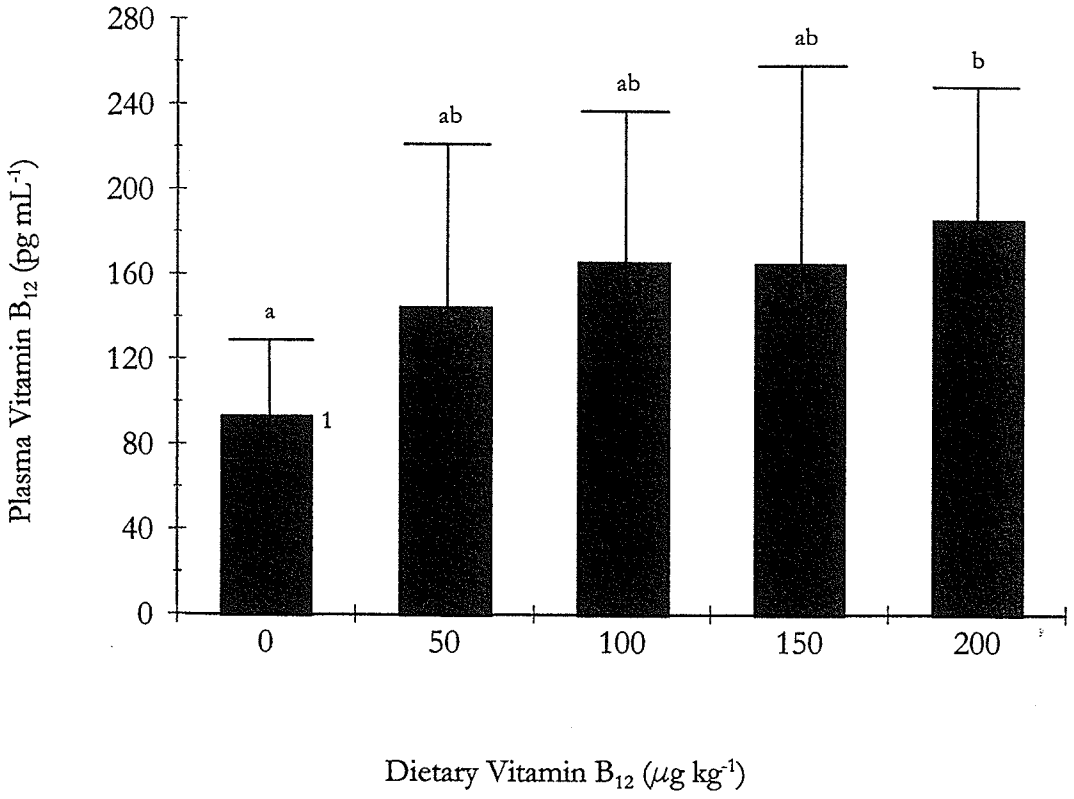
[1] Treatment by pen interaction was not significant at $P>0.05$

[2] Mean ± Standard Deviation (n=12)



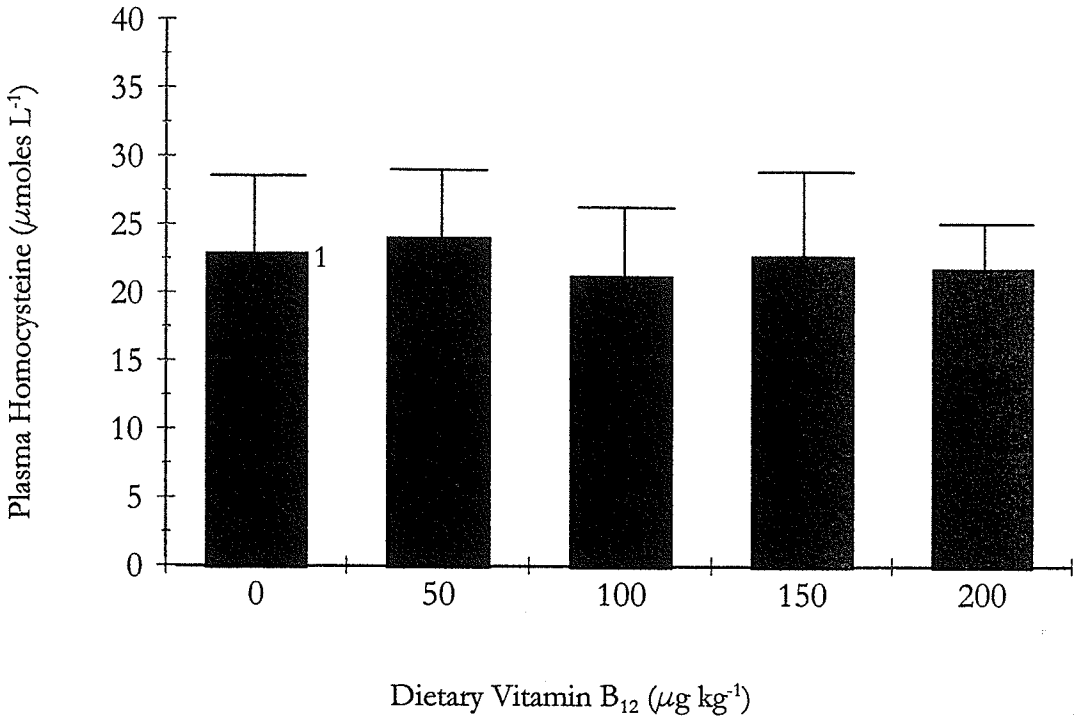
[1] Mean±Standard Deviation (n=12)

Figure 16. Impact of dietary vitamin B_{12} concentration on packed cell volume in early weaned pigs in experiment 4.



[1] Mean±Standard Deviation (n=12)

Figure 17. Impact of dietary vitamin B_{12} concentration on plasma vitamin B_{12} in early weaned pigs in experiment 4. Means with different letters indicate significant differences: Student-Newman-Keuls' ($P<0.05$).



[1] Mean±Standard Deviation (n=12)

Figure 18. Impact of dietary vitamin B_{12} concentration on plasma homocysteine in early weaned pigs in experiment 4.

6.0 DISCUSSION

This thesis focuses on the water-soluble nutrient, vitamin B_{12}. Vitamin B_{12} is an essential component of the enzymes, methionine synthase and L-methylmalonyl-CoA mutase. These enzymes stand at the intersection of the remethylation of methionine and conversion of L-methylmalonyl-CoA to succinyl-CoA. Thus, vitamin B_{12} functions in the metabolism of protein and energy. Protein and energy are essential for lean tissue accretion. The quantity of vitamin B_{12} required by the early weaned pig is therefore of considerable importance.

In experiment 1, growth performance parameters (ADG, ADFI, and FCE) were not affected at any time by the addition of 35, 70, 105, or 140 μg kg^{-1} of added vitamin B_{12} compared to the vitamin B_{12}-unsupplemented diet. Similarly, no significant differences were observed in ADG and ADFI among pigs fed 0, 10, 20, 30, and 40 μg of vitamin B_{12} kg^{-1} of diet in experiment 3. Clearly, experiment 3 was conducted to further establish the early weaned pig's requirement for vitamin B_{12} because the graded levels of vitamin B_{12} used in experiment 1 were too broad. Moreover, no apparent differences were detected in ADG, ADFI, and FCE among pigs in experiment 4 fed 0, 50, 100, 150, and 200 μg of vitamin B_{12} kg^{-1} of diet. Experiment 4 was essentially conducted to validate the findings in experiment 1. The reason is as follows.

Prior to conducting experiment 4, samples from all dietary treatments in experiments 1 and 3 were analyzed for concentrations of vitamin B_{12}. Analyzed values showed considerable variation from calculated values. The dietary treatments were described as containing 0, 35, 70, 105, and 140 μg vitamin B_{12} kg^{-1} of diet in experiment 1, but according

to radioimmunoassay, they contained 4, 179, 437, 655, and 876 μg vitamin B_{12} kg^{-1} of diet. The dietary treatments in experiment 3 calculated to contain 0, 10, 20, 30, and 40 μg vitamin B_{12} kg^{-1} of diet, in fact, contained 33, 65, 260, 300, and 404 μg vitamin B_{12} kg^{-1} of diet. Based on these findings, the dietary treatments in experiment 4 were formulated to contain 0, 50, 100, 150, and 200 μg vitamin B_{12} kg^{-1} of diet. For this experiment, the vitamin B_{12} was manufactured by the primary investigator using 99 % pure vitamin B_{12}, in the form of cyanocobalamin (Sigma Chemical Co., St. Louis, MO, USA). Analysis later revealed the dietary treatments contained 15, 73, 100, 133, and 110 μg vitamin B_{12} kg^{-1} of diet.

