B-VITAMINS FOR CATTLE: AVAILABILITY, PLASMA LEVELS, AND IMMUNITY

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CHAPTER I

INTRODUCTION

B-vitamin requirements of ruminants must be met by the combination of 1) dietary B-vitamins that escape degradation in the rumen, and 2) microbial B-vitamin synthesis. Dietary supplementation studies have been the standard approach to investigate B-vitamin nutrition in ruminants as well as other livestock species. Yet dietary supplementation may not consistently alter duodenal B-vitamin supply and B-vitamin status of the ruminant. Ruminal degradation of dietary B-vitamins can be extensive; this forces the animal to rely on microbial synthesis of some B-vitamins. Microbial B-vitamin synthesis in the rumen tends to be inversely related to dietary concentrations.

Due to microbial synthesis, overt B-vitamin deficiencies in ruminants are unknown except for thiamin and for vitamin B_{12}. B_{12} becomes deficient when feeds contain inadequate cobalt. However, marginal B-vitamin deficiencies may be important in highly productive or stressed ruminants.

The first study was designed to assess the availability of eight B-vitamins in ruminal contents from steers fed high-concentrate diets. Semi-purifed diets lacking in single B-vitamins were fed to 10-day-old broiler chicks for 2 weeks. The ability of added ruminal contents to meet

chick B-vitamin requirements was measured based on growth, feed efficiency, and survival of chicks.

The second study consisted of a survey of plasma B-vitamin concentrations in suckling beef calves, transport-stressed cattle, feedlot cattle and high-producing dairy cows. From this study, baseline data were obtained for normal concentrations and ranges of ascorbic acid, folic acid, vitamin B_{12}, B_6 vitamers and pantothenic acid in cattle blood plasma. Concentrations were contrasted with those measured in other species.

Requirements by nonruminants for certain B-vitamins (vitamin B_6, folic acid, pantothenic acid, and ascorbic acid) are markedly increased by physical stress. Depletion of these vitamins in transport-stressed cattle could affect immunity and increase the incidence of bovine respiratory disease during the first few weeks after arrival in feedyards. To study this, calves were stressed by weaning, confinement in metabolism stalls, and restricted feeding followed by a 3-day fast. These processes duplicated some of the conditions undergone by transport-stressed calves. B-vitamins were supplemented by intramuscular injection of half of the calves. Effects of B-vitamin injections on plasma vitamin concentrations, resistance to bovine herpesvirus-1 infection, and various aspects of immune function were measured.

CHAPTER II

LITERATURE REVIEW

Introduction

All animals have a metabolic requirement for certain B-vitamins. These include thiamin (B_1), riboflavin (B_2), niacin, pyridoxine (B_6), pantothenic acid, folic acid, cyanocobalamin (B_{12}), and biotin. In the ruminant, the supply of B-vitamins is determined primarily by microbial synthesis and(or) destruction in the rumen. Unlike other livestock species, ruminant B-vitamin status normally is unaffected by dietary B-vitamin supply. In most cases, supplemental B-vitamins in the diet have had little effect on the ruminant's B-vitamin status; presumably, this is because most B-vitamins can be synthesized and destroyed in the rumen.

Ruminal B-vitamin synthesis was first suggested by Theiler and coworkers in South Africa (1915 as cited by McElroy and Goss, 1941a). Theiler et al. (1925) fed a diet low in thiamin to cattle for 44 to 52 weeks with no evidence of thiamin deficiency. Bechdel et al. (1926) proved that calves could grow to maturity and produce normal offspring on a diet so deficient in B-vitamins that it would not support life in rats. Using rat growth assays, Bechdel et al. (1928) demonstrated that ruminal contents contained B-

vitamins, and that a bacterial isolate from the rumen synthesized B-vitamins in vitro.

Between 1925 and 1930, vitamin B, considered a single entity, was subdivided into several components. The term B_1 (later called thiamin) was reserved for the antineuritic component. First isolated in 1926, it was synthesized and completely characterized in 1936. The next factor identified was B_2, also known as riboflavin. In the 1930's and 1940's, niacin, vitamin B_6 (pyridoxine and related vitamers), pantothenic acid, folic acid and vitamin B_{12} were discovered to be essential B-vitamins. Development of assays for the specific B-vitamins speeded progress in understanding vitamin requirements. Initially, the vitamin assays used microbiological or rat and chick bioassays.

In several studies, vitamin content of dietary and ruminal dry matter (DM) were compared to estimate ruminal vitamin synthesis. McElroy and Goss (1939; 1940a,b; 1941a,b) first demonstrated that riboflavin, vitamin B_6, thiamin and pantothenic acid were synthesized in the rumen. McElroy and Jukes (1940) similarly demonstrated biotin synthesis. Enrichment of the ruminal DM with riboflavin, nicotinic acid, pantothenic acid, pyridoxine and biotin was shown for both a heifer fed a vitamin-deficient synthetic diet and a heifer fed 6 diets containing various amounts of grain, linseed meal, and urea (Wegner et al., 1940, 1941). Thiamin clearly was more concentrated in ruminal contents than in the diet when a synthetic diet was fed, but not when

a practical diet was fed. Supplementation of this synthetic diet with 200 mg thiamin per day increased ruminal concentrations of all 6 B-vitamins tested. Hunt et al. (1941) found that thiamin concentration was greater in ruminal DM than in feed DM at 4 hours post-feeding in 2 out of 3 trials but it was lower when rumen samples were taken 12 or more hours after feeding. They also observed that net synthesis of ruminal riboflavin in steers was positive when a mixed diet was fed but not when alfalfa hay alone was fed. Subsequently, further evidence was provided for synthesis of riboflavin but not of thiamin (Hunt et al., 1943). Ruminal synthesis of folic acid and biotin were demonstrated first by Lardinois et al. (1944) and Hale et al. (1950), respectively.

Net B-vitamin synthesis was readily apparent when ruminants were fed purified diets (Agrawala et al., 1953; Virtanen, 1967). Comparison of B-vitamin intake with output in urine and feces of sheep, and in urine, feces and milk of cattle also confirmed that B-vitamins were synthesized in the gastrointestinal tract (Pearson et al., 1953; Porter, 1961).

B-vitamin synthesis occurs quite early in life of young ruminants given access to solid feed. In 8 to 32-day-old calves, the ruminal concentrations of thiamin, riboflavin, niacin, and folic acid were several times higher than in the alfalfa hay plus calf starter diet (Kesler and Knodt, 1951). Similarly, the ruminal concentrations of thiamin, ribo-

flavin, and niacin were greater in lambs 3 weeks of age than in lambs 1 week of age (Buziassy and Tribe, 1960b). B-vitamin synthesis also occurs in the large intestine in ruminants as in most other species.

Quantification of B-vitamin Production. Early studies of ruminal B-vitamins provided a qualitative but not a quantitative picture of B-vitamin production. Buziassy and Tribe (1960a) attempted to quantitate rumen microbial synthesis by comparing daily B-vitamin consumption with the total ruminal contents of thiamin, riboflavin and nicotinic acid 90 minutes after feeding. They used three different methods to estimate ruminal volume. Synthesis was calculated as the difference between intake and ruminal content of each B-vitamin, ignoring effects of outflow, absorption from the rumen, and residues from previous meals. These workers also differed from McElroy and Goss (1940, 1941), Wegner et al. (1940, 1941), and Hunt et al. (1941, 1943) in using more tedious chemical rather than simpler microbiological assays. Buziassy and Tribe (1960a) showed that microbial synthesis of B-vitamins in the rumen was very low when the diet was rich in vitamins, but it was high when the diet was vitamin-deficient.

Zinn et al. (1987) may have been the first group to quantitate ruminal escape and net microbial synthesis for each B-vitamin. They used three 194 kg. steer calves with cannulas in the rumen and proximal duodenum in a 3 X 3 Latin square. The three treatments consisted of a control diet

(34.5% alfalfa hay, 44.5% steam-flaked corn, sudangrass, molasses, fat, urea, and salt), and this diet with supplemental vitamins at one (X) and ten times (10X) the estimated requirement.

Their calculations were based on the assumption that vitamin intake and source (feed versus supplemental B-vitamins) did not alter either microbial synthesis or the percentage of each vitamin destroyed in the rumen. This assumption conflicts with the conclusions of Buziassy and Tribe (1960a). The extent of ruminal escape of vitamins was estimated from treatment means using the slope-ratio procedure: vitamin escape (%) = 100 X (duodenal flow of vitamin with nonsupplemented diet)/(vitamin intake with high level of supplementation - vitamin intake with non-supplemented diet). Net microbial synthesis of various B-vitamins was estimated by difference: vitamin synthesis (mg/kg digestible organic matter intake) =((duodenal vitamin flow - (vitamin escape/100) X vitamin intake)/kg digestible organic matter intake.

In general, the daily flow of B-vitamins to the small intestine was increased noticeably with the 10X supplementation level but not with the X level. No difference between the control and X treatments were detected for any B-vitamin except that duodenal flow of niacin was non-significantly depressed (from 277.4 mg/day to 207.5 mg/day) when intake was increased from 67.0 mg (control) to 267 mg (X). In contrast, the 10X level of supplementation

increased duodenal flow of thiamin, B_6, pantothenic acid, and biotin by 5 to 41 times. Niacin flow increased by 44.5% and folic acid flow increased by 345%, whereas the flow of riboflavin and B_{12} were not affected. These results indicate how ruminal synthesis and(or) microbial destruction can negate the effects of supplemental dietary B-vitamins on duodenal flow, except possibly for thiamin, B_6, pantothenic acid, and biotin when fed at extremely high levels.

Factors affecting B-vitamin production

Intake. Zinn et al. (1987) also fed 4 steers an 80% concentrate diet at 1.2, 1.5, 1.8, and 2.1% of bodyweight. The intestinal supply of each of 5 B-vitamins measured was correlated positively with feed intake. Daily intestinal supplies of thiamin, riboflavin, niacin, B_6, and B_{12} were 14.2, 29.4, 262.0, 19.7 and 7.5 mg, respectively, at the lowest feed intake versus 33.2, 49.8, 616.4, 58.1 and 12.3 mg, respectively, at the highest intake level.

Protein and Energy. Ruminal levels of B-vitamins often appear remarkably constant, despite marked differences in the diet and in experimental conditions. However, if microbial growth is limited by the supply of nutrients, particularly nitrogen or energy, microbial B-vitamin synthesis will be depressed. Nitrogen source, whether nonprotein N or protein, also may influence niacin synthesis.

Ruminal concentrations of thiamin, riboflavin, and niacin were markedly lower when the supply of N in the diet was low. Yet, total substitution of urea for dietary protein did not affect thiamin and riboflavin concentrations even though niacin concentration was depressed (Buziassy and Tribe, 1960a).

For 16 sheep fed 8 different diets, the ruminal levels of riboflavin, nicotinic acid, and pantothenic acid were lowest when prairie hay was fed. Protein content of the diet and feed intake also were lowest for this diet. Supplementation with corn and urea increased ruminal concentrations of these vitamins, and substituting soybean meal for urea further increased niacin content (Hollis et al., 1954). Similarly, addition of readily-available carbohydrate (corn, molasses, or starch) and nitrogen supplementation (urea or casein) increased the amounts of riboflavin, niacin, and pantothenic acid in the rumen (Lardinois et al., 1944).

When mature grass hay was fed to young dairy calves, addition of grain to the diet increased ruminal B-vitamin levels. However, ruminal concentrations were not affected by the grain:hay ratio when a good quality alfalfa hay was fed (Conrad and Hibbs, 1954).

Hayes (1966) compared ruminal B-vitamin levels for steers fed 6 corn/soy rations. Corn processing method (flaked versus ground) and forage (none, long hay, ground hay) were compared in a 2 X 3 factorial design. Flaked corn

or ground corn (6.3 to 7.5 kg daily) each were fed to steers with no hay, 1.8 kg long hay, or 1.8 kg ground hay. A soybean meal supplement was included in all 6 dietary treatments. Riboflavin, thiamin, pantothenic acid, folic acid, niacin, and vitamin B_{12} concentrations in ruminal fluid varied markedly with diet. Thiamin concentration (16.8 µg/100 ml) was higher ($P<0.05$) when the ground corn diet was fed than when the other 5 diets were fed; lowest levels (.3 µg/100 ml) were observed when the ground corn/long hay diet was fed. Pantothenic acid and niacin were higher in rumen contents of steers fed diets without hay, and higher when steers were fed corn in the ground rather than the flaked form. Folic acid was highest in ruminal contents of steers fed diets without hay; its concentration was negatively correlated with ruminal pH. B_{12} was highest in rumen contents of steers fed the ground corn no-hay diet. Biotin levels varied from 2.9 to 4.8 ug/100 mls, with no significant differences detected. Unfortunately, they tested no high-forage diets.