The microbiological assay is the method of choice for measuring feed vitamin B_{12} concentrations (Casey *et al.* 1982). The microbiological assay, performed by Maxxam Analytics Inc., revealed the vitamin B_{12}-unsupplemented diet in experiment 1 contained 36.5 μg of vitamin B_{12} kg^{-1} of diet. However, due to the cost of the microbiological assay, an attempt was made to use a commerical radioimmunoassay to measure the vitamin B_{12} concentration in the experimental diets. However, the reader should bear in mind the radioimmunoassay was specifically designed to measure plasma vitamin B_{12}, not feed vitamin B_{12}. The radioimmunoassay method used by Casey *et al.* (1982) on fortified and unfortified food products was not identical to the radioimmuoassay method used by the present primary investigator. Due to the extreme variation of analyzed and calculated vitamin B_{12} values within and between experiments, the feed vitamin B_{12} data is considered erroneous. Thus, only the calculated values will be referred to herein.

The growth performance results suggest that with diets similar in composition to the one used herein, added vitamin B_{12} is not necessary for the early weaned pig from 17 to 32

days of age, which reflects the length of a starter pig phase 1 diet. However, it is worthwhile to consider that the duration of the experiments were only 15 days. This period of time may not have been sufficient to allow for the true effects of the vitamin B_{12} deficient diet(s) to be manifested. It is plausible, but speculative, that there may be sufficient storage of vitamin B_{12} in these early weaned pigs, based upon the growth performance parameters, to produce effective nutritional "carry-over" for the 15 days. Sow's milk contains about 2 ng of vitamin B_{12} mL^{-1} (Ford *et al.* 1975) which is effectively absorbed from the intestine and retained within the suckling pig, mostly in the liver (Ford *et al.* 1975; Trugo *et al.* 1985).

Earlier research uncovered that there is sufficient storage of vitamin B_{12} to produce effective nutritional "carry-over" for 6 to 8 weeks (Anderson and Hogan 1949; Richardson *et al.* 1951). The greater capacity for lean tissue accretion in modern genetic strains of pigs, as compared to pigs evaluated in the former experiments, would presumably force them to utilize vitamin B_{12} stores more rapidly than in 6 to 8 weeks. It was not the function of this thesis to address this issue.

The ADG target for early weaned pigs on commercial farms is 250 g day^{-1} from 5.5 to 8 kg and 430 g day^{-1} from 8 to 14 kg of body weight (Mavromichalis and Baker 1999). Thus, the overall ADG from 5.5 to 8.5 kg of body weight was lower in experiments 1, 2, and 3 (200 g day^{-1}, 248 g day^{-1}, and 120 g day^{-1}, respectively) than under commercial farm conditions or in the experiments of Hill *et al.* (2000) and Owsu-Asideu (1998), who reported 306 and 315 g day^{-1}, respectively. The low overall ADG of the present experiments were not an unexpected finding. After all, the basal diet was not formulated to maximize the growth potential of the pigs. It was disturbing, however, to observe broad differences in overall ADG between the

experiments. The reason for this difference is not clear. Pigs did not differ in age, genetic background, or health status between the experiments. In addition, the basal diets were identical.

One possible explanation for the overall ADG in experiment 4 (120 g day^{-1}) being considerably lower than the overall ADG in experiments 1 and 3 (200 and 248 g day^{-1}, respectively), is the omission of ASP250 from the basal diet. ASP250, an antibiotic, is used in feed to maintain weight gains and stimulate appetite during periods of stress (Compendium of Medicating Ingredient Brochures 1998), such as weaning. Indeed, the overall ADFI was lower in experiment 4 compared to the overall ADFI in experiments 1 and 3. This explanation seems reasonable because this 120 g day^{-1} is more similar to 150 g day^{-1}, the overall ADG of pigs fed the vitamin B_{12}-unsupplemented diet devoid of ASP250 in experiment 2. Experiment 2 was conducted to determine the impact of ASP250, an antibiotic, on vitamin B_{12} status of the early weaned pig.

Only 1 scientific paper, after an exhaustive search, could be found on the impact of ASP250 on vitamin B_{12} status in pigs. The research in this scientific paper was conducted in 1951 by Richardson *et al.* on pigs weighing 45 kg. It was concluded that pigs receiving a vitamin B_{12} devoid diet with ASP250 gained significantly less than pigs receiving a vitamin B_{12} devoid diet without ASP250. It appears that the antibiotics adversely affected the microorganisms which synthesize vitamin B_{12}. Vitamin B_{12} synthesis in the colon was inhibited.

It was anticipated that the findings of the present experiment would mimic the published findings of Richardson *et al.* (1951). However, this was not the situation. Significant

differences in ADG, ADFI, or FCE were not observed. Similarly, packed cell volume, plasma vitamin B_{12}, and plasma homocysteine were not significantly affected by the addition of 10 g ASP250 kg^{-1} of vitamin B_{12}-unsupplemented diet.

Although not anticipated, the findings of the present experiment do complement the literature on vitamin B_{12} metabolism. Numerous research (Hendrickx *et al.* 1964; Keys and DeBarthe 1974; Cullen and Oace 1977; Varel 1987; Varel and Yen 1997) emphasizes that the colon harbors plenty of microorganisms. These microorganisms are capable of synthesizing vitamin B_{12}. However, the cubilin receptor for IF-B_{12} is located on the apical brush border of ileal enterocytes (Donaldson, Jr. *et al.* 1967; Xu and Fyfe 2000). There is no way in which this synthesized vitamin B_{12} can return to the ileum. Unexplainable is the evidence of colonic uptake of vitamin B_{12} observed by Henderickx *et al.* (1964) and the findings of Richardson *et al.* (1951).

The low ADG in experiments 1, 3, and 4 is presumably due to the low level of feed intake by the pigs. The feed intake targets for early weaned pigs on commercial farms is 260 g day^{-1} from 5.5 to 8 kg and 570 g day^{-1} from 8 to 14 kg of body weight (Mavromichalis and Baker 1999). Post-weaning stress and diarrhea are factors that are known to reduce feed intake and depress growth in early weaned pigs. Diarrhea was present among the pigs at pen entry. It is known that certain dietary ingredients such as spray-dried porcine plasma, which was not present in the basal diet, can counteract these effects. Feed ingredients of animal origin contain measurable amounts of vitamin B_{12} (NRC 1998). Omission of spray-dried porcine plasma from the basal diet was necessary to create a vitamin B_{12} devoid diet.

Weaver *et al.* (1995) summarized 25 experiments in which the effects of added spray-

dried porcine plasma to starter pig diets were examined and concluded that on average, daily gain had been improved by 39 %, daily feed intake was increased by 32 %, and FCE was improved by 5.4 %. Since spray-dried porcine plasma is considered essential in the diet of the early weaned pig (Thacker 1999), it stands to reason that excluding it from the basal diet in these experiments resulted in the low level of feed intake, and subsequent low ADG. Spray-dried porcine plasma was included in the experiments of Hill *et al.* (2000) and Owusu-Asiedu (1998) along with some combination of skim milk, whey protein concentrate, fish meal, spray-dried blood meal, and soyabean meal. The latter ingredients are common to majority of starter pig phase 1 diets.

Despite the low ADG and ADFI, FCE for experiment 1 and 3 was more than acceptable and within FCE targets for early weaned pigs. The FCE targets for early weaned pigs on commercial farms is 0.96 from 5.5 to 8 kg and 0.73 from 8 to 14 kg of body weight (Mavromichalis and Baker 1999). Thus, the pigs effectively converted the feed into lean tissue. Analysis of FCE in experiment 3 revealed significant differences among pigs fed 0, 10, 20, 30, and 40 μg of vitamin B_{12} kg^{-1} of diet. The overall FCE was lower in pigs fed 10 μg of vitamin B_{12} kg^{-1} of diet. This pattern was also observed for the period, day 10 to 15 post-weaning. This observation, not regarded with particular importance, may be attributed to the variability of the data set. However, the FCE of pigs in experiment 4 was below the targets established by Mavromichalis and Baker (1999). This can be attributed to the low ADG verses the high ADFI of the pigs.