Hunt et al. (1954) studied the effects of various dietary components and chlortetracycline on 4 B-vitamins in a series of in vitro experiments. Supplemental sulfur stimulated riboflavin synthesis but inhibited pantothenic acid synthesis. Starch stimulated the synthesis of all the B-vitamins studied. Cellulose stimulated synthesis of riboflavin more than of pantothenic acid. Chlortetracycline

inhibited synthesis of pantothenic acid, niacin and riboflavin but not of B_{12}.

Miller et al. (1986a) examined the effects of two concentrate:forage ratios and of grain source on duodenal flow of four B-vitamins. The high concentrate (HC) diet contained 88.9% corn and 10% alfalfa meal; the low concentrate (LC) diet contained 29.5% corn and 70% alfalfa meal. Both diets were fed pelleted, and resulted in similar ruminal OM digestibilities; thus, the LC diet would not be representative of typical high-forage diets. Thiamin, niacin, riboflavin, and biotin duodenal flows were not affected ($P<0.05$) by diet. Concentrate diets (85.5% grain) from corn, wheat, oats, and barley indicated a net loss of thiamin in the rumen; only with sorghum grain was net production positive although ruminal OM digestibility was much lower for this diet. Ruminal pH was not affected by diet. Although grain source altered intake of riboflavin, niacin and biotin, it did not affect duodenal flow of these vitamins.

Diet changes can alter ruminal B-vitamin levels rapidly. Phillipson and Reid (1957) measured ruminal thiamin levels in sheep when the diet was changed. In 15 out of 20 dietary changes, a new plateau in ruminal thiamin concentration was reached by the fourth day after the diet change. When sheep were moved to pasture at any time of summer, ruminal thiamin levels increased initially, regardless of subsequent levels.

Feed additives. By altering ruminal fermentation, feed additives can affect microbial synthesis and degradation of B-vitamins. Effects of some dietary antibiotics have been studied. Feeding either 40 or 56 g/day of sulfathalidine increased urinary and fecal thiamin concentrations, but neither affected niacin, riboflavin and pantothenic acid concentrations (Teeri et al, 1950). Feeding 200 mg of procaine penicillin daily did not markedly affect ruminal concentrations of riboflavin, pyridoxine, biotin and B_{12} in steers fed a mixed diet (Kon and Porter, 1953). Chance et al. (1953 a,b) measured the effects of dietary chlortetracycline (CTC) at .5 and 1 g/day for 15 days in steers. Although total bacterial counts were unaffected, the lower amount reduced riboflavin but increased niacin and panthothenate concentrations in ruminal contents. CTC at 7.8 mg/kg diet, about 50 mg/d per steer, in a corn-based pellet did not detectably affect thiamin, ribo-flavin, or niacin at the duodenum, ileum, or feces; however, it increased duodenal and ileal biotin concentrations (Miller et al., 1986b). In a second trial, CTC fed at a higher level (70 mg/day) increased ileal ($P<0.01$) but not duodenal thiamin flow (Miller et al, 1986b).

The effects of ionophores on ruminal B-vitamin metabolism have not been investigated extensively. Ionophores are substances that promote transfer of ions from an aqueous medium into a hydrophobic phase (Dobler, 1981). Two commonly fed carboxylic ionophores, monensin and lasalocid,

are produced by Streptomyces cultures. Almost all feedlot diets and many supplements, minerals and mixed feeds fed to growing cattle, beef cows and non-lactating dairy cows contain one of these ionophores. Monensin and lasalocid may alter the microbial population in the rumen, affecting the extent and products of ruminal fermentation. They increase efficiency of feed use and reduce the incidence of coccidiosis and bloat.

Miller et al. (1986b) measured dietary, duodenal, and ileal supply and fecal output of 4 vitamins in 8 steers fed diets supplemented with either 0 or 22 mg monensin/kg diet. Monensin had little effect on thiamin, except for increasing ileal thiamin flow (1.7 mg/d vs .8 mg/d, $P<0.20$); it also tended to reduce apparent ruminal thiamin destruction (34 vs 41%). Monensin tended to reduce ruminal niacin synthesis. In contrast, monensin increased riboflavin synthesis, tending to increase duodenal flow of riboflavin (84.3 vs 60.8 mg/d). Monensin did not affect duodenal or ileal biotin flows, but it tended to increase fecal biotin output.

Thiamin

Thiamin occurs primarily in the form of phosphate esters in animal tissues, with about 80% as thiamin pyrophosphate (TPP), the active coenzyme form. Another 10% occurs as thiamin triphosphate, with trace amounts of monophosphate and free thiamin (Gubler, 1984). Wet tissue concentrations of thiamin content are highest in heart,

kidney, liver, brain and nervous tissue. Although muscle concentration is relatively low, muscle contains 40 to 50% of total body thiamin.

In mammals, thiamin is involved with three major functions--energy metabolism, synthetic mechanisms and nerve conduction. The first two functions employ TPP as a coenzyme to transfer an activated aldehyde group. TPP is required for oxidative decarboxylation of α-ketoacids. These reactions are essential for the generation of ATP from pyruvate in the TCA cycle (pyruvate → acetyl CoA; α-ketoglutarate → succinyl CoA), and also for the oxidation of the keto-analogs of amino acids (leucine, isoleucine, valine) in the TCA cycle. By inhibiting carbohydrate metabolism, a deficiency of thiamin elevates blood lactate, pyruvate and oxaloacetate levels.

The transketolase reaction of the hexose monophosphate shunt also requires TPP. This pathway produces pentoses for RNA and DNA synthesis, as well as NADPH for biosynthetic reactions.

Independent of its effects on energy metabolism, thiamin is essential for function of the central nervous system; this involves thiamin triphosphate (TTP) instead of TPP. The brain contains a unique enzyme system which catalyzes the synthesis of TTP from TPP (Nishino and Itokawa, 1983). Thiamin levels in brain are relatively stable and fall only after 3 to 5 weeks of severe depletion.

In all species, the prominent symptoms of thiamin deficiency are anorexia and weight loss, and irregularities of the heart and nervous system (Gubler, 1984). In ruminants, clinical signs of non-acute thiamin deficiency include transient scouring, ill-thrift and anorexia; these are associated with production losses in cattle and sheep (Rammell and Hill, 1986).

Clinical signs of a central nervous system disorder occur at a later stage of thiamin deficiency. The disease is most common in cattle, sheep and dogs, but it also affects goats, horses, deer and other species. The terms cerebrocortical necrosis (CCN) or polioencephalomalacia (PEM) describe the disease in ruminants. Outbreaks are sporadic with morbidity rates from less than 10% to 50% but high mortality (Loew and Dunlop, 1972a). Clinical signs of CCN include rapid onset, aimless wandering, circling, hyperexcitability, incoordination, opisthotonus, recumbency, blindness, convulsions and coma. Because these clinical signs are not specific, CCN must be differentiated from lead poisoning, encephalitic listeriosis, bacterial meningitis, aflatoxicosis, hypomagnesemia, enterotoxemia, pregnancy toxemia, and idiopathic coccidiosis. Multiple necrotic foci in the cerebral cortex, and a characteristic fluorescence observed under the meninges in the outer cortex of a transverse brain section under UV illumination, confirm the diagnosis (Edwin et al., 1979).

Various dietary and management conditions can alter the delicate balance between thiamin production and destruction in the rumen and cause CCN. Thiaminases of both plant and microbial origin destroy thiamin. Outbreaks of CCN in the U.K. and Australia have resulted from livestock grazing fronds or rhizomes of ferns rich in thiaminase I (Evans, 1975; Evans et al., 1975).

Endogenous thiaminase enzymes produced by gut microflora also can cause thiamin deficiency; these have been isolated from ruminal contents or feces from normal animals. Ruminal thiaminase can destroy up to 1 mg thiamin per minute per kg of rumen digesta (Edwin and Jackman, 1982). Two types of microbial thiaminase, I (EC 2.5.1.2) and II (EC 3.5.99.2), have been isolated from ruminal contents (Evans, 1975; Edwin and Jackman, 1970, 1982). Thiaminase I forms thiamin derivatives, which may act as thiamin antagonists, exacerbating a thiamin deficiency (Brent and Bartley, 1984; Edwin et al., 1976b). Thiaminase I is markedly inhibited by thiamin and must be activated by a cosubstrate; cosubstrate affinity varies depending on the enzyme source. Suitable cosubstrates include δ-1-pyrroline, cysteine, proline, hypotaurine, lysine, nicotinic acid, pyridoxine, and imidazole, all of which are found in ruminal fluid. Availability of specific cosubstrates may be critical to the development of CCN (Bakker et al., 1980). CCN has been precipitated by administration of levamisole hydrochloride or thiabendazole,

anthelminths that can act as thiaminase I cosubstrates (Linklater et al., 1977).

CCN occurs most frequently in grain-fed animals susceptible to lactic acidosis; diets rich in readily fermented carbohydrates have been fed to induce CCN. Many bacterial species including Bacillus thiaminolyticus, Clostridium sporogenes, and Megasphaera elsdenii, produce thiaminase I. The bacterial thiaminase I is an exoenzyme bound to the cell surface; little or no activity occurs in the cell-free fluid from normal animals at pH 6.8; in fact, net synthesis of thiamin may be positive. But, if ruminal contents are "acid shocked" in vitro to pH 4.5 and then adjusted to pH 6.8, free thiaminase is found in ruminal fluid (Sapienza, 1981). In sheep affected with CCN, the enzyme was found primarily in the supernatant fluid, presumably due to lactic acidosis (Brent and Bartley, 1984). Lactic acidosis also increases histamine in ruminal contents; histamine is an active cofactor for thiaminase I.

Thiaminase II is a hydrolytic enzyme that cleaves thiamin into pyrimidin and thiazole components without forming analogs; it does not require a cosubstrate (Harmeyer and Kollenkirchen, 1989). While thiaminase II has been identified in ruminal contents (Edwin and Jackman, 1982), Bakker et al. (1980) and Edwin and Jackman (1970) isolated only thiaminase I from animals affected with CCN.

High sulfate levels in the diet or water can increase the incidence of CCN. The sulfite ion, produced during the

microbial reduction of sulfates to sulfides, can destroy thiamin (Zoltewicz et al., 1982). CCN has occurred in cattle fed grain preserved with sulfur dioxide (Gibson et al., 1986) and in cattle fed diets containing calcium sulfate (gypsum) or $MgKSO_4$ as an intake limiter (Brent and Bartley, 1984).

Amprolium, a feed additive used to prevent coccidiosis in ruminants and chicks, is a thiamin analog. It has a pyrimidine ring similar to thiamin but, lacking the hydroxyl group, it does not act as a substrate for thiamin pyrophosphokinase; hence, it does not inhibit TPP-dependent enzymes. Nevertheless, amprolium inhibits thiamin transport across microbial cell walls; this is the basis of its anticoccidial action. Amprolium also can affect thiamin transport across the intestinal wall, causing thiamin deficiency and PEM (Loew and Dunlop, 1972b; Gubler, 1984).

Several methods have been used to assess thiamin status in animals and man; these include blood thiamin, pyruvate and lactate levels, the response of blood transketolase to added thiamin (TPP effect), and urinary thiamin excretion.

Blood thiamin levels can be useful if they reflect tissue levels, as has been shown in nonruminant animals fed deficient diets. Blood contains about .8% of the total body thiamin, and the concentration is very low (6 to 12 μg/100 ml). Most of the blood thiamin is located in the erythrocytes and leukocytes, so serum levels are even lower. As a result of technical difficulties in measuring serum levels,

the indirect method of assessing thiamin status through transketolase activity became most popular and is still extensively used in human nutrition. However, numerous HPLC methods for measuring thiamin in blood and tissue are available.