Analogous to growth performance parameters, packed cell volume in experiments 1 and 4 was not significantly different between the various levels of vitamin B_{12}

supplementation at the end of the 15 days. This was not an unexpected finding. Many researchers (Newmann *et al.* 1950; Stabler *et al.* 1996; Stabler *et al.* 1988; Lindenbaum *et al.* 1990; Stabler *et al.* 1990; Savage *et al.* 1994) have discovered that vitamin B_{12} deficient subjects have normal or mildly normal values for packed cell volume. In fact, Savage *et al.* (1994) claims that both homocysteine and methylmalonic acid are elevated in >89 % of the episodes in which the packed cell volume was normal. An explanation for this finding can not be provided.

Contrary to the above observation, packed cell volume in experiment 3 differed among the dietary treatments. Interpretation and significance of the results are unclear. Basically, packed cell volume was lowest in pigs fed 0 μg of vitamin B_{12} kg^{-1} of diet which was significantly different from pigs fed 30 μg of vitamin B_{12} kg^{-1} of diet. The packed cell volume of all pigs in experiment 3, roughly 33 %, was equivalent to that of experiment 1.

Multiple researchers now recognize that plasma metabolite levels are sensitive indicators of a vitamin B_{12} deficiency (Stabler *et al.* 1986; Chu and Hall 1988; Stabler *et al.* 1988; Allen *et al.* 1990; Lindenbaum *et al.* 1990; Moelby *et al.* 1990; Stabler *et al.* 1990; Savage *et al.* 1994; Snow *et al.* 1999). Because of this growing evidence, special consideration was given in this thesis to the possible use of such indicators as criteria of adequacy in the early weaned pig. The findings of the experiments partially confirm and extend the observations of the latter researchers. The level of plasma vitamin B_{12}, but not homocysteine, was significantly altered by the addition of 35, 70, 105, and 140 μg kg^{-1} of added vitamin B_{12} in experiment 1. This same pattern was observed in experiment 4. Furthermore, the levels of methylmalonic acid and cystathionine were drastically elevated in pigs fed the vitamin B_{12}-unsupplemented

diet versus those supplemented with 140 μg of vitamin B_{12} kg^{-1} of diet in experiment 1. These plasma metabolite levels were not determined in the subsequent experiments due to the cost of the analysis. Contrary to experiments 1 and 4, a significant difference in plasma homocysteine was detected in experiment 3 along with plasma vitamin B_{12}. All of the researchers cited previously used humans to determine if plasma metabolite levels are sensitive indicators of a vitamin B_{12} deficiency. To our knowledge no other researchers have used pigs to determine if plasma metabolite levels are sensitive indicators of a vitamin B_{12} deficiency.

In the experiments, change in plasma metabolite levels developed before any change in growth performance parameters. Hence, the measurement of plasma metabolite levels is proposed as a better means of diagnosing a vitamin B_{12} deficiency and establishing a vitamin B_{12} requirement in the early weaned pig from 17 to 32 days of age.

The reason pigs fail to show change in growth performance parameters is not clear, although the quantity of stored vitamin B_{12} in the tissues at the beginning of the experimental period may play a role as mentioned earlier. Measurable quantities of vitamin B_{12} are maintained in the liver, kidneys, heart, lungs, spleen, pancreas, and brain (Ford *et al.* 1975; Trugo *et al.* 1985). The estimates for the period of protection afforded by vitamin B_{12} stores remain unsolved. Although earlier research suggests the period of protection is 6 to 8 weeks (Anderson and Hogan 1949; Richardson *et al.* 1951). A careful reader will no doubt conclude that further research is required to determine how long tissue vitamin B_{12} stores can cover need in modern, leaner genetic strains of pigs.

Since the concentration of vitamin B_{12} in plasma reflects both the tissue vitamin B_{12}

stores and absorbed dietary vitamin B_{12} (Dietary Reference Intakes 1998), it is reasonable to believe that plasma metabolite levels would be altered before growth performance parameters. With inadequate dietary vitamin B_{12} intake, growth performance could be maintained at the expense of vitamin B_{12} in the tissue.

The concentration of plasma vitamin B_{12} in experiment 1 reached plateau with a supplement of 35 μg of vitamin B_{12} kg^{-1} of diet. In experiment 4, the concentration of plasma vitamin B_{12} reached plateau with a supplementation of 50 μg kg^{-1} of diet. This would suggest that the objective of experiment 4 was accomplished; validating the findings of experiment 1. The plateau was more difficult to determine in experiment 3. After careful review and analysis, plasma vitamin B_{12} is suggested to plateau at 20 μg of vitamin B_{12} kg^{-1} of diet. Although not supported statistically, the plasma homocysteine levels support this interpretation. Taken together, these results would seem to suggest a saturation of cublin receptor sites on the ileum; this saturation indicating that the requirement for vitamin B_{12} by the early weaned pig from 17 to 32 days of age was met with 20 μg of vitamin B_{12} kg^{-1} of diet. A requirement is defined by Dietary Reference Intakes (1998) as the lowest continuing intake level of a nutrient that will maintain a defined level of nutriture in an individual. According to Donaldson, Jr. *et al.* (1973) and Hooper *et al.* (1973), there is a limited number of cublin receptor sites on the ileum that bind IF-B_{12}.

For confirmation of normal plasma metabolite concentrations, all pigs in experiment 2 were subject to blood collection at pen entry. The results imply that the normal plasma vitamin B_{12} concentration in these pigs is 262 pg mL^{-1}. This value is identical to experiment 3 (264 pg mL^{-1}), but different from experiments 1 and 4 (164 pg mL^{-1} and 145 pg mL^{-1},

respectively). Following the 15 day experiment, the plasma vitamin B_{12} concentration of pigs receiving 0 μg of vitamin B_{12} kg^{-1} of diet in experiment 2 was the same as those pigs receiving 0 μg of vitamin B_{12} kg^{-1} of diet in experiment 1; this plasma vitamin B_{12} concentration was approximately 50 pg mL^{-1}.

Surprisingly, such a response was not observed in the concentration of plasma homocysteine for experiments 1 and 4. The plasma homocysteine concentrations in experiment 1 were 29, 23, 26, 25, and 23 μmoles of homocysteine L^{-1} of plasma for pigs fed 0, 35, 70, 105, and 140 μg of vitamin B_{12} kg^{-1} of diet. The plasma homocysteine concentration of pigs fed 0, 50, 100, 150, and 200 μg of vitamin B_{12} kg^{-1} of diet in experiment 4 were 23, 24, 21, 23, and 22 μmoles L^{-1}. These values are moderately higher than the 18 to 22 μmoles L^{-1} reported by Stangl *et al.* (2000) to be normal plasma concentration of homocysteine in pigs. The results of experiment 2 would imply that the normal plasma concentration of homocysteine is 30 μmoles L^{-1}.

It is noteworthy that 2 outliers were removed, via studentized residuals, prior to statistical analysis of the data set of experiment 1. These included 62 and 67 μmoles of homocysteine L^{-1} of plasma for pigs fed 0 and 140 μg of vitamin B_{12} kg^{-1} of diet, respectively.

It was a surprise that plasma homocysteine concentrations remained unchanged. The literature emphasizes the sensitivity and specificity of homocysteine and methylmalonic acid in diagnosing a vitamin B_{12} deficiency (Stabler *et al.* 1986; Stabler *et al.* 1988; Lindenbaum *et al.* 1990; Moelby *et al.* 1990; Allen *et al.* 1993; Savage *et al.* 1994; Moller *et al.* 1999; Bolann *et al.* 2000) relative to plasma vitamin B_{12}. However, Allen *et al.* (1990) and Lindenbaum *et al.* (1990) suggests an elevation in methylmalonic often precedes the elevation in

homocysteine. This is in agreement with Savage *et al.* (1994) who reported a 98.4 % verses 95.9 % detection rate of vitamin B_{12} deficient patients using methylmalonic acid and homocysteine, respectively. The reason for this difference is not clear, although a possible explanation is that L-methylmalonyl-CoA metabolism proceeds uniquely by means of the vitamin B_{12}-dependent enzyme, L-methylmalonyl-CoA mutase. Homocysteine, on the other hand, is metabolized by either the remethylation or transsulfuration pathway.

This lack of response by plasma homocysteine is thought to be attributed to the poor growth rate of pigs in experiments 1 and 4. Theoretically, high vitamin B_{12} intake would not be needed if growth rate was poor. If growth rate was ample, on the other hand, high vitamin B_{12} intake would be needed. If higher intake was not supplied, plasma and tissue vitamin B_{12} stores would be used to maintain growth rate. The vitamin B_{12} in plasma would be depleted before tissue stores. Therefore, pigs with poor growth rates would not use tissue vitamin B_{12} stores as rapidly as pigs with ample growth rates. Since homocysteine metabolism occurs in the tissues, it is logical that the plasma homocysteine concentration of pigs was not altered in experiments 1 and 4.

Homocysteine only showed a response to various levels of vitamin B_{12} supplementation in experiment 3. Plasma homocysteine was significantly increased in pigs fed 0 and 10 μg of vitamin B_{12} kg^{-1} of diet in experiment 3. The concentration of plasma homocysteine decreased and reached plateau with a supplementation of 20 μg of vitamin B_{12} kg^{-1} of diet. This observation confirmed that the requirement for vitamin B_{12} by the early weaned pigs from 17 to 32 days of age was met with 20 μg of vitamin B_{12} kg^{-1} of diet. Considering the statements just communicated, this response is warranted. Plasma and tissue

vitamin B_{12} stores were sacrificed to maintain the growth rate of pigs in experiment 3, which was greater than pigs in experiment 1 and 4. Consequently, homocysteine metabolism was compromised in the tissues. This resulted in elevated tissue and plasma homocysteine concentrations. This information would seem to suggest that plasma homocysteine can be used as a criterion for estimating the vitamin B_{12} requirement of the early weaned pig from 17 to 32 days of age. However, change in plasma homocysteine may be dependent on growth rate. Plasma homocysteine is not a sensitive indicator of a vitamin B_{12} deficiency when growth rate is poor.

It was proposed earlier, based on the results obtained in experiments 1, 2, and 4, that the normal plasma homocysteine concentration of pigs may be 21 to 30 μmoles L^{-1}. Certainly, the results of experiment 3 complement this proposal. Plasma homocysteine concentrations were 22, 21, and 22 μmoles L^{-1} when pigs were fed 20, 30, and 40 μg of vitamin B_{12} kg^{-1} of diet respectively.

Plasma concentrations of methylmalonic acid in experiment 1 were markedly elevated in pigs fed the unsupplemented diet versus those on the 140 μg of vitamin B_{12} kg^{-1} diet. The conversion of L-methylmalonyl CoA to succinyl CoA requires vitamin B_{12}; therefore, a deficiency of vitamin B_{12} causes an increases in the plasma level of methylmalonic acid. The normal concentration of methylmalonic acid, 215 nmole L^{-1}, published by Allen *et al.* (1993) is half of the concentration observed in the present experiment. This inconsistency may be explained by differences in age, genetic background, or diet of the pigs. These considerations were not detailed by Allen *et al.* (1993). Additional research is needed to determine the normal plasma concentration of methylmalonic acid in pigs. Since methylmalonic acid accumulated

when dietary vitamin B_{12} intake was inadequate, as determined by plasma vitamin B_{12} levels, methylmalonic acid holds promise as a criterion for estimating the vitamin B_{12} requirement of the early weaned pig from 17 to 32 days of age.

In addition to plasma vitamin B_{12} and methylmalonic acid, plasma cystathionine levels were elevated in pigs fed the unsupplemented diet. There observations are consistent with those of recent experiments of Stabler *et al.* (1993) and Stabler *et al.* (1997), whom reported elevations of cystathionine in humans with a vitamin B_{12} deficiency. However, the basal plasma cystathionine level in the present experiment, 1096 nmol L^{-1}, is far greater than 430 nmol L^{-1}. Preliminary research (Stabler *et al.* 1993) found 430 nmol L^{-1} to be the basal plasma cystathionine level in pigs. Justification for this difference is not known.

In the transsulfuration pathway homocysteine and serine are condensed by an enzyme, cystathionine β-synthase to form cystathionine. Cystathionine is cleaved by another vitamin B_6 dependent enzyme, cystathionine-γ-lyase, to form cysteine and α-ketobutyrate. Therefore, homocysteine is at a branch point where it can either converted to methionine or cystathionine. The latter forming cysteine. If homocysteine were prevented in participating in remethylation, as in a vitamin B_{12} deficiency, it is conceivable that there would be an increase through the transsulfuration pathway resulting in an increase in cystathionine levels.

7.0 CONCLUSION

After careful review and analysis of all 4 experiments, the vitamin B_{12} requirement of the early weaned pig from 17 to 32 days of age is concluded to be 20 μg kg^{-1} of diet Furthermore, ASP250 does not alter the vitamin B_{12} of the early weaned pig. The pigs received no benefit from microbial synthesis of vitamin B_{12} in the colon.

To estimate this requirement, the primary focus was on the amount of vitamin B_{12} needed for the maintenance of normal plasma metabolite levels (plasma vitamin B_{12} and plasma homocysteine). Changes in plasma metabolite levels developed before any change in growth performance parameters. Hence, the measure of plasma metabolite levels is proposed as a better means of diagnosing a vitamin B_{12} deficiency and establishing a vitamin B_{12} requirement in the early weaned pigs from 17 to 32 days of day. To our knowledge no other researcher have used pigs to determine if plasma metabolite levels are sensitive indicators of a vitamin B_{12} deficiency.

However, change in plasma homocysteine appears to be dependent on growth rate. Plasma homocysteine appears not to be a sensitive indicator of a vitamin B_{12} deficiency when growth rate is poor. It is proposed that the normal plasma homocysteine concentration of pigs is 21 to 30 μmoles L^{-1}. The normal plasma vitamin B_{12} concentration of pigs appears to be >160 pg mL^{-1}.

The findings also show that elevation of plasma methylmalonic acid was an early event, preceding an elevation of plasma homocysteine. Since plasma methylmalonic acid accumulated when dietary vitamin B_{12} intake was inadequate in experiment 1, plasma

methylmalonic acid values hold promise as a criterion for estimating the vitamin B_{12} requirement of the early weaned pigs for 17 to 32 days of age.

8.0 RESEARCH RECOMMENDATIONS FOR VITAMIN B_{12}

A study in a field environment is needed to determine if early weaned pigs will respond in a similar manner or not, as in this particular research environment. For practical application of this research, the basal diet should also contain some combination of skim milk, whey protein concentrate, fish meal, spray-dried blood plasma, and soyabean meal. The latter feed products are common to starter pig phase 1 diets.

Topics of research related to vitamin B_{12} that also merit attention are the following:

- Improved, economical, and sensitive methods to detect vitamin B_{12} in feed products.
- The contribution of sow's milk to vitamin B_{12} stores in early weaned pigs.
- The period of protection afforded by vitamin B_{12} stores.
- The contribution of bacterial synthesis in the gastrointestinal tract to vitamin B_{12} status.
- The attachment of vitamin B_{12} to TC II during the proposed receptor-mediated endocytosis.
- The basal plasma homocysteine, methylmalonic acid, and cystathionine levels in pigs.
- The distribution of homocysteine between remethylation and transsulfuration *in vivo*.
- The cause of elevated homocysteine, in a fed not fasting state, with a vitamin B_6 deficiency.

9.0 LITERATURE CITED

Allen, R. H., Seetharam, B., Podell, E. and Alpers, D.H. 1978. Effect of proteolytic enzymes on the binding of cobalamin to R protein and intrinsic factor. In vitro evidence that a failure to partially degrade R protein is responsible for cobalamin malabsorption in pancreatic insufficiency. J. Clin. Invest. **61**:47-54.

Allen, R. H., Stabler, S. P., Savage, D. G. and Lindenbaum, J. 1993. Elevation of 2-Methylcitric Acid I and II levels in serum, urine, and cerebrospinal fluid of patients with cobalamin deficiency. Metabolism. **42**(8):978-988.