Riboflavin

Riboflavin occurs in feeds in the form of free riboflavin, FMN, and FAD. Any of these three forms can fulfill the vitamin requirement. In mammalian tissues, riboflavin exists primarily as FAD and FMN although riboflavin is found free in the eye and the urine.

The riboflavin coenzymes FMN and FAD act as intermediates in the transfer of electrons in biological oxidation-reduction reactions. Those that function aerobically are called oxidases; those that function anaerobically are called dehydrogenases.

The oxidases are soluble enzymes found in the cell cytoplasm; they transfer hydrogen to molecular oxygen to form hydrogen peroxide. The best known oxidase, Warburg's old yellow enzyme, is an FMN-containing flavoprotein that oxidizes glucose to gluconic acid.

The flavoproteins that function anaerobically link substrate oxidation with phosphorylation and ATP synthesis. This pathway usually involves NAD, cytochromes, and the transfer of hydrogen from substrate oxidation to form water. Two ATP are generated for each hydrogen passed directly from

the substrate to a flavoprotein (e.g., succinic dehydrogenase) whereas 3 ATP are formed when hydrogen is transfered first from the substrate to NAD and later to FAD and the cytochrome system (e.g., cytochrome reductase). Other important flavoproteins include the acyl-coenzyme A dehydrogenases for fatty acid oxidation. Mitochondrial 2-glycerophosphate dehydrogenase and lactic acid dehydrogenase are part of shuttle mechanisms in which reducing equivalents are passed from the cytoplasm to the mitochondria.

Pyridoxine

Vitamin B_6 is the generic term for all 3-hydroxy-2-methyl pyridine derivatives. The trivial names and acronyms for the 3 principal forms of vitamin B_6 and their phosphate esters are: pyridoxine or pyridoxol, PN; pyridoxal, PL; pyridoxamine, PM; pyridoxine 5'-phosphate, PNP; pyridoxal 5'-phosphate, PLP; and pyridoxamine 5'-phosphate, PMP. These forms of vitamin B_6 are interconvertible in animal tissues.

Pyridoxal phosphate is the coenzyme form of vitamin B_6. It participates in reactions of more than 100 enzymes, nearing half involving transamination reactions. During transamination reactions, pyridoxal phosphate is transformed reversibly into pyridoxamine phosphate (Snell, 1986). PLP is involved in numerous other reactions in the metabolism (synthesis and catabolism) of α-amino acids. These include

nonoxidative deaminations, decarboxylations, and desulfhydration reactions.

PLP affects gluconeogenesis in two ways: first as the coenzyme for transamination reactions, and second as a component of glycogen phosphorylase. In rats, a deficiency of vitamin B_6 has decreased liver and muscle glycogen phosphorylase (Angel and Moller, 1974).

PLP affects erythrocyte function and metabolism. PLP is the coenzyme for δ-aminoleuvulinic acid synthetase; this synthetase forms δ-aminoleuvulinic acid, a precursor of heme. Consequently, a vitamin B_6 deficiency in animals may result in a hypochromic, microcytic anemia. PLP and PL bind to specific chains of hemoglobin, altering O_2 binding affinity. PLP also is the coenzyme for various erythrocyte transaminases; this forms the basis for several enzymatic assays of vitamin B_6 status.

The immune system can be influenced by PLP. Vitamin B_6 deficiency can depress lymphocyte production (van den Berg et al., 1988), antibody response (Chandra and Puri, 1985), and cell mediated immunity (Cheslock and McCully, 1960). PLP is the coenzyme for a key enzyme involved in 1-carbon metabolism, serine transhydroxymethlase, which affects nucleic acid synthesis. Via this mechanism, vitamin B_6 status may alter immune function.

PLP participates in multiple enzymatic reactions in the complex tryptophan-niacin pathway. On a low vitamin B_6 diet, niacin formation from tryptophan is depressed.

Thereby, urinary excretion of niacin metabolites after a tryptophan load can be used to evaluate vitamin B_6 status in humans (Leklem, 1991).

Vitamin B_6 affects the nervous system. Neurotransmitters including serotonin, dopamine, norepinephrine, and histamine are synthesized by PLP-dependent enzymes. Decarboxylation of glutamic acid to GABA, and degradation of GABA, both involve PLP-dependent enzymes. GABA is involved in the regulation of synaptic transmission in the nervous system (Driskell, 1984). Vitamin B_6 deficiency results in convulsions, abnormal EEGs, and structural and biochemical changes in the brain (Leklem, 1991).

Lipid metabolism also is affected by vitamin B_6 status although the mechanism is poorly understood. Vitamin B_6 may modify methionine metabolism, indirectly affecting phospholipid and fatty acid metabolism (Loo and Smith, 1986) and carnitine synthesis (Cho and Leklem, 1990).

Recently, PLP has been shown to modulate steroid action (Litwack et al., 1985; Cidlowski and Thanassi, 1981). PLP reacts with steroid receptors for estrogen, androgen, progesterone and glucocorticoids. B_6 status may alter sensitivity of the end-target tissue to steroids. The physiological significance of this alteration has not been established (Leklem, 1991).

Vitamin B_6 is required or stimulatory for certain ruminal microbes including Ruminococcus albus, R. flavefaciens, Butyrivibrio fibrisolvens, and Megasphaera

elsdenii. Bacteroides succinogenes and B. amylophilus do not require vitamin B_6 (Wolin and Miller, 1988).

The effect of diet composition on ruminal B_6 synthesis has not been studied in detail. Increasing the nitrogen concentration of the feed did not affect vitamin B_6 in the rumen in two studies (Wegner et al., 1941; Lardinois et al., 1944). Ruminal vitamin B_6 concentrations have ranged from 8 to 10 mg/kg rumen DM (McElroy and Goss, 1940b) and 3 to 12 mg/kg DM (Wegner et al., 1940, 1941).

Dietary vitamin B_6 appears to escape ruminal degradation; in addition, microbial B_6 is synthesized in the rumen (Zinn et al., 1987). Comparison of ruminal levels or duodenal flows of vitamin B_6 with non-ruminant requirements reveals that vitamin B_6 requirements should normally by supplied. However, Horst and Reinhardt (1983) speculated that a transient deficiency of vitamin B_6 could potentiate the negative effect of glucocorticoids on intestinal calcium absorption, increasing the incidence of milk fever in cows.

Physical symptoms of deficiency in nonruminants include symmetrical scaling dermatitis, poor growth, muscle weakness, hyperirritability, and altered activity and curiosity scores. Anemia, fatty liver, nerve degeneration, and decreased reproduction also are seen. Depressed antibody, nucleic acid, and protein synthesis are characteristic (Driskell, 1984).

Vitamin B_6 deficiency has not been identified in adult ruminants under practical conditions. Vitamin B_6 deficiency

symptoms in the preruminant calf included loss of appetite, and slowing and cessation of growth. Diarrhea and "fits" occurred in some calves. Post-mortem examinations showed enteritis, inflammation of lymph nodes, and demyelination of peripheral nerves (Johnson et al., 1950).

Pyridoxine and pyridoxamine and their phosphorylated forms are the predominant vitamin B_6 forms found in plants. PLP predominates in most animal feeds. Other conjugated forms of pyridoxine occur, such as the glycosylated form in wheat bran. In humans, the level of glycosylated vitamin B_6 in foods was correlated inversely with vitamin B_6 bioavailability (Leklem, 1991).

Phosphorylated forms of vitamin B_6 are hydrolyzed by alkaline phosphatase in the intestine. Absorption in rats appears to be a nonsaturable, passive process primarily restricted to the small intestine. Recently, an active absorption process was proposed (Middleton, 1985).

The liver is the organ primarily responsible for metabolism of vitamin B_6. The B_6 vitamers are inter-converted in the liver; the active form, PLP, is released into the circulation for other tissues (Lummeng and Li, 1980; Lumeng et al., 1974). PLP formed in liver and other tissues binds via a Schiff base reaction with proteins. This PLP binding may metabolically trap vitamin B_6 in cells (Lummeng and LI, 1980).

PLP released from the liver is found in the plasma bound to albumin. Binding to albumin protects PLP from

hydrolysis and enables it to be delivered to other tissues. PLP is the most abundant B_6 vitamer found in the plasma, but PN, PMP and PM also are found.

Erythrocytes are involved in metabolism and transport of vitamin B_6. PL and PN are absorbed rapidly by the erythrocyte through simple diffusion; PL binds more tightly to hemoglobin than to albumin. The PL concentration is 4 to 5 times greater in the erythrocyte than in plasma. PN and PL are converted to PLP in the human erythrocyte. The role of the erythrocyte in transporting vitamin B_6 is not clear, particularly due to the tight binding of hemoglobin to PLP and PL (Leklem, 1991).

Research with humans suggests that most (85 to 90%) of the vitamin B_6 in the body is located in muscle, mainly as PLP bound to glycogen phosphorylase (Krebs and Fischer, 1964; Coburn et al., 1988). In contrast, only 10% of liver vitamin B_6 is bound to glycogen phosphorylase. Muscle may be a storage site for vitamin B_6. Studies in animals and humans suggest that muscle B_6 is released during times of decreased caloric intake or increased gluconeogenesis, not as a direct response to the vitamin B_6 requirement (Black et al., 1978; Russell et al., 1985; Leklem, 1985).

Vitamin B_{12}

Vitamin B_{12} is the generic name for all corrinoids exhibiting the biological activity of cyanocobalamin. Cyanocobalamin is both widely available and most stable, so is the primary form of supplemental B_{12} used.

Cobalamins are primitive coenzymes that were in their prime when oxygen was scarce and have been declining in biological significance since that time (Bradbeer, 1982). Higher plants do not use cobalamins; this is fortunate as these coenzymes are light and oxygen sensitive; animals have retained only two cobalamin coenzymes. However, cobalamin-dependent reactions prevail in microbes, particularly those in dark, anaerobic environments.

Of all B-vitamins, Vitamin B_{12} is the largest and most complex molecule. Many different analogs and derivatives exist. Althought many of the naturally occurring analogs have biological activity for microorganisms, most analogs are inactive in animals. Analogs of B_{12} may interfere with (1) cobalamin uptake, (2) formation of coenzyme forms or (3) binding action of the coenzymes.

Coates and his colleagues at the National Institute for Research in Dairying in England initiated most of the early work with vitamin B_{12} analogs. They had found that the B_{12} in ruminal contents and feces was less active for chicks than had beem indicated by the mutant E. coli assay. This led to the identification of three compounds structurally similar to cyanocobalamin. Originally termed factors A,B, and C, they differed from cyanocobalamin in having 2-methyladenine, no base, and guanine, respectively, as the base of the nucleotide instead of 5,6-dimethylbenzimidazole.

In spite of ruminal production of vitamin B_{12} analogs, B12 deficiency in ruminants has not been described except when cobalt is deficient in the feed.

The coenzyme forms of vitamin B_{12} in animals are adenosylcobalamin and methylcobalamin. These plus hydroxocobalamin are the major cobalamins found in animal tissues. Only three B_{12}-dependent enzymes are known in mammalian systems. Methylmalonyl-CoA CoA-carbonyl mutase (E.C. 5.4.99.2) is crucial to the metabolism of propionate by animal tissue. It isomerizes R-methylmalonyl CoA (derived from propionate, and also from the degradation of isoleucine, branched chain fatty acids, and fatty acids with an odd number of carbons) to succinyl CoA. A cytoplasmic enzyme, 5-methyl-H_4-folate homocysteine methyltransferase is required for the biosynthesis of methionine from homocysteine. L-leucine-2,3-aminomutase is widely distributed in nature and important in leucine metabolism, although its functions in mammals have not been clarified.

B-Vitamins in Immune Response

The direct relationship of B-vitamin nutriture and immunocompetence has not been explored in ruminant animals. Therefore, we must rely on data from non-ruminant animals.

In non-ruminant animals, certain B-vitamin deficiencies increase susceptibility to bacterial infection. Specific changes in the metabolism of B-vitamins also can occur early in the development of chronic disease. Pyridoxine, pantothenic acid and folic acid are especially important B-

vitamins in the immune response. Body stores of all three, and especially pyridoxine, are readily depleted.

Pyridoxine status has a profound impact on the immune system due to its critical role as pyridoxal-5-phosphate. Pyridoxine deficiency reduces DNA synthesis; consequently, RNA (particularly mRNA) and protein synthesis are reduced. Because the immunologic response to an antigen requires synthesis of protein, RNA and DNA for cell transformation and replication, the immune response can be retarded or blocked when pyridoxine is deficient.