Allen, R. H., Stabler, S. P., Savage, D. G. and Lindenbaum, J. 1990. Diagnosis of cobalamin deficiency I: Usefulness of serum methylmalonic acid and total homocysteine concentrations. Am. J. Hematol. **34**:90-98.

Anderson, G. C. and Hogan, A. G. 1950. Requirement of the pig for vitamin B_{12}. J. Nutr. 50:243-253.

Ardeman, S., Chanarin, I. and Berry, V. 1965. Studies on human gastric intrinsic factor. Observations on its possible absorption and enterohepatic circulation. Br. J. Haematol. **11**:11-14.

Association of Official Analytical Chemists (A. O. A. C.). 1990. Official Methods of Analysis 15th Edition. Association of Official Analytical Chemists, Inc. Arlington, Virginia, USA.

Batuman, V., Verroust, P. J., Navar, G. L., Kaysen, J. H., Goda, F. O., Campbell, W. C., Simon, E., Pontillon, F., Lyles, M., Bruno, J. and Hammond, T. G. 1998. Myeloma light chains are ligands for cubilin (gp280). Am. J. Physiol. Renal Physiol. **275**:F246-F254.

Birn, H., Fyfe, J., Jacobsen, C., Mounier, F., Verroust P. J., Ørskov, H. Willnow, T., Moestrup, S. K. and Christensen, E. I. 2000. Cubilin is an albumin binding protein important for renal tubular albumin reabsorption. J. Clin. Invest. **105**(10):1353-1361.

Birn, H., Verroust, P. J., Nexø, E., Hager, H., Jacobsen, C., Christensen, E. I. and Moestrup, S. K. 1997. Characterization of an epithelial ~460-kDa protein that facilitates endocytosis of intrinsic factor-vitamin B_{12} and binds receptor-associated proteins. J. Biol. Chem. **272**:26497-26504.

Baker, D. H. 1986. Critical Review: Problems and Pitfalls in Animal Experiments Designed to Establish Dietary Requirements for Essential Nutrients. J. Nutr. **116**:2339-2349.

Begley, J. A., Colligan, P. D., Chu, R. C. and Hall, C. A. 1993. Cobalamin metabolism in cultured human chorionic villus cells. J. Cell. Physiol. **156**:43-47.

Bender, D. A. 1992. Nutritional Biochemistry of Vitamins. Cambridge University Press. New York, NY.

Bolann, B. J., Solli, J. D., Schneede, J., Grottum, K. A., Loraas, A., Stokkeland, M., Stallemo, A., Schjøth, A., Bie, R. B., Refsum, H., and Ueland, P. M. 2000. Evaluation of indicators of cobalamin deficiency defined as cobalamin-induced reduction in increased serum methylmalonic acid. Clin. Chem. **46**(11):1744-1750.

Canadian Council on Animal Care. 1993. Guide to the care and use of experimental animals. Olfert, E.D., Cross, B.M., and McWilliam, A.A., eds. Vol. l, 2nd Edition.

Casey, P. J., Speckman, K. R., Ebert, R. J. and Hobbs, W. E. 1982. Radioisotope dilution technique for determination of vitamin B12 in foods. J. Assoc. Off. Anal. Chem. **65**(1):85-88.

Catron, D. V., Richardson, D., Underkofler, L. A., Maddock, H. M. and Friedland, W. C. 1952. Vitamin B_{12} requirement of weanling pigs. II Performance on low levels of vitamin B_{12} and requirement for optimum growth. J. Nutr. **47**:461-468.

Christensen, E. I. and Birn, H. 2001. Megalin and cubilin: synergistic endocytic receptors in renal proximal tubule. Am. J. Physiol. Renal Physiol. **280**:F562-F573.

Chu, R. C. and Hall, C. A. 1988. The total serum homocysteine as an indicator of vitamin B_{12} and folate status. Am. J. Clin. Pathol. **90**:446-449.

Cline, T. R. 1991. Feeding pigs weaned at three to four weeks of age. Pages 497-508 *in* E. R. Miller, D. E. Ullrey and A. J. Lewis. Swine Nutrition. Butterworth-Heinemann. Stoneham, MA.

Compendium of Medicating Ingredient Brochures 8th Edition. 1998.

Cook, D. A. and Easter, R. A. 1991. The water-soluble vitamins in swine nutrition. Pages 252-254 *in* E. R. Miller, D. E. Ullrey and A. J. Lewis. Swine Nutrition. Butterworth-Heinemann. Stoneham, MA.

Cooper, B. A. and White, J. J. 1968. Absence of intrinsic factor from human portal plasma during $^{57}CoB_{12}$ absorption in man. Brit. J. Haemat. **14**:73-78.

Cullen, R. W. and Oace, S. M. 1978. Methylmalonic acid and vitamin B_{12} excretion of rats consuming diets varying in cellulose and pectin. J. Nutr. **108**:640-647.

Dan, N. and Culter, D. F. 1994. Transcytosis and processing of intrinsic factor-cobalamin

in Caco-2 cells. J. Biol. Chem. **269**:18849-18855.

De La Haba, G. and Cantoni, G. L. 1959. The enzymatic synthesis of S-adenosyl-L-homocysteine from adenosine and homocysteine. J. Biol. Chem. **234**:603-608.

De Passillé, A. M. B., Bilodeau, R. R., Girard, C. L. and Matte, J. J. 1989. A study on the occurance of coprophagy behaviour and its relationship to B-vitamin status in growing-finishing pigs. Can. J. Anim. Sci. **69**:299-306.

Delgado-Reyes, C. V., Wallig, M. A. and Garrow, T. A. 2001. Immunohistochemical detection of betaine-homocysteine S-methyltransferase in human, pig, and rat liver and kidney. Arch. Biochem. Biophys. **393**(1):184-186.

Donaldson, Jr., R. M., Mackenzie, I. L. and Trier J. S. 1967. Intrinsic factor-mediated attachment of vitamin B_{12} to brush border and microvillous membranes of hamster intestine. J. Clin. Invest. **46**:1215-1228.

Donaldson, Jr., R. M., Small, D. M., Robins, S. and Mathan, V.I. 1973. Receptors for vitamin B_{12} related to ileal surface area and absorptive capacity. Biochim. et Biophys. Acta. **311**:477-481.

Doscherholmen, A. and Hagen, P. S. 1957. A dual mechanism of vitamin B_{12} plasma absorption. J.Clin.Invest. **36**:1551-1557.

Easter, R. A. 1988. Acidification of diets pigs. Pages 61-72 *in* W. Haresign and D. J. A. Cole. Recent advances in animal nutrition. Butterworths. London.

El Kholy, S. Gueant, J. L., Bressler, L. Djalali, M. Boissel, P. Gerard, P. and Nicolas, J. P. 1991. Portal and biliary phases of enterohepatic circulation of corrinoids in humans. Gastroenterology **101**:1399-1408.

Ellenbogan, L. and Cooper, B. A. 1991. Vitamin B12. Pages 491 - 536 *in* Machlin, L. J. Handbook of Vitamins 2nd Edition. Marcel Dekker, Inc. New York, NY.

Ellenbogan, L. and Highley, D. R. 1970. Enzymatic digestion of intrinsic factor and vitamin B_{12}-intrinsic factor complex. Fed. Proc.(abstract) **29**:633.

Ensminger, M. E., Oldfield, J. E. and Heinemann, W. W. 1990. Feeds and Nutrition 2nd Edition. The Ensminger Publishing Company. Clavis, CA.

Finkelstein, J. D. 1990. Methionine metabolism in mammals. J. Nutr. Biochem. **1**:228-237.

Finkelstein, J. D. and Chalmers, F. T. 1970. Pyridoxine effects on systathionine synthase in rat liver. J. Nutr. **100**:467-469.

Finkelstein, J. D. and Martin, J. J. 1984. Methionine metabolism in mammals. Distribution of homocysteine between competing pathways. J. Biol. Chem. **259**(15):9508-9513.

Finkelstein, J. D., Kyle, W. E. and Harris, B. J. 1971. Methionine metabolism in mammals. Regulation of homocysteine methyltransferase in rat tissue. Arch. Biochem. Biophys. **146**:84-92.

Frederick, G. L. and Brisson, G. L. 1961. Some observations on the relationship between vitamin B_{12} and reproduction in swine. Can. J. Anim. Sci. **41**:212-219.