Pyridoxine deficiency affects lymphoid tissues, especially the thymus. Deficiency causes thymic involution, and lymphocyte depletion in the thymus, spleen and lymph nodes (Clark and Stoerk, 1956); peripheral blood lymphopenia often is observed (Gross and Newberne, 1976).

Antibody production is extremely sensitive to pyridoxine deficiency. Many investigators, using a variety of experimental species and antigens, have observed this impairment of the antibody response. Pyridoxine is required during both the primary and secondary phase of the antibody response to diphtheria toxoid in rats. Moreover, deficiency during the primary immunization phase caused an impairment of the secondary phase which could not be corrected with pyridoxine therapy (Pruzansky and Axelrod, 1955). In a study using the thymus-dependent antigen, sheep red blood cells (SRBC), splenic antibody-forming cells in rats were markedly reduced after primary immunization with SRBC (Kumar

and Axelrod, 1968). A combination of pyridoxine and pantothenate deficiencies virtually eliminated the antibody response (Hodges et al., 1962).

T-lymphocyte function and cell-mediated immunity also are impaired by pyridoxine deficiency, as evidenced by prolonged allograft survival, reduced mixed leukocyte reactivity, and poor delayed type hypersensitivity reactions (Axelrod et al., 1958; Robson and Schwartz, 1975).

Pyridoxine supplementation in excess of requirements can prove beneficial. In mice, high dietary pyridoxine increased oxygen radical production of both blood and spleen phagocytic cells, and increased B-lymphocyte proliferation response to polysaccharide (Gebhard et al., 1990). These workers suggested that tumor inhibition by high dietary pyridoxine may be mediated by T lymphocyte-dependent mechanisms, because it did not occur in athymic mice. High dietary pyridoxine also enhanced cell-mediated immunity measured by footpad swelling of mice; response was decreased in deficient mice. In vitro, pyridoxine supplementation of the culture medium increased stimulation of spleen cells by phytohaemagglutinin (Gridley et al., 1988).

Pantothenic Acid deficiency also can depress antibody production. Antibody responses to Salmonella pullorum antigen and human O+RBC were markedly depressed in pantothenate-deficient swine (Harmon et al., 1963). Both the primary and secondary antibody response to diphtheria toxoid was impaired by a pantothenate deficiency in rats

(Axelrod, 1971). In deficient rats, the primary antibody-forming cell response and primary hemolysin antibody response to SRBC were severely decreased. Also, primary splenic antibody-forming cells were reduced (Lederer et al., 1975). These workers found that the secretion of proteins into extracellular space was blocked by a pantothenic acid deficiency. T-lymphocyte and cell-mediated immunity, however, were not affected by pantothenate deficiency.

Folic Acid functions in the metabolism of nucleic acids, protein, amino acids and phospholipids. Because folic acid is critical to cell growth and reproduction, a deficiency affects host resistance and immune function. Folate-deficient experimental animals are more susceptible to bacterial infections (Chvapil, 1973; Chvapil, 1976). Folate deficiency impairs both T-lymphocyte and B-lymphocyte functions, depresses antibody production, and reduces delayed type hypersensitivity responses (Stinnett, 1983). Even a marginal folic acid deficiency may cause a measurable drop in mitogenic response, cytotoxic killing function, and T-cell-dependent B-cell function (Gross and Newberne, 1980).

Effects of Stress and Disease on B-vitamin Requirements

With the exception of vitamin B_{12}, B-vitamins are not warehoused in the body. They are depleted rapidly during periods of increased demand or reduced availability. B-vitamin flow at the duodenum increased as feed intake increased, mainly due to increased microbial synthesis (Zinn et al, 1987). Therefore, animals with reduced feed intake

are particularly at risk, even if ruminal metabolism is normal.

Little information is available on the influence of stress on B-vitamin requirements in livestock. In general, poultry and swine under field conditions will not thrive when fed only the minimum NRC recommended levels for vitamins; animals grown under unstressed research conditions may require less than animals produced commercially (McNaughton, 1990). Levels of B-vitamins used commercially often exceed the NRC requirements by up to ten times; this is an attempt to meet added needs due to high production, stress and subclinical disease. In support of an altered requirement, a summary of research with humans indicates that requirements for some B-vitamins are markedly increased above normal by even "moderate injury" (Mueller and Thomas, 1975). Folic acid and pantothenic acid requirements were increased 15 and 4-fold, respectively by moderate injury; the pyridoxine requirement was increased 20-fold with severe injury. Stress-induced immunosuppression in patients awaiting surgery was assessed by lymphocyte proliferation. Oral treatment with 600 mg thiamin disulfide, 1200 mg pyridoxine hydrochloride, and 1.2 mg cyanocobalamin weekly for 7 weeks prevented immunosuppression (Lettko and Meuer, 1990). In severe illness, much of the plasma pyridoxal 5'-phosphate (PLP) may be tightly bound and unavailable; free plasma PLP concentration alone has been used successfully to predict survival of seriously ill surgical patients.

Survival has been improved with pyridoxine supplementation (Enriquez et al., 1988; Keniston et al., 1990). Increased need for B_6, folic acid and pantothenic acid under stress conditions makes them of particular concern, especially considering their involvement in the immune response.

In cattle, stress is one of the major components in the etiology of the Bovine Respiratory Disease complex (BRD). The Bovine Respiratory Disease Complex is responsible for approximately 75% of morbidity and mortality in feedlot cattle (Jensen et al., 1976). This disease results in economic losses due to added veterinary costs and medications, as well as reduced rate and efficiency of gain, and death. The total cost to the cattle industry in the U.S. may exceed one-half billion dollars annually (McMillan, 1983).

BRD involves infection by any of ten bovine viruses (primarily bovine herpesvirus-1, parainfluenza-3, and bovine viral diarrhea) followed usually by infection with Pasteurella haemolytica (Rosenquist, 1984). As in other species including man, virus infections predispose cattle to bacterial infections (Babiuk, 1984; Jakab, 1984).

Numerous stressors during the shipping/marketing cycle predispose cattle to a high incidence of BRD during the first 3 weeks after arrival at the feedyard (Kelly and Janzen, 1986). Stress can increase disease susceptibility through several mechanisms. Adrenocorticotropic hormone is released from the anterior pituitary gland in response to

stress; it stimulates the adrenal cortex to increase the synthesis and excretion of cortisol (hydrocortisone). Cortisol and other glucocorticoids have been shown to suppress the immune response in cattle and other species (Roth, 19854; Golub and Gershwin, 1985). Shipping is a primary stressor; serum cortisol levels were increased for 4 to 7 days by trucking compared to less than 2 days by weaning (Von Tungeln, 1986; Crookshank et al., 1979). Cattle frequently are shipped long distances in the U.S.; in one survey, more than half of cattle in Texas feedyards had been shipped more than 1000 miles (McMillan, 1984).

Cortisol mediates immunosuppression through an interaction with glucocorticoid receptors on leukocytes (Roth, 1985). High plasma cortisol concentrations, by impairing neutrophil function, inhibit the first line of defense against infection. Macrophages and monocytes also are sensitive to glucocorticoids. Furthermore, concentrations of serum antibodies and various soluble immunoregulatory factors may be affected by glucocorticoids.

One role for vitamin B_6 is modulating the cortisol-induced effects of stress; vitamin B_6, by blocking cortisol as it blocks other steroid hormones, may modulate hormonal action. Steroid hormones affect expression of specific target genes through interaction with specific receptor proteins to form a complex. This complex localizes to the nucleus, associating there with specific DNA sequences to modulate expression of certain genes (Allgood et al., 1990).

Pyridoxal phosphate affects biochemical properties of steroid hormone receptors, and affects subcellular localization and DNA binding of these receptors. Animal studies indicate that translocation of steroid receptor complexes from the cytoplasm to the nucleus is enhanced by a vitamin B_6 deficiency and decreased by elevated vitamin concentrations (Holley et al., 1983; Symes et al., 1984; Bunce and Vessal, 1987).

Stress affects immunity in various ways in addition to cortisol. Transport and marketing indirectly affect disease susceptibility through nutrition. Immunity depends on cell proliferation; therefore, it is intensely sensitive to nutrient deficiencies or imbalance (Golub and Gershwin, 1985). Transport and marketing involve periods of feed and water deprivation, dietary changes, weight loss and dehydration. Other stresses such as transport exacerbate these problems (Von Tungeln, 1986). Extent of weight loss during transit (shrink) appears to be correlated with morbidity and mortality in feedlot cattle (Griffin, 1983).

Nutrition problems may continue during the first weeks after arrival. Digestive capacity is lost during shipping and marketing due to reduced ruminal volume, altered microbial populations, and impaired function (Damron et al., 1979; Hamlett et al., 1983; Koers et al., 1974). As a result, appetite and intake recover slowly.

Various studies have shown that disease resistance of receiving cattle can be influenced by management and

nutrition prior to shipping and during the critical 14 to 28 days post-arrival (Hutcheson and Cole, 1986). However, the role of individual nutrients, including B-vitamins is unclear; most studies have involved the comparison of feeds or feed combinations. Studies involving the addition of B-vitamin mixtures to the diet have had variable results (Cole et al., 1979; Lee et al., 1985; Zinn et al., 1987). Reasons include 1) the low B-vitamin levels fed, 2) ruminal degradation of dietary B-vitamin sources, and 3) failure to influence B-vitamin status of the animal. B-vitamin status in response to supplementation has not been measured. Considering these problems, the several reports that supplemental B-vitamins have reduced morbidity of receiving calves are encouraging.

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CHAPTER III

RUMEN CONTENTS AS A SOURCE OF B-VITAMINS FOR CHICKS

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ABSTRACT

Chick growth and survival were monitored as indices of the B-vitamins supplied by wet ruminal contents. Ten diets were formulated containing all, none, or all but one of each of eight B-vitamins. These diets were fed with and without addition of liquid ruminal contents to test the ability of the ruminal contents to meet the chick's requirement for each B-vitamin. Compared to requirements for chicks, ruminal contents of steers fed concentrate diets contained 37, 500, 160, 244, 309, 207, 72 and 15,300% of the chick's total needs for thiamin, riboflavin, vitamin B_6, niacin, pantothenic acid, folic acid, choline and vitamin B_{12}. Based on chick requirements, the concentration of B-vitamins in ruminal contents was low for thiamin and choline but adequate for all the other B-vitamins. Added ruminal contents increased gain and efficiency for each vitamin deficient diet as expected except for the vitamin B_{12} and pantothenate-deficient diets. Vitamin status of cattle fed high concentrate diets may be impaired by the antagonism of vitamin B_{12} analogs, the low availability of pantothenic acid, and the low concentrations of thiamin.

Introduction

Because dietary supplementation typically has failed to improve performance of ruminants, nutritionists have concluded that extensive microbial production of B-vitamins in the rumen exceeds ruminant requirements. Consequently, the many dietary factors affecting ruminal B-vitamin synthesis have not been well characterized. Further, the availability of B-vitamins in rumen contents has not been investigated, particularly for cattle fed high-concentrate diets.

Ruminal production and degradation of B-vitamins have been studied previously at Oklahoma State (Zinn et al., 1987). Duodenal flow of pantothenic acid and folic acid were less than 10% of estimated requirements. In contrast, supply of other B-vitamins (thiamin, riboflavin, niacin, pyridoxine and vitamin B_{12}) grossly exceeded estimated requirements at normal feed intake levels. Whether or not these vitamins were all present in a form available for animals, whether heat drying duodenal contents prior to analysis might have altered calculated supplies, and whether duodenal flow fully appraises ruminal B-vitamin yield, is not clear.

Generally, B-vitamin requirements of ruminants are higher when animals are young and growing rapidly. Mechanisms of absorption, post-absorptive transport, metabolism and excretion are thought to be similar in

ruminants and non-ruminants (Harmeyer and Kollenkirchen, 1989). We used chicks to assess the adequacy of available B-vitamins in ruminal contents. Chicks have been used as a bioassay for many nutrients, including many B-vitamins. Because of their rapid growth, chicks exhibit deficiencies rapidly. Using chicks, we attempted to identify which B-vitamins potentially might limit performance of rapidly-growing young ruminants fed a high-concentrate diet.