Ford, J. E., Scott, K. J., Samsom, B. F. and Taylor, P. J. 1975. Some observations on the possible nutritional significance of vitamin B_{12}-and folate-binding proteins in milk. Absorption of [^{58}Co]cyanocobalamin by suckling piglets. Br. J. Nutr. **34**:4679-491.

Glass, G. B. J. 1963. Gastric intrinsic factor and its function in the metabolism of vitamin B_{12}. Physiol. Rev. **43**:529-849.

Gräsbeck, R. 1967. Intrinsic factor and the transcobalamins with reflection on the general function and evolution of soluble transport proteins. Scand. J. Clin. Lab. Invest. Suppl. **95**:7-17.

Gräsbeck, R. 1969. Intrinsic factor and the other vitamin B_{12} transport proteins. Prog. Hematol. **6**:233-260.

Gräsbeck, R. 1959. Influence of specific group inhibitors and enzymes of intrinsic factor activity. Acta. Physiol. Scand. **45**:88-95.

Gräsbeck, T., Nyberg, W. and Reizenstein, P. 1958. Biliary and fecal vitamin B_{12} excretion in man. An isotope study. Proc. Soc. Exp. Biol. Med. **97**:780-784.

Green, R. and Jacobsen, D. W. 1995. Clinical implications of hyperhomocysteinemia. Pages 75-124 *in* Bailey, L. E. Folate in Health and Disease. M. Dekker. New York, NY.

Green, R., Jacobsen, D. W., Van Tonder, S. V., Kew. M. C. and Metz, J. 1981. Enterohepatic circulation of cobalamin in the nonhuman primate. Gastroenterology **81**:773-776.

Hall, C. A. 1979. The transport of vitamin B_{12} from food to use within the cells. J. Lab. Clin. Med. **94**(6):811-816.

Hall, C. A. 1984. The uptake of vitamin B_{12} by human lymphocytes and the relationship to the cell cycle. J. Lab. Clin. Med. **103**:70-81.

Hall, C. A. and Finkler, A. E. 1965. The dynamics of transcobalamin II. A vitamin B_{12}

binding substance in plasma. J. Lab. Clin. Med. **65**:459-468.

Hall, C. A. and Finkler, A. E. 1966. Function of transcobalamin II: A B_{12} binding protein in human plasma. Proc. Soc. Exp. Biol. Med. **123**:55-58.

Hammad, S. M., Stefansson, S., Twal, W. O., Drake, C. J., Fleming, P., Remaley, A., Brewer, H. B. and Argraves, W. S. 1999. Cubilin, the endocytic receptor for intrinsic factor-vitamin B_{12} complex, mediates high-density lipoprotein holoparticle endocystosis. Proc. Natl. Acad. Sci. USA. **96**:10158-10163.

Henderickx, H. K., Teague, H. S., Redman, D. R. and Grifo, Jr., A. P. 1964. Absorption of vitamin B_{12} from the colon of the pig. J. Anim. Sci. **23**:1036-1038.

Herbert, V. 1988. Vitamin B-12: plant sources, requirements, and assay. Am. J. Clin. Nutr.. **48**:852-858.

Herbert, V. 1996. Vitamin B-12. Pages 191-202 *in* E. E. Ziegler and L. J. Filer Jr. Present Knowledge in Nutrition 7th Edition. ILSI Press. Washington, DC.

Hill, G. M., Cromwell, G. L., Crenshaw, T. D., Dove, C. R., Ewan, R. C., Knabe, D. A., Lewis, A. J., Libal, G. W., Mahan, D. C., Shurson, G. C., Southern, L. L. and Veum, T. L. 2000. Growth promotion effects and plasma changes from feeding high dietary concentrations of zinc and copper to weanling pigs (region study). J. Anim. Sci. **78**:1010-1016.

Hines, J. D., Rosenberg, A. and Harris, J. W. 1968. Intrinsic factor-mediated radio-B12 uptake in sequential incubation studies using everted sacs of guinea pig small intestine: Evidence that IF is not absorbed into the intestinal cell. Proc. Soc. Exp. Biol. Med. **129**:653-658.

Hippe, E. 1970. Changes in stokes radius on binding of vitamin B_{12} to human intrinsic factor and transcobalamins. Biochim. Biophys. Acta. **208**:337-339.

Hippe, E., Haber, E. and Olesen, H. 1971. Nature of vitamin B_{12} binding. II. Steric orientation of vitamin B_{12} on binding and number of combining sites of human intrinsic factor and the transcobalamins. Biochimica et Biophysica Acta. **243**:75-82.

Hølleland, G., Schneede, J., Ueland, P. M., Lund, P. K., Refsum, H., and Sandberg, S. 1999. Cobalamin deficiency in general practice. Assessment of the diagnostic utility and cost-benefit analysis of methylmalonic acid determination in relation to current diagnostic strategies. Clin. Chem. **45**(2):189-198.

Hooper, D. C., Alpers, D. H., Burger, R. L., Mehlman, C. S. and Allen, R. H. 1973. Characterization of ileal vitamin B_{12} binding using homogeneous human and hog intrinsic factors. J. Clin. Invest. **52**:3074-3083.

House, J. D., Jacobs, R. L., Stead, L. M., Brosnan, M. E. and Brosnan, J. T. 1999. Regulation of homocysteine metabolism. Advan. Enzyme Regul. **39**:69-91.

Jeffries, G. H. and Sleisenger, M. H. 1965. The pharmacology of intrinsic factor secretion in man. Gastroenterology **48**:444-448.

Jenkins, W. J., Empson, R., Jewell, D. P. and Taylor, K. B. 1981. Subcellular localisation of vitamin B_{12} during absorption in the guinea-pig ileum. Gut **22**:617-622.

Jensen, M. S., Jensen, S. K. and Jakobsen, K. 1997. Development of digestive enzymes in pigs with emphasis on lipolytic activity in the stomach and pancreas. J. Anim. Sci. **75**:437-445.

Keys Jr., J. E. and DeBarthe, J. V. 1974. Cellulose and hemicellulose digestibility in the stomack, small intestine and large intestine of swine. J. Anim. Sci. **39**(1):53-56.

Kozyraki, R., Kristiansen, M., Silahtaroglu, A., Hansen, C., Jacobsen, C., Tommerup, N., Verroust, P. J. and Moestrup, S. K. 1998. The human intrinsic factor-vitamin B_{12} receptor, *cubilin*: Molecular characterization and chromosomal mapping of the gene to 10p within the autosomal recessive megaloblastic anemia (*MGA 1*) region. Blood **91**:3593-3600.

Lassiter, J. W. and Edwards Jr., H. M. 1982. Animal Nutrition. Reston Publishing Company, Inc. Reston, Virgina.

Lehninger, A. L., Nelson, D. L., and Cox, M. M. 1993. Principles of Biochemistry 2nd Edition. Worth Publishers. New York, NY.

Lien, E. L., Ellenbogen, L., Law, P. Y. and Wood, J. M. 1974. Studies on the mechanism of cobalamin binding to hog intrinsic factor. J. Biol. Chem. **249**(3):890-894.

Lindenbaum, J., Savage, D. G., Stabler, S. P., and Allen, R. H. 1990. Diagnosis of cobalamin deficiency: II. Relative sensitivies of serum cobalamin, methylmalonic acid, and total homocysteine concentrations. Am. J. Hematol. **34**:99-107.

Lutz and Stahly, T. S. 1997. ISU Swine Research Report, ASL-R1478.

MacKenzie, I, L. and Donaldson, Jr., R. M. 1972. Effect of divalent cations and pH on intrinsic factor-mediated attachment of vitamin B_{12} to intestinal microvillous membranes. J. Clin. Invest. **51**:2465-2471.

Mavromichalis I. and Baker, D. H. 1999. Considerations in constructing diets for nursery pigs. PorkNet.

McDonald, P., Edwards, R. A., Greenhalgh, J. F. D. and Morgan, C. A. 1995. Animal Nutrition 5th Edition. Longman Group Ltd. United Kingdom.

McDowell, L. R. 2000. Vitamin B_{12}. Pages 323-347 *in* Vitamins in Animal and Human Nutrition 2nd Edition. Iowa State University Press. Ames, Iowa.