Materials and Methods

Preliminary Study. A preliminary study was conducted to test 1) the semi-purified diet formula and 2) the consumption of this diet mixed with ruminal contents. Dry matter intake, survival, and weight gain were measured for chicks fed semi-purified diets supplemented with all or none of the B-vitamins, and fed either dry alone or mixed with wet ruminal contents. Chicks readily consumed all four diets. Ten-day-old chicks thrived when fed for 2 weeks on a diet complete in B-vitamins, whether dry or mixed with ruminal contents. The B-vitamin deficient diet resulted in poor weight gain and feed efficiency, with substantial mortality occurring in chicks after one week on the diet. Addition of ruminal contents to the deficient diet improved performance and reduced mortality significantly ($P < .05$).

Chicks. One thousand one day old Cobb X Cobb (broiler) cockerels were purchased and raised in batteries on a standard corn-soybean meal diet until they were 9 days of

age. The vitamins used were provided at typical industry levels in a broiler premix manufactured by Hoffman LaRoche (Table 1). Each chick was wing banded for identifcation and 640 chicks were randomly allocated to 80 pens in 8 batteries in the OSU. Chick Lab, with 4 pens of 8 chicks fed each diet. The chicks were fed their test diets for 14 days from 10 to 25 days of age.

Diets. Ten semi-purified diets were formulated from corn starch, vitamin-free casein, minerals, vitamins and corn oil (Table 2). A cornstarch premix was prepared for each B-vitamin to provide the same supplemental B-vitamin levels as in the starter corn/soy diet when added at .33% of the diet (Table 2). Vitamin-deficient diets were prepared by replacing these premix(es) with cornstarch. The COMPLETE diet was supplemented with all B-vitamins; the DEVOID diet was devoid of all supplemental B-vitamins with the exception of biotin. The eight other diets were formulated to be deficient only in one B-vitamin (either thiamin, riboflavin, niacin, pyridoxine, pantothenic acid, folic acid, vitamin B_{12}, or choline). Each diet was fed in two forms, 1) dry or without added ruminal contents (RC-) and 2) mixed with wet ruminal contents (RC+) at a rate of about 55 g wet ruminal contents to 45 g dry semipurified diet.

Ruminal contents were obtained from 5 mature 600 kg. rumen-cannulated beef cattle adapted to a high concentrate diet. Each animal was fed 7 kg. concentrate plus .9 kg prairie hay daily in 2 feedings, and had access to salt and

Table 1. Composition of starter diet

Ingredient	Amount (%)
Corn, ground	48.00
Soybean meal 49%	41.90
Fat, animal & vegetable	6.00
Dicalcium phosphate	2.00
Limestone	1.21
Salt	.25
DL-methionine 99%	.30
Vitamin premix[a]	.25
Trace mineral premix	.10

[a]Vitamin premix provided per kg feed: 10,000 IU vitamin A, 2,500 IU vitamin D_3, 50 IU vitamin E, 2 mg menadione, 2.0 ppm thiamin, 6.6 ppm riboflavin, 33 ppm niacin, 3.96 ppm pyridoxine, 11 ppm pantothenic acid, 1.1 ppm folic acid, .019 ppm B12, and .35 ppm biotin.

Table 2. Composition of complete diet

Ingredient	Amount (grams)
Corn starch	54.35
Vitamin-free casein	26.40
Glista salts[a]	5.37
Corn oil	5.00
Solka-floc (cellulose)	3.00
Citric acid[b]	1.00
Arginine	1.20
DL-methionine	0.40
Glycine	0.20
Choline bitartrate	0.31
Vitamin ADEK premix[c]	0.10
Thiamine hydrochloride premix[d]	0.33
Riboflavin premix[d]	0.33
Niacinamide premix[d]	0.33
Pyridoxine hydrochloride premix[d]	0.33
Calcium D-pantothenate premix[d]	0.33
Folic acid premix[d]	0.33
Vitamin B_{12} premix[d]	0.33
Biotin premix[e]	0.33

[a]Glista salts (TD#88178, Teklad Premier Laboratory Diets, Madison, WI) provided dietary levels of 1.16% Ca, .64% P, .404% K, .35% Na, 600 ppm Mg, 212 ppm Mn, .1 ppm Se, 56 ppm Zn, 10 ppm Cu, 5 ppm I, 84 ppm Fe, 5350 ppm Cl, 0.058% S, and .2 ppm Co.

[b]Citric acid (anhydrous) was included at 1% of diet to prevent microbial growth.

[c]Vitamin ADEK premix (Teklad) provided 10,000 IU vitamin A, 2,500 IU vitamin D_3, 50 IU vitamin E and 2 mg menadione per kg.

[d]In the complete diet, added B-vitamins were supplied at 2.0 ppm thiamin, 6.6 ppm riboflavin, 33 ppm niacin, 3.96 ppm pyridoxine, 11 ppm pantothenic acid, 1.1 ppm folic acid, and .019 ppm B_{12}. In vitamin deficient diets were made by replacing the vitamin premix(es) with an equivalent amount of corn starch.

[e]Biotin was present in all diets at .35 ppm.

water. Ruminal contents (approximately 50 liters) were sampled from 2 of the 5 animals each day; the same animal was not used on 2 successive days.

Froth was skimmed off the rumen contents; then, the fluid was squeezed through 2 layers of cheesecloth. The solids caught by the cheesecloth were ground in a Waring blender, and remixed with the remaining fluid. Ruminal contents were sampled and analyzed for dry matter; samples were frozen, lyophilized and composited for B-vitamin analysis. B-vitamin concentrations in ruminal contents were analyzed (Woodson-Tenent Laboratories, Inc., Memphis, TN).

Preparation and analysis of diets. Each diet containing ruminal contents (RC+) was mixed fresh daily. Each diet was sampled in duplicate daily and analyzed for dry matter. On the average, RC+ diets contained (wet basis) 42.75% diet and 57.25% ruminal contents. The RC+ diets had an average dry matter concentration of 46.4%. Based on the DM percentage in ruminal contents (10%), only 12.4% of the diet DM was supplied by ruminal contents.

Data Collection. The chicks were weighed at 10, 17 and 24 days of age (d0, d7 and d14 of the study) after an overnight fast. Mortality was recorded daily. Daily dry matter intake per pen was calculated from initial and final feed weights, and duplicate dry matter analyses of orts.

Results and Discussion

B-vitamin concentrations in ruminal contents, the chick B-vitamin requirement, and the percentage of the chick

requirements supplied by ruminal contents are shown in Table 3. Nutrient requirements are highest for young animals gaining at a relatively high percentage of their body weight. Per unit of dry matter, requirements for many nutrients such as B-vitamins therefore decline as animals mature because requirements for maintenance are lower than requirements for weight gain or production. B-vitamin requirements per unit of metabolic weight should be somewhat lower for cattle, depending on the stage of growth, than for the 10-day-old chick. However, assuming adequate bioavailability, comparison of ruminal B-vitamin concentrations with chick requirements on a metabolic basis (Table 3) indicates that supply of all B-vitamins in pure ruminal fluid DM (except for thiamin) is in excess. Other researchers have occasionally observed very low concentrations of thiamin in ruminal contents despite the absence of clinical deficiency signs (Hunt et al., 1943; Phillipson and Reid, 1957; Hayes et al., 1966). These observations suggest that subclinical deficiencies of thiamin may depress performance without inevitably leading to polioencephalomalacia.

Average daily gain (ADG), dry matter intake (DMI), feed efficiency (G/F) and survival of the chicks fed various diets are presented in Table 4. Despite the objectionable odor of ruminal contents, adding ruminal contents to the vitamin-free diets increased ($P < .05$) DMI with six of the eight single vitamin-deficient diets, as well as with the COMPLETE and DEVOID diets. Addition of ruminal contents

Table 3. B-vitamin composition of ruminal contents compared to chick requirements

Vitamin	Ruminal supply concentration ruminal contents in RC + diets ppm	Chick requirement[a] ppm	Fraction of requirement supplied by rumen contents in RC + diets
Thiamin	.04	1.8	4.6
Riboflavin	2.25	3.6	62.0
Pyridoxine	.06	3.0	19.8
Niacin	8.2	27.0	30.3
Pantothenate	3.8	10.0	38.6
Folic Acid	.14	.55	25.7
B_{12}	.17	.009	1900
Choline	114	1300	8.9

[a]Requirements for chicks 0 to3 weeks of age (NRC, 1984).

Table 4. Chick average daily gain, dry matter intake, gain/feed and survival

Vitamin diet[a]	Ruminal fluid	ADG g/d	DM Intake g/d	Gain/ feed	Survival, % to end
Complete	+	27.0[b]	44.5[b]	.607[b]	100
Complete	-	20.5[c]	31.1[c]	.659[c]	97
Folic acid	+	25.4[b]	42.2[b]	.601[b]	97
Folic acid	-	19.1[c]	28.4[c]	.672[c]	97
Niacin	+	24.5[b]	42.3[b]	.578[b]	100
Niacin	-	16.9[b]	27.0[b]	.624[b]	95
Choline	+	15.1[b]	27.6[b]	.549[b]	100
Choline	-	16.7[b]	25.5[b]	.654[b]	97
Riboflavin	+	23.2[b]	39.6[b]	.586[b]	97
Riboflavin	-	7.1[3c]	17.5[c]	.407[c]	100
Pyridoxine	+	19.8[b]	34.0[b]	.582[b]	87
Pyridoxine	-	7.24[c]	15.4[c]	.469[c]	93
Thiamin	+	15.1[b]	30.7[b]	.491[b]	97[a]
Thiamin	-	2.94[c]	11.1[c]	.257[c]	20[b]
B_{12}	+	0.12[b]	18.1	.004[b]	100
B_{12}	-	.38[c]	14.7	.093[c]	100
Pantothenate	+	-1.30[b]	15.2[b]	-.086	96
Pantothenate	-	-2.01[c]	9.67[c]	-.209	94
Devoid	+	10.5[b]	23.4[b]	.448	100[a]
Devoid	-	3.30[c]	9.37[c]	.341	13[b]

[a]The vitamin treatment describes the B-vitamin that was omitted from the diet; + indicates that ruminal contents were mixed with the diet whereas - indicates the diet had no ruminal contents added.

[b,c]Means within a vitamin without a common superscript differ.

increased dry matter intake by 48%, 56%, 57%, 120%, 127%, 150% and 178% for the folic acid, niacin, pantothenic acid, pyridoxine (vitamin B_6), riboflavin, thiamin and the B-vitamin devoid (DEVOID) diets, respectively. Chick DMI often is depressed when vitamins are deficient; therefore some of the increase in feed intake by chicks fed the RC+ vitamin-deficient diets probably was due to provision of the limiting B-vitamin(s) by the ruminal contents. However, dry matter intake also was increased by a mean of 43% for the RC+ COMPLETE diet compared to the dry (RC-) COMPLETE diet, indicating that other characteristics of the diets with ruminal fluid added increased intake even though dietary vitamin concentrations were adequate.

For six of the eight vitamin-deficient diets tested, addition of ruminal contents numerically increased ADG or decreased weight loss. The improvement was significant for folic acid, riboflavin, pyridoxine, thiamin, pantothenic acid, and DEVOID diets. Therefore, ruminal contents presumably provided substantial amounts of these B-vitamins for growth in addition to stimulating dry matter intake. Alternatively, ruminal contents may have provided other nutrients which spared deficient vitamins or delayed the onset of B-vitamin deficiencies.

The diet devoid of all B-vitamins (DEVOID) resulted in lower dry matter intake, ADG and survival compared to the diet supplemented with all B-vitamins (COMPLETE) confirming that the DEVOID diet was deficient in at least some of the

B-vitamins tested. With chicks fed the DEVOID diet, mortality was first observed on day 8 and totalled 87.5% at the end of the trial. The most prevalent deficiency signs were similar to those for thiamin-deficient birds, specifically opisthotonus. Ruminal contents increased dry matter intake, ADG, G/F and survival for chicks fed the DEVOID diet, but failed to restore growth to that of the COMPLETE diet.

Performance of chicks fed the RC- folic acid, niacin and choline-deficient diets was almost equal to that of chicks fed the RC- COMPLETE diet. For choline, a deficiency may be slow to develop or slow to affect growth. The severity of choline deficiency lesions is markedly affected by other dietary factors such as methionine, vitamin B_{12}, and folic acid, the nature of dietary fat and protein, and the choline source (Chan, 1991). In birds, choline deficiency retards growth and induces perosis. Leg problems (perosis, short and thick legs, and thickened, red hocks) occurred in some birds on both RC+ and RC- choline- diets, but a choline deficiency had little effect on ADG or dry matter intake.

Responses to addition of ruminal contents by chicks fed riboflavin and pyridoxine deficient diets were similar. ADG, DMI, and G/F were reduced on the RC- diets; both responded to the addition of ruminal contents. Performance on both RC+ diets was at least as good as on the RC- COMPLETE diet. Leg problems, including curled toes and inability to stand, were common in the RC- riboflavin-

birds, and probably reduced intake. Feathering was poor in the RC- pyridoxine- birds. Survival was not affected with either RC- diet, although dry matter intake was greatly reduced in the final few days of the experiment.

Thiamin deficiency severely reduced performance even though the chicks gained weight during the first week. Dry matter intake increased for the first 4 days, then dropped to 5 grams or less from day 8 onward. Mortality began to occur on day 8. Birds were very weak and exhibited opisthotonus, a classic thiamin-deficiency sign also observed in thiamin-deficient cattle. Added ruminal contents improved ($P < .05$) ADG, DMI, G/F and survival. With ruminal contents, dry matter intake increased from days 1 to 6 of the study, and decreased from days 8 to 14, indicating that ruminal contents only partially met the chick's thiamin requirements. This might be expected because ruminal contents provided only 4.6% of estimated thiamin requirements (Table 3); nevertheless, it delayed the onset of deficiency symptoms. Polioencephalomalacia, a thiamin deficiency disease which can occur in feedlot cattle, has been associated with ruminal thiamin destruction or production of antimetabolites (Brent and Bartley, 1984; Harmeyer and Kollenkirchen, 1989). In this study, the low ruminal concentrations measured may have been related either to marginal synthesis or ruminal degradation. However, the positive chick response to ruminal contents indicates that antimetabolites were not a major problem. Chicks previously

have been used to assess thiamin concentration of ruminal contents, resulting in analyzed values of 2 to 12 ppm for ruminal dry matter (McElroy and Goss, 1939; Wegner et al, 1940, 1941); those are much higher than the thiamin concentration in our study (.67 ppm).

Performance was very poor for birds fed both the B_{12} and pantothenic acid deficient diets; these birds were lively and appeared to be healthy. Feed intake was very low, highly variable, and virtually ceased within a week. Ruminal contents added to the B_{12}-deficient diet did not significantly improve intake, in fact, added ruminal contents decreased ($P < .05$) ADG and G/F. Depressed ADG and G/F for RC+ compared to RC- suggest that ruminal contents exacerbated the B_{12} deficiency, even though survival was not depressed. Survival on both B_{12}-deficient diets was high; compared to other B-vitamins, B_{12} is retained tenaciously in the body. Also, reduced intake may be a mechanism to enhance survival.

According to microbial analysis, ruminal contents from our steers contained 1.38 ppm B_{12}; therefore, ruminal contents should have provided 1900% of requirements for B_{12} in the RC+ diet. However, rumen contents may contain several B_{12} analogs, especially in cattle fed high-grain diets (Sutton and Elliot, 1972). Ruminal production of B_{12} analogs, e.g. pseudovitamin B_{12}, Factor A, and Factor B, can exceed ruminal B_{12} synthesis (Bigger et al., 1976). Natural vitamin B_{12} analogs exhibit little or no activity in animals

even though they are active in microbial systems (Elliot, 1980). Consequently, they may be incorrectly identified as B_{12} by the microbial B_{12} assay. Whether B_{12} analogs are detrimental to ruminants is not known.

Birds fed pantothenate-deficient diets, with or without ruminal contents, had low dry matter intakes and lost weight although survival was quite high. Added ruminal contents increased dry matter intake and G/F, and decreased weight loss. However, even though ruminal contents contributed 38.6% of pantothenic acid requirements, dry matter intake, ADG and feed efficiency were much poorer for both RC+ and RC- diets than for any other diets, even though mortality was considerably higher in the RC-thiamin- and RC-DEVOID diets. The RC+ pantothenate-deficient diet was the only RC+ diet that resulted in weight loss, perhaps due to the B_{12} analogs in ruminal contents, as B_{12} supply may be important in pantothenic acid deficiency. B_{12} has a sparing action on pantothenic acid requirements in poultry (Yacowitz, 1951). Other nutritional interactions also may be important. Pantothenic acid requirements are influenced by dietary concentrations of biotin, ascorbic acid, folic acid, fat, protein and copper (Fox, 1991).

Another possible reason for the inability of pantothenic acid from ruminal contents to better meet chick requirements could be due to destruction of pantothenic acid during and after mixing ruminal contents with the dry feed. Pantothenic acid is one of the least stable B-vitamins. It

must be stabilized for use as a feed additive by conversion into the calcium salt. Finlayson and Seeley (1983) measured "total", Coenzyme A-bound pantothenate, and free pantothenic acid in ruminal contents, using a Lactobacillus plantarum assay. Total pantothenic acid flow to the duodenum ranged from 11.4 to 39.6 mg/day for 50 kg sheep fed 9 diets. Most of the pantothenate (9.6 to 34.5 mg/day) was in the form of Coenzyme A; the coenzyme should be more stable than free pantothenic acid. However, pH and heat are known to affect stability of ruminal pantothenate. Wegner et al. (1943) used a microbiological assay to analyze pantothenic acid in ruminal contents from a heifer. When a sample was split and dried at 40 to 50^{o}C without acid or after adjusting pH to 4.7, the original sample assayed at 24.4 ppm compared to only 8.0 ppm for the acid-treated sample. Because the chick diet was slightly acidic due to the inclusion of 1% citric acid, this may have increased destruction of pantothenic acid. Further investigation is needed to determine if the growth-depressing effect of ruminal fluid as a pantothenic acid source was due to low availability or degradation of the vitamin after mixing with the dry diet.

Conclusions

A 14-day chick bioassay was used in which chicks were fed vitamin-deficient diets in order to assess the availability/supply of ruminal B-vitamins. The test was insensitive for folic acid, niacin and choline; dry matter intake, ADG and feed efficiency were not depressed by these

deficiencies under the conditions of this study. Single deficiencies of thiamin, riboflavin, vitamin B_6 and pantothenic acid reduced dry matter intake, ADG, and feed efficiency. Addition of ruminal contents increased ADG or dry matter intake for chicks fed these vitamin deficient diets. According to microbial analysis, ruminal contents provided 62.0%, 19.8%, 4.6% and 38.6% of the chick requirements for riboflavin, pyridoxine, thiamin and pantothenic acid. However, growth response to pantothenate in ruminal contents was very low.

In contrast, no benefit from added ruminal contents was evident with the B_{12}-deficient diet. By microbial assay, ruminal contents provided 1900% of the chick's requirements for this vitamin. True B_{12} may have been only a small fraction of the analyzed B_{12} content. Depression of growth from addition of rumen contents to the B_{12} deficient diet suggests that B_{12} adequacy may be marginal for cattle fed concentrate diets due to low availability or presence of analogs. Also, the low thiamin concentration in ruminal contents indicates that the supply of thiamin may be inadequate for rapid growth.

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CHAPTER IV

BLOOD PLASMA LEVELS OF B-VITAMINS IN DIFFERENT CLASSES OF CATTLE

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ABSTRACT

Blood plasma samples from suckling beef calves, dairy cows, feedlot cattle, and stressed weaned calves were analyzed for vitamin C, folic acid, pantothenic acid, and vitamin B_{12}. The B_6 vitamers also were analyzed in blood plasma samples from one group of stressed calves. Differences in blood plasma vitamin levels were detected between groups. Plasma vitamin C was highest in suckling calves, but similar for dairy cows, feedlot cattle and stressed calves. The lowest vitamin C concentrations were found in stressed calves and dairy cows; these would be considered deficient based on standards for humans. Plasma folic acid levels were highest in feedlot cattle and lowest in suckling calves. Vitamin B_{12} plasma levels were similar to levels in humans, except for feedlot cattle, some of which would be considered deficient based on guidelines for humans. Low B_{12} in plasma of feedlot cattle reflects the high ruminal production of B_{12} analogs when cattle are fed high concentrate diets. In the radioassay for B_{12} we used, purified intrinsic factor served as the binding protein; therefore, we measured only

B_{12}. Many previous assays have included inactive B_{12} analogs together with B_{12}, resulting in higher plasma levels. Using a highly sensitive technique for measurement of blood plasma pantothenic acid, concentrations were found to be markedly lower than in plasma from humans or rats. Plasma pantothenate was higher ($P < .05$) in feedlot cattle and suckling calves than in stressed calves and dairy cows. Pyridoxal phosphate and total B_6 concentrations in stressed calves were extremely low compared to values published for a limited number of healthy calves.

(Key Words: B-Vitamins, Stress, Vitamin C, Folic, Pantothenic, B_{12}.)

Introduction

Microbial synthesis of B-vitamins generally has been considered sufficient to meet requirements for cattle. However, production responses to various supplemental dietary B-vitamins have been demonstrated in several classes of cattle. For example, niacin supplementation of dairy cows in early lactation has alleviated ketosis (Fronk and Schultz, 1979) and increased milk production (Muller et al., 1986). Niacin has improved performance of cattle fed some growing and finishing diets (Byers, 1981). Thiamin is used therapeutically to treat polioencephalomalacia in cattle (Thomas, 1986), and also frequently is added in combination with niacin to diets for starting calves. Stressed, sick or anorexic calves often are injected with commercial B-vitamin mixtures, though the popularity of this practice has

declined recently due to the current focus on injection-site lesions. Formulas for injectable B-vitamin complexes are not based on any research findings.

In the absence of data on blood or tissue B-vitamin levels, it is difficult to determine whether responses to dietary supplementation can be ascribed to changes in post-ruminal vitamin flow or alterations in ruminal fermentation. Assessment of blood B-vitamin levels should allow one to evaluate B-vitamin status and, hence, the effectiveness of supplementation strategies. Data establishing normal plasma levels of B-vitamins in cattle are needed in order to detect responses to an increase in the supply and to determine when cattle might benefit from supplementation.

In this study, blood plasma samples were obtained from dairy cows, feedlot cattle, suckling beef calves, and three truck-loads of tranport-stressed calves. We measured plasma concentrations of B-vitamins in these different classes of cattle, focussing on B-vitamins critical to the immune response of stressed cattle, i.e., vitamin B_6, folic acid, vitamin C and pantothenic acid. Vitamin B_{12} also was measured. Our objective was to develop baseline and comparative data on the mean and the range of blood plasma concentrations of these B-vitamins in specific classes of cattle. These may prove useful for evaluating B-vitamin status by 1) comparison with the nonruminant literature and 2) comparison with other classes of cattle.

Materials and Methods

Animals. Suckling calves from 2 crossbred beef cattle herds on native range, two groups of high-producing dairy cows, feedlot cattle, and three groups of stressed calves were sampled. Numbers and description are provided in Table 5.

Blood Samples. Blood samples were collected by jugular venipuncture in 10 ml evacuated glass tubes (Vacutainer, Sherwood Medical, St. Louis, MO). For each animal sampled, three tubes were collected with potassium EDTA as an anticoagulant and two tubes with sodium heparin as an anticoagulant.

Vitamin Assays. Multiple samples of plasma were frozen at -20^{o}C in appropriate aliquots for subsequent analysis of the water soluble vitamins. Samples were processed under subdued lighting to minimize photodegradation; this is a problem primarily with the B_6 vitamers and vitamin C. Heparinized plasma for vitamin C analysis was added 1:1 to 10% (w/v) metaphosphoric acid prepared immediately before use; dilution was lower than the 1:5 with 6% metaphosphoric acid that is used for human plasma due to lower concentrations of vitamin C in livestock plasma. These samples were vortexed and frozen immediately.