McKeever, M. P., Weir, D. G., Molloy, A., and Scott, J. M. 1991. Betaine-homocysteine methyltransferase: organ distribution in man, pig and rat and subcellular distribution in the rat. Clin. Sci. **81**:551-556.

Miller, J. W., Nadeau, M. R., Smith, D. and Selhub, J. 1994. Vitamin B-6 deficiency vs folate deficiency: comparison of responses to methionine loading in rats. Am. J. Clin. Nutr. **59**:1033-1039.

Miller, J.W., Ribaya-Mercado, J. D., Russell, R. M., Shepard, D. C., Morrow, F. D., Cochary, E. F., Sadowski, J. A., Gershoff, S. N. and Selhub, J. 1992. Effect of vitamin B-6 deficiency on fasting plasma homocysteine concentrations. Am. J. Clin. Nutr. **55**:1154-1160.

Minet, J., Bissé, E., Aebischer, C., Beil, A., Wieland, H. and Lütschg, J. 2000. Assessment of vitamin B-12, folate, and vitamin B-6 status and relation to sulfur amino acid metabolism in neonates. Am. J. Clin. Nutr. **72**(3):751-757.

Moelby, L., Rasmussen, K., Jenson, M. K., Pedersen, K. O. 1990. The relationship between clinically confirmed cobalamin deficiency and serum methylmalonic acid. J. Inter. Med. **228**:373-378.

Moestrup, S. K., Birn, H., Fischer, P. B., Petersen, C. M., Verroust, P. J., Sim, R. B., Christensen, E. I. and Nexo, E. 1996. Megalin-mediated endocystosis of transcobalamin-vitamin-B_{12} complexes suggest a role of the receptor in vitamin-B_{12} homeostasis. Proc. Natl. Acad. Sci. USA **93**:8612-8617.

Møller, J., Rasmussen, K., and Christensen, L. 1999. External quality assessment of methylmalonic acid and total homocysteine. Clin. Chem. **45**(9):1536-1542.

Monsen, A. B., Ueland, P. M., Vollset, S. E., Guttormsen, A. B., Markestad, T., Solheim, E. and Refsum, H. 2001. Determinants of cobalamin status in newborns. Pediatrics **108**(3): 624-630.

National Research Council (NRC). 1998. Nutrient Requirements of Swine. 10th Edition. National Academy of Science. Washington, DC.

National Research Council (NRC). 1988. Nutrient Requirements of Swine. 9th Edition. National Academy of Science. Washington, DC.

National Research Council (NRC). 1987. Vitamin Tolerance of Animals. National Academy of Science. Washington, DC.

Nesheim, R. O., Krider, J. L. and Johnson, B. C. 1950. The quantitative crystalline vitamin B_{12} requirement of the baby pig. Arch. Biochem. **27**:240-242.

Neumann, A. L., Johnson, B. C. and Thiersch, J. B. 1950. Crystalline Vitamin B_{12} in the nutrition of the baby pig. J. Nutr. **40**:403-414.

Neumann, A. L., Thiersch, J. B., Krider, J. L., James, M. F. and Johnson, B. C. 1950. Requirement of the baby pig for vitamin B_{12} fed as a concentrate. J. Anim. Sci. **9**:83-89.

Newberry, R. C. and Wood-Gush, D. G. M. 1988. Development of some behaviour patterns in piglets under semi-natural conditions. Animal Production **46**:103-109.

Noblet, J. and Perez, J. M. 1993. Prediction of digestibility of nutrients and energy values of pig diets from chemical analysis. J. Anim. Sci. **71**:3389-3398.

Owusu-Asiedu, A. 1998. Utilization of peas by early weaned pigs. M.Sc. Thesis. University of Manitoba. Winnipeg, MB.

Pekas, J. C. 1991. Pages 37-73 *in* E. R. Miller, D. E. Ullrey and A. J. Lewis. Swine Nutrition. Butterworth-Heinemann. Stoneham, MA.

Pfeiffer, C., Smith, S. J., Miller, D. T. and Gunter, E. W. 1999. Comparison of serum and plasma methylmalonic acid measurements in 13 laboratories: An international study. Clin. Chem. **45**(12):2236-2242.

Ramanujam, K. S., Seetharam, S., Ramasamy, M. and Seetharam, B. 1991. Cellular expression of cobalamin transport proteins and cobalamin transcytosis in human colon adenocarcinoma cells. Am. J. Physiol. **260**:G416-422.

Richardson, D., Catron, D. V., Underkofler, L. A., Maddock, H. M. and Friedland, W. C. 1951. Vitamin B_{12} requirement of male weanling pigs. J. Nutr. **40**:371-379.

Saito, A., Pietromonaco, S., Loo, A. K., and Farquhar, M. G. 1994. Complete cloning and sequencing of rat gp330/"megalin", a distinctive member of the low density lipoprotein receptor gene family. Proc. Natl. Acad. Sci. **91**:9725-9729.

Savage, D. G., Lindenbaum, J., Stabler, S. P., and Allen, R. H. 1994. Sensitivity of serum methylmalonic acid and total homocysteine determinations for diagnosing cobalamin and folate deficiencies. Am. J. Med. **96**:239-246.

Scott, J. M., Molloy, A. M., Kennedy, D. G., Kennedy, S. and Weir, D. G. 1994. Effects of the disruption of transmethylation in the central nervous system: an animal model. Acta. Neurol. Scand. **154**:27-31.

Seetharam, B. 1999. Receptor-mediated endocytosis of cobalamin (vitamin B_{12}). Annu. Rev. Nutr. **19**:173-195.

Seetharam, B., Bose, S. and Li, N. 1999. Cellular import of cobalamin (vitamin B-12). J. Nutr. **129**:1761-1764.

Selhub, J. 1999. Homocysteine metabolism. Annu. Rev. Nutr. **19**:217-246.

Seligman, P. A. and Allen, R. H. 1978. Characterization of the receptor for transcobalamin II isolated from human placenta. J. Biol. Chem. **253**(6):1766-1772.

Sizer, F. and Whitney, E. 1997. Nutrition: concepts and controversies 7th Edition. Wadsworth Publishing Company. Belmont, CA.

Stabler, S. P., Allen, R. H., Savage, D. G., and Lindenbaum, J. 1990. Clinical spectrum and diagnosis of cobalamin deficiency. Blood **76**(5):871-881.

Stabler, S. P., Lindenbaum, J., Savage, D. G. and Allen, R. H. 1993. Elevation of serum cystathionine levels in patients with cobalamin and folate deficiency. Blood **81**(12):3404-3413.

Stabler, S. P., Marcell, P. D., Podell, E. R., Allen, R. H., Savage, D. G. and Lindenbaum, J. 1988. Elevation of total homocysteine in the serum of patients with cobalamin or folate deficiency detected by capillary gas chromatography-mass spectrometry. J. Clin. Invest. **81**:466-474.

Stabler, S. P., Marcell, P. D., Podell, E. R., Allen, R. H. and Lindenbaum, J. 1986. Assay of methylmalonic acid in the serum of patients with cobalamin deficiency using capillary gas chromatography-mass spectrometry. J. Clin. Invest. **77**:1606-1612.

Stabler, S. P., Sampson, D. A., Wang, L. and Allen, R. H. 1997. Elevations of serum cystathionine and total homocysteine in pyridoxine-, folate-, and cobalamin-deficient rats. J. Nutr. Biochem. **8**:279-289.

Stahly, T. S., Williams, N., Swenson, S. G., and Ewen, R. C. 1995. ISU Swine Research Report, ASL-R1263.

Stahly, T. S. and Cook, D. 1996. ISU Swine Research Report. ASL-R1373.

Stangl, G. I., Roth-Maier, D. A. and Kirchgessner, M. 2000. Vitamin B-12 deficiency and hyperhomocysteinemia are partly ameliorated by cobalt and nickel supplementation in pigs. J. Nutr. **130**:3038-3044.

Statistical Analysis System (SAS) Institute, Inc. 1986. SAS User's Guide. Statistics 1986 Edition. SAS Institute, Inc. Cary, NC.

Sturman, J. A., Cohen, P. A. and Gaull, G. E. 1969. Effects of deficiency of vitamin B_6 on transsulfation. Biochem. Med. **3**:244-251.