Vitamin C in heparinized plasma was measured colorimetrically using derivitization with 2,4-dinitrophenylhydrazine (McCormick, 1986). Ascorbic acid was oxidized by Cu(II) to form dehydroascorbic acid; this reacts with acidic 2,4-dinitrophenylhydrazine to form a red bis-

Table 5. Description of cattle used in B-vitamin survey

Class	Number	Description
Suckling beef calves	22	Fallborn British x Limousin steer calves, 6-8 months, 225 kg, dormant native range, 4/8/91
	13	Fall born Angus/Hereford steer calves, 6-8 months, 190 kg, separated from dams 20 h before sampling, dormant winter range, 3/27/91
Dairy cows	32	OSU Holsteins, average 7-day production 36 kg
	14	OSU Holsteins, average 7-day production 36.7 kg, (range 28.1 - 52.0)
Feedlot cattle	16	Mixed crossbred steers, 475 kg, corn-based finishing diet
Stressed calves	15	Mixed crossbred heifers, Arkansas, 218 kg
	20	Mixed crossbred heifers, Missouri, 186 kg
	10	Mixed crossbred heifers, Georgia, 195 kg

hydrazone which was measured at 520 μM. In order to minimize reaction of the 2,4-dinitrophenylhydrazine with aldehydes or ketones in the sample (e.g., glucose, pyruvate) a 40 hour incubation period at 15 to 18 ^{o}C was used instead of the more usual 3 to 4 h at 37 ^{o}C; at low temperatures coupling is much faster with dehydroascorbic acid than with other compounds. This also allows more complete reaction of the dehydroascorbic acid (E. Norkus, Our Lady of Mercy Medical Center, Bronx, Personal Communication, 1991). Using thiourea increases the specificity (Strohecker and Henning, 1965; Roe, 1967), and incubating for 1/2 hour after addition of the H_2SO_4 also helps destroy non-specific binding (Pelletier, 1985).

Folate and vitamin B_{12} plasma concentrations were measured in K_2EDTA plasma using a radioassay method for human samples (Quantaphase Folate and Vitamin B_{12} Radioassay, Bio-Rad Clinical Division, Hercules, California); this had been validated for folate quantitation in bovine blood samples by Girard et al. (1989).

Pantothenic acid in heparinized plasma was measured using a sensitive indirect ELISA assay (Song et al., 1990) at Dr. Song's laboratory at Michigan State University. The antibody is not available commercially; it is prepared in rabbits by immunization with pantothenic acid conjugated to bovine serum albumin. Each sample was assayed by two technicians, and results were averaged.

The B_6 vitamers were analyzed in heparinized plasma using a cation-exchange HPLC procedure (Coburn and Mahuren, 1983) at Dr. Coburn's laboratory in the Fort Wayne State Development Center. Plasma, treated with trichloroacetic acid to remove protein and free protein-bound forms, was extracted with redistilled ethyl ether. The aqueous portion, filtered through a .45 μm membrane, was mixed just prior to injection with an internal standard (2-amino-5-chlorobenzoic acid). Post-column injection of a phosphate buffer containing bisulfite permitted all seven major forms of vitamin B_6 to be detected with good sensitivity at a single wavelength (Coburn and Mahuren, 1983).

An ion-exchange HPLC method for simultaneous detection of thiamin, riboflavin and the B6 vitamers previously used for human blood plasma was evaluated for use with bovine plasma samples (Bötticher and Bötticher, 1987). This procedure was not useful as the very low thiamin and riboflavin concentrations in plasma could not be detected with UV detection. Also, the procedure involved use of the multi-enzyme mixture Clara Diastase to release protein and degrade the phosphate ester linkage; the enzyme is difficult to obtain, frequently contaminated with B-vitamins of interest, and produced an extremely high background when incubated with bovine plasma. However, vitamin B_6 could be detected when the procedure was modified using potato acid phosphatase instead of Clara Diastase. The C18 solid phase extraction columns in this technique was very useful in

sample cleanup, and may improve sample cleanup for some B-vitamins in other HPLC techniques. However, the method provides less information for the B_6 vitamers than other procedures (Coburn and Mahuren, 1983) which allow separate detection of each B_6 vitamer.

Statistical Analysis. Plasma vitamin data were analyzed by analysis of variance models testing the effect of animal class. Means were separated using Duncan's Multiple Range Test.

Results

Blood plasma vitamin concentrations are shown in Table 6 and Table 7. Plasma ascorbic acid, folic acid, vitamin B_{12} and pantothenic acid concentrations were affected by class of cattle ($P < .001$).

Plasma ascorbic acid was higher ($P < .05$) in suckling calves than in feedlot cattle, stressed weaned calves, and dairy cows (1.04 vs .49, .44, and .42 mg/dL; $P < .05$).

Plasma folate averaged 26.0, 13.1, 9.7 and 6.8 ng/ml in feedlot cattle, dairy cattle, stressed calves and suckling calves, respectively.

Vitamin B_{12} concentrations in plasma were similar in suckling calves, stressed calves and dairy cows (229, 251, and 286 pg/ml respectively). However, plasma from feedlot cattle was lower in vitamin B_{12} (160 pg/ml; $P < .05$).

Plasma pantothenic acid concentrations were similar ($P > .05$) in feedlot cattle and suckling calves, averaging 0.143 and 0.128 nm/mL, respectively. Concentrations were

Table 6. Plasma vitamin levels in cattle

		Class of Cattle				
Vitamin	Item	Beef Calf	Stressed Calf	Feedlot	Dairy cows	SE
Vitamin C	n	35	41	16	44	
(mg/dL)	mean	1.04[a]	.44[b]	.49[b]	.42[b]	.09
	range	.54-2.12	.22-.76	.34-.83	.13-.76	
Folic Acid	n	35	13	14	31	
(ng/mL)	mean	6.8[d]	9.7[c]	26.0[a]	13.1[b]	1.7
	range	3.2-11.0	6.4-14.6	14.2-35.2	7.1-25.4	
Vitamin B_{12}	n	34	13	14	17	
(pg/mL)	mean	229[a]	251[a]	160[b]	286[a]	35
	range	113-277	116-396	97-297	150-396	
Pantothenic Acid	n	31	25	16	14	
(nm/mL)	mean	.128[a]	.095[b]	.143[a]	.089[b]	.019
	range	.039-.195	.027-.167	.062-.201	.023-.186	

[a,b,c,d]Means in the same row with different superscript differ ($P < .05$).

Table 7. Plasma B_6 vitamers in stressed calves

	Vitamer (nM)				
Animal	PLP	PL	4PA	PN	Total B_6
1	101	9	35	ND	145
2	46	ND	43	ND	89
3	95	ND	27	ND	122
4	64	ND	39	ND	103
5	74	6	33	ND	113
6	147	ND	31	ND	178
7	148	8	31	ND	187
8	102	ND	28	ND	130
9	222	47	37	62	368
Mean[a]	97	2.9	33.4	0	133
SE[a]	37	4.1	5.4	0	35

[a]Includes data from animals 1 to 8, omitting 9, which may have been recently injected with pyridoxine.

similar ($P > .05$) for stressed calves and dairy cows, (0.095 and 0.089 nm/mL respectively), but were lower than values for feedlot cattle and suckling calves ($P < .05$).

Plasma pyridoxal phosphate (PLP) averaged 97 nM in a group of eight stressed calves (Table 7). Pyridoxal (PL) averaged 2.9 nM in these calves. The B6 metabolite, 4-pyridoxic acid (4PA), averaged 33.4 nM. No pyridoxine (PN) was detected in plasma from these calves. Data from animal 9 was not used to calculate means and standard errors for the B6 vitamers, because high PLP, total B6, and presence of pyridoxine in its serum suggest it may have been injected with pyridoxine. Plasma from animal 9 contained 62 nM pyridoxine. Total B6 averaged 133 nM in the eight stressed calves when 4PA was included. However, some researchers do not include 4PA in the total plasma B6, because it is not available.

Discussion

Ascorbic Acid. Numerous methods can be used to measure either total ascorbic acid, or ascorbic acid, dehydroascorbic acid, and 2,3-diketogulonic acid. Common methods include chemical, electrochemical, and chromatographic (ion-exchange, ion-pair, and partition) procedures.

Ascorbic acid is easily and reversibly oxidized to dehydroascorbic acid, a compound which is less stable but still physiologically active. Dehydroascorbic acid is irreversibly oxidized to 2,3-diketogulonic acid; this acid

has no ascorbic acid activity and can be further cleaved or excreted intact. Ascorbic acid in serum is rapidly degraded at room temperature, but it can be stored for 3 weeks at -70 ^{o}C without a preservative, or at -10 to -15 ^{o}C when mixed with a metaphosphoric acid solution (Pelletier, 1985). In animals, ascorbic acid normally occurs entirely or nearly entirely in its reduced form; animal tissues contain systems to reduce dehydroascorbic acid to ascorbic acid. Therefore, it is assumed that values obtained by the dinitrophenyl-hydrazine method are entirely ascorbic acid (Roe, 1953).

Whole blood, serum, and plasma concentrations are used as biochemical indicators of ascorbic acid status. Human blood or plasma levels reflect recent intake, whereas peripheral blood leukocyte levels reflect tissue reserves. In humans, plasma ascorbic acid levels normally range from .8 to 1.4 mg/dL (Jaffe, 1984). Plasma levels lower then .3 mg/dL are considered deficient (Sauberlich, 1984).

Diet is not an important source of ascorbic acid for ruminants. Dietary ascorbic acid is rapidly degraded in the rumen. Cows' milk contains low levels of ascorbic acid, e.g., 1 to 2 mg/100 grams; human babies develop scurvy when fed solely cows' milk because it contains less ascorbic acid than human breast milk (Moser and Bendich, 1991). However, a beef calf consuming 5 kg milk/day would receive 50 to 100 mg ascorbic acid daily; milk could contribute substantially to its ascorbic acid balance because milk can bypass the rumen. Cattle, unlike humans, possess a key enzyme for

ascorbic acid synthesis, L-gulonolactone oxidase (GLO, EC 1.1.3.8) in their liver.

The reason for the higher ascorbic acid concentration in suckling calves than other classes of cattle is not clear. It may reflect higher blood glucose levels in these animals. Compared to other classes of cattle, calves are more similar to nonruminants. Plasma concentrations in suckling calves ranged from .54 to 2.12 mg/dL; compared to normal concentrations in humans, none of these calves would be considered deficient in ascorbic acid.

Ascorbic acid may be released from the liver and adrenals during stress, including procedures such as blood sampling; therefore, cattle unaccustomed to handling temporarily may have elevated blood ascorbic acid levels due to sampling. However, no clear correlation between plasma ascorbic acid and ease of sampling either within a class of cattle or between classes was evident. The group of 22 calves that were handled most easily had higher ascorbic acid levels than calves that had been separated from their dams for 20 hours and were very difficult to sample (Table 5).

Mean ascorbic acid levels were similar in the stressed calves, feedlot cattle and dairy cows, but all these classes had lower ascorbic acid concentrations than suckling calves and normal humans. Of the stressed calves and high-producing dairy cows, 20 and 14 percent would be considered deficient based on human standards. If a plasma ascorbate

concentration <.40 mg/dL is considered to indicate a marginal deficiency, another 27% of stressed calves and 30% of lactating dairy cows are also at risk. Both dairy cows in early lactation and stressed calves should have been in negative energy balance for some period of time prior to sampling. Possibly the low plasma levels in some animals in these two classes could reflect a negative energy balance because D-glucose is the initial precursor for ascorbic acid. Alternatively, lower levels may reflect increased requirements in these animals due to production or physical stress.

Folic acid, the common name for the commercial form of the vitamin, does not exist alone in nature. Folic acid (PteGlu) is composed of a pteridine linked through a methylene group at the C-6 position to a p-aminobenzoyl glutamate group. The naturally-occurring folates include over 100 structurally related compounds; they all are pteroyloligo-γ-L-glutamates ($PteGlu_n$), where n indicates a chain of one to nine glutamates.

Serum and plasma folate levels have been used extensively to assess folate status in humans. Conveniently, serum contains only one major folate, 5-Methyl-H_4PteGlu, the transport form of the vitamin; therefore concentrations reflect recent dietary intake in humans. In contrast, red cell folate reflects body stores at the time the red cell was formed; thereby, erythrocyte folate may be a more accurate, less variable index of folate status in humans

(Brody, 1991). Folate levels are much higher in red cells; hemolysis must be avoided if plasma or serum is analyzed. Although much of the serum folate is nonspecifically bound, it can be analyzed without extraction, indicating that the pteridine moiety is sterically available to participate in other reactions (Rothenberg et al., 1975).