Sullivan, L. W., Herbert, V. and Castle, W. B. 1963. *In vitro* assay for human intrinsic factor. J. Clin. Invest. **42**(9):1443-1457.

Sunden, S. L. F., Renduchintala, M. S., Park, E. I., Miklasz, S. D. and Garrow, T. A. 1997. Betaine-homocysteine methyltransferase expression in porcine and human tissues and chromosomal localization of the human gene. Arch. Biochem. Biophys. **345**(1):171-174.

Swendseid, M. E., Villalobos, J. and Friedrich, B. 1964. Free amino acids in plasma and tissues of rats fed a vitamin B6-deficient diet. J. Nutr. **82**:206-208.

Thacker, P. A. 1999. Nutritional requirements of early weaned pigs: A review. Pigs New and Information **20**(1):13N-24N.

Toskes, P. P., Deren, J. J., Fruiterman, J. and Conrad, M. E. 1973. Specificity of the correction of vitamin B_{12} malabsorption by pancreatic extract and its clinical significance. Gastroenterology **65**:199-294.

Trugo, N. M. F., Ford, J. E. and Sansom, B. F. 1985. Vitamin B_{12} absorption in the neonatal piglet. 1. Studies in vivo on the influence of the vitamin B_{12}-binding proteins from sows' milk on the absorption of vitamin B_{12} and related compounds. Br. J. Nutr. **54**:245-255.

Ubbink, J. B., van der Merwe, A., Delport, R., Allen, R. H., Stabler, S. P. and Riezler, R. 1996. The effect of a subnormal vitamin B-6 status on homocysteine metabolism. J. Clin. Invest. **98**(1):177-184.

Ueland, P. M., Refsum, H., Stabler, S. P., Malinow, M. R., Andersson, A. and Allen, R. H. 1993. Total homocysteine in plasma or serum: Methods and clinical applications. Clin. Chem. **39**:1764-1779.

Varel, V. H. 1987. Activity of fiber-degrading microorganisms in the pig large intestine. J. Anim. Sci. **65**:488-496.

Varel, V. H. and Yen, J. T. 1997. Microbial perspective on fibre utilization by swine. J. Anim. Sci. **75**:2715-2722.

Vester, B. and Rasuseen, K. 1991. High performance liquid chromatography method for rapid and accurate determination of homocysteine in plasma and serum. Eur. J. Clin. Chem. Clin. Biochem. **29**:549-554.

Vilaseca, M. A., Moyano, D., Ferrer, I. and Artuch, R. 1997. Total homocysteine in pediatric patients. Clin. Chem. **43**(4):690-692.

Winter, C. A. and Mushett, C. W. 1950. Absence of toxic effects from single injections of crystalline vitamin B_{12}. J. Am. Pharm. Assoc. **39**:360-361.

Woodworth, J. C., Goodband, R. D., Nelssen, J. L. Tokach, M. D., Musser, R. E., Loughmiller, J. A., Moser, S. A., Grinstead, G. S. and O'Qinn, P. R. 1997. Pyridoxine, but not thiamin, improves growth performance of weanling pis. Swine Day.
u, D. and Fyfe, J. C. 2000. Cubilin expression and posttranslational modification in the canine gastrointestinal tract. Am. J. Physiol. Gastrointest. Liver Physiol. **279**:G748-G756.

Yamaguchi, N., Rosenthal, W. S. and Glass, G. B. J. 1970. Study of the intestinal absorption of ^{51}Cr-labeled intrinsic factor. Am. J. Clin. Nutr. **23**(2):156-164.

Youngdahl-Turner, P. and Rosenberg, L. E. 1978. Binding and uptake of transcobalamin II by human fibroblasts. J. Clin. Invest. **61**:133-141.

Appendix 1. Proximate analysis of dietary treatments in experiment 1.

	Vitamin B_{12} inclusion rate, μg of vitamin B_{12} kg^{-1} of diet				
Parameter	0	35	70	105	140
Dry Matter (%)	91.5	91.5	91.7	91.1	90.8
Ash (%)	5.0	4.9	4.9	5.0	4.8
Crude Fat (%)	4.7	4.2	5.1	5.0	4.3
Neutral Detergent Fibre (%)	6.5	6.0	5.8	5.6	5.3
Crude Protein (%)	20.7	20.4	19.5	20.5	20.0
Gross Energy (MJ kg^{-1})	4095	4108	4087	4086	4070
Digestible Energy (Kcal kg^{-1})[1]	3698	3734	3725	3727	3736

[1] Calculated according to the equation of Noblet and Perez (1993).

Appendix 2. Proximate analysis of dietary treatments in experiment 2.

Parameter	Treatments	
	No ASP250	ASP250
Dry Matter (%)	90.6	89.9
Ash (%)	5.6	5.0
Crude Fat (%)	4.5	4.6
Neutral Detergent Fibre (%)	6.6	5.9
Crude Protein (%)	21.9	20.6
Gross Energy (MJ kg^{-1})	4075	4053
Digestible Energy (Kcal kg^{-1})[1]	3653	3690

[1] Calculated according to the equation of Noblet and Perez (1993).

Appendix 3. Proximate analysis of dietary treatments in experiment 3.

	Vitamin B_{12} inclusion rate, μg of vitamin B_{12} kg^{-1} of diet				
Parameter	0	10	20	30	40
Dry Matter (%)	91.4	91.1	91.1	90.8	91.4
Ash (%)	5.0	5.1	5.1	5.0	5.0
Crude Fat (%)	5.1	4.6	4.6	4.5	4.5
Neutral Detergent Fibre (%)	5.9	6.1	6.3	6.5	5.7
Crude Protein (%)	21.4	21.5	21.5	20.8	20.2
Gross Energy (MJ kg^{-1})	4055	4016	3981	3992	3990
Digestible Energy (Kcal kg^{-1})[1]	3691	3649	3612	3617	3648

[1] Calculated according to the equation of Noblet and Perez (1993).

Appendix 4. Proximate analysis of dietary treatments in experiment 4.

	Vitamin B_{12} inclusion rate, μg of vitamin B_{12} kg^{-1} of diet				
Parameter	0	50	100	150	200
Dry Matter (%)	92.3	91.6	91.7	90.0	92.1
Ash (%)	6.3	5.6	5.6	5.6	5.0
Crude Fat (%)	5.3	5.2	5.0	5.2	5.3
Neutral Detergent Fibre (%)	8.6	7.4	6.9	7.4	6.7
Crude Protein (%)	20.2	21.7	20.9	20.0	21.2
Gross Energy (MJ kg^{-1})	3988	4032	4079	3998	4100
Digestible Energy (Kcal kg^{-1})[1]	3472	3586	3637	3559	3694

[1] Calculated according to the equation of Noblet and Perez (1993).

Appendix 5. Vitamin B_{12} concentration ($\mu g\ kg^{-1}$) of some commonly used feed ingredients in swine starter diets.

Ingredient	Amount, ($\mu g\ kg^{-1}$)
Cereal Grains:	
Corn, grain	0.79
Wheat, grain	0.00
Wheat, middlings	0.94
Barley, grain	0.43
Protein Sources:	
Soyabean meal, dehulled	6.43
Canola meal	2.64
Propulse 975 pea protein	0.36
Blood plasma, spray-dried	3.36
Fish meal, menhaden	229.02
Lactose	4.94
Whey, dried	10.58
Casein	30.29
Skim milk	24.60
Amino Acids:	
L-Lysine	2.36
L-Threonine	2.22
DL-Methionine	2.22
Other:	
Vegetable oil	1.73
Dicalcium phosphate	6.85
Calcium carbonate	0.00
Salt, deiodized	0.48
ASP250 (Superchlor)	613.73 - 1429.58

Appendix 6. Quality control data from radioimmunoassays used to measure plasma vitamin B_{12}.[1]

	Experiment			
	1	2	3	4
Total Counts (cpm)	16013	15446	17929	18402
Maximum Binding (%)	53	47	62	66
Nonspecific Binding (%)	3.5	3.5	4.4	3.0
Sensitivity at 95% Binding (pg mL^{-1})	16	15	16	17
Intraassay Coefficient of Variation (%)	< 14 (n=6)[2]	< 18 (n=6)	< 3 (n=6)	< 7 (n=4)

[1]Interassay Coefficient of Variation was 19 %
[2]n represents the number of replicas