The radioassay method used in this study is one of many techniques available which utilize a natural specific binder of folate which occurs in milk. Radioassays that use competitive protein-binding are simpler than microbiological assaus and are not affected by antibiotics. Serum folate levels may vary depending on the assay method or radioassay kit used; therefore, normal levels need to be established for each laboratory.

In humans, serum folate levels of less than 3.0 ng/mL are considered deficient (high risk), levels between 3.0 and 5.9 ng/dL are low (medium risk), and levels > 6.0 are considered acceptable. Folate and B_{12} frequently are measured simultaneously in human medicine. In a primary vitamin B_{12} deficiency, serum folate can be elevated while red cell levels are depressed. In a folic acid deficiency, both serum and red cell folate levels are depressed (Sauberlich, 1975).

Ruminal bypass of dietary folate appeared negligible according to Zinn et al. (1987); this suggests that folic acid status of cattle is dependent solely on ruminal microbial synthesis. They also estimated that net microbial

synthesis of folate was .42 mg/kg total tract digestible organic matter. Total duodenal flow of folate was estimated at 1.1 to 1.2 mg/day in 200 kg steers; this is very low compared to estimated requirements of 10 mg/day extrapolated from swine requirements. By comparison, Hayes et al. (1966) measured folate levels ranging from .080 to .186 mg/liter ruminal fluid, which, with a ruminal volume of 40 l and a .05/h dilution rate would yield 3.2 to 7.4 mg folic acid/day.

Plasma folic acid levels in cattle in this study do not support the conclusions of Zinn et al. (1987) that folate is deficient. Indeed, blood plasma folate levels tended to be high, except for suckling calves, which were lower and more similar to typical levels in humans. Microbial synthesis appears to be a generous source of folic acid, The technique used by Zinn et al. (1987) may have underestimated total folate due to destruction of folate in samples during drying at 65^{o}C. Folates in solution are sensitive to oxygen, light, and pH extremes (Brody, 1991). Bell (1974) suggested that samples containing labile vitamins such as folic acid should be homogenized in a blender and assayed immediately without drying or freeze-drying.

Plasma folate was highest in the feedlot cattle, being twice as high as in dairy cattle. Similarly, Hayes et al. (1966) observed that folate concentration in ruminal fluid was twice as high for yearling feedlot steers fed all-concentrate diets than for steers fed similar diets with 1.8

kg alfalfa hay added. Folic acid in ruminal fluid was negatively correlated with rumen pH in that study. The folate status of dairy cows may be lower than that of feedlot cattle due either to higher forage concentration in the diet and higher rumen pH, or due a a higher folate requirement for milk production. Fresh bovine milk contains .05 to .12 μg folate/g wet wt compared to .08 μg/g wet wt for raw ground chuck (Brody, 1991). At a daily production of 30 kg of milk, 1.5 to 3.6 mg would be secreted daily, values equal to half of daily ruminal production.

The lower folate concentrations in plasma from stressed calves compared to feedlot and dairy cattle may be due to impaired ruminal synthesis due to low feed intake, and increased metabolism due to stress.

Blood plasma folate levels were lowest in the suckling calves; some would be considered marginally deficient based on human standards. Although these suckling calves all were at the age to ruminate, milk still could form a sizeable part of their diet. Because milk is digested primarily post-ruminally and is a relatively poor source of folate, total folate supply may be marginal in suckling calves. In our study, mean plasma folate was 6.8 ng/mL; values ranged from 3.2 to 11.0 ng/mL for suckling calves. Similarly, Girard et al. (1989) found that mean serum folate was 8.2 ng/mL in 2 week old dairy heifers fed whole milk and concentrates. Older heifers (120 days in age and fed 1 kg

concentrate twice daily plus hay and silage) had a mean serum folate concentration of 14.8 ng/mL.

Vitamin B_{12}. Vitamin B_{12} deficiencies in ruminants are not apparent except when dietary cobalt is inadequate for ruminal B_{12} synthesis. Ruminant organs, particularly liver, brain, kidney and heart, are excellent dietary B_{12} sources for man (Ellenbogen and Cooper, 1991). Vitamin B_{12} is stored in these organs. In man, turnover is very low, about .1% of body stores/day; hence, it can take years to deplete body stores during a vitamin B_{12} deficiency.

Both microbiological and radioassay methods have been used to assess vitamin B_{12} status in humans. In microbiological assays, the ability of cobalamins to stimulate growth is quantitated by turbidimetry or by measuring carbon monoxide production. Specificity of the assay for B_{12} depends on the organism used. Ochromonas malhamensis utilizes cobalamins almost exclusively, whereas E. coli, Lactobacillus leichmannii and Euglena gracilis are less specific and will also utilize corrins that are unavailable to mammalian species (Nexo and Olesen, 1982). Microbiological methods have been replaced largely by radioassays.

Isotope dilution methods are easier, faster, more precise and have lower detection limits than microbiological methods. By this method, bound cobalamin is extracted from either serum, heparinized or EDTA plasma, converted to cyanocobalamin, and mixed with a known quantity of radioactive cyanocobalamin. Endogenous B_{12} in the sample or a

standard competes with the radioactive B_{12} for binding with a cobalamin-binding protein. Since 1980, radioassay kits sold in the U.S. have used purified intrinsic factor as the binding protein. Historically, numerous binding proteins were used, including transcobalamin, saliva binder, and a chicken serum protein (Nexo and Olesen, 1982). These proteins have lower affinities for B_{12} than does intrinsic factor; lower affinity allows nonspecific binding to B_{12} analogs. The purity of the intrinsic factor is important; less purified preparations contain proteins which can bind inactive B_{12} analogs in serum (Ellenbogen and Cooper, 1991).

Because of these problems, caution must be used when interpreting earlier studies using earlier assay methods. A serum sample assayed at 95 pg B_{12}/mL was tested in a U.K. quality control study. Participating laboratories determined levels ranging from 50 to 730 pg B_{12}/mL by microbiological assays, and 50 to 1750 pg/mL using radioassays (England and Linnell, 1980). Hence, the reliability of commercial cobalamin assays still is contested (Ellenbogen and Cooper, 1991).

Less information is available for normal blood B_{12} levels in cattle than in sheep. Levels in sheep can be markedly higher than in cattle, so values may not be useful as a guideline for assessing bovine B_{12} status. For example, serum B_{12} in cobalt-deficient lambs was 100 to 256 pg/mL, compared to 982 to 1613 pg/mL in cobalt-supplemented lambs (Stebbings and Lewis, 1986). However, these workers

used a nonspecific analytical method which may have measured B_{12} analogs as well as B_{12}. In another study, serum B_{12} levels in marginally cobalt-deficient lambs averaged 320 ± 111 pg/mL (Millar and Lorentz, 1979).

Plasma B_{12} levels averaged 70 pg/mL in cobalt-deficient steers fed diets containing .03 to .04 ppm cobalt, compared to 180 pg/mL in cattle supplemented with cobalt (Bentley et al., 1954). These workers used the L. leichmannii assay.

Our results were similar to those of Bentley et al. (1954); plasma B_{12} levels in cattle were not high when compared to normal levels in humans. Standards for B_{12} status in humans were established by Bio-Rad (Clinical Division, Hercules, CA) for their radioassay method using data from 283 healthy adults and 105 patients diagnosed as B_{12} deficient. Normal was defined as serum B_{12} >200, marginal as 160 to 200, and deficient as < 160 pg B_{12}/mL. Using these standards, average concentrations in suckling calves, stressed calves, and dairy cattle were normal, whereas plasma vitamin B_{12} in feedlot cattle is low. Approximately 57% of feedlot cattle, 23% of suckling calves, 38% of stressed calves and 6% of lactating dairy cows would be considered deficient based on human standards. Implications of this are not known. Cobalt levels in Oklahoma feeds have not been defined although cobalt is supplemented routinely throughout the US and Canada. Nonetheless, some of these differences may reflect the adequacy of the cobalt-supplementation programs.

The mean plasma B_{12} concentration in feedlot cattle was 160 pg/mL, indicating a borderline deficiency. The lowest plasma B_{12} concentration, 97 pg/mL, was observed in a feedlot steer. The lower plasma B_{12} in feedlot cattle may be related to B_{12} analog production on the high concentrate diet. For most diets, only 15 to 20% of the B_{12}-related compounds produced in the rumen exhibit B_{12} activity in animals (Dryden and Hartman, 1971). Feeding dairy cows a high concentrate diet resulted in lower liver and milk B_{12} in spite of higher total (nonspecific) B_{12} in serum (Walker and Elliot, 1972). In sheep, a high corn diet (60% corn, 40% timothy) markedly reduced B_{12} but not analog production. Total serum B_{12} measured nonspecifically increased with dietary corn level, but actual B_{12} in serum decreased (Sutton and Elliot, 1972). Possibly analog production at the expense of B_{12} is even more extensive on a typical feedlot diet containing 80% or more concentrate.

More research is needed to determine whether the low B_{12} levels in plasma are physiologically important. Also, cobalt concentration of the diets should have been measured. Performance responses to B_{12} injections by feedlot steers have been variable.

Pantothenic Acid. Pantothenic acid is found primarily in the form of coenzyme A in red blood cells, and as free pantothenic acid in plasma. Red blood cell pantothenic acid concentrations are higher than in plasma; red blood cell concentrations also are more responsive to dietary

pantothenic acid (Fox, 1991), but serum levels will respond to extreme differences in intake.

As pantothenic acid deficiencies are not observed in humans except during severe malnutrition, the volume of research on assessment of pantothenic acid status is minimal compared to that for other vitamins. Pantothenic acid deficiencies have been described in numerous animal species fed deficient diets, including the preruminant calf (Sheppard and Johnson, 1957). However, parameters of normal pantothenic acid status have not been defined for livestock.

Methods for assessment of pantothenic acid status in humans include radioimmunoassay (Wyse et al., 1979), gas chromatography-mass fragmentography (Banno et al., 1990) and indirect enzyme linked immunosorbent assay (Song et al., 1990) of blood, plasma or tissue. Microbiological methods also are used but are tedious and time consuming. Microbiological and chick assays are useful in measuring levels in feedstuffs and foods.

An extremely wide range of total pantothenic acid levels in tissue samples of normal individuals has been reported, e.g., 1.1 to 12 μM for whole blood. Difficulties in enzymatic hydrolysis of bound forms and use of enzyme preparations containing pantothenic acid can explain this variation. The liberation of pantothenic acid from coenzyme A requires three enzyme activities: pyrophosphatase, orthophosphatase, and a specific amidase, pantetheinase, which is not available commercially. Enzymatic methods used

to liberate pantothenic acid often result in unacceptable levels of error, and frequently overestimate pantothenic acid levels (Wittwer et al., 1989).

In order to avoid these problems, pantothenic acid was measured using the ELISA, which does not require enzymatic preparation and is more sensitive than other methods (Song et al., 1990). This method may be sensitive to short-term changes in animal pantothenic acid status. Marked depressions of plasma or serum pantothenic acid have been noted previously only for rats fed pantothenic acid deficient diets for at least 4 weeks (Reibel et al., 1982); in humans, plasma pantothenic acid may not sensitively reflect pantothenic acid intake (Song et al., 1985). However, when plasma pantothenic acid was measured using the ELISA in rats fed a pantothenic acid deficient diet, it was depressed as early as 1 1/2 weeks (0.86 ±0.31 vs 1.56 ± 0.28 nM in deficient and control rats, respectively; Song et al., 1990).

Plasma pantothenic acid levels in cattle were found to be markedly lower than levels in rat or human plasma analyzed by the same technique in the same laboratory. Bovine plasma pantothenic acid concentrations have not been reported previously. Plasma levels in humans normally are about 1.0 nM. The mean plasma pantothenic acid for our 86 cattle was 0.115 nM.

Plasma pantothenic acid was affected ($P < .002$) by cattle class. It was highest ($P < .05$) in feedlot cattle

and suckling calves (0.143 and 0.128 nM), and lowest in dairy cows and transport-stressed calves (.089 and .095 nM).

The higher levels in feedlot cattle may be explained by the observation that ruminal pantothenic acid production is maximal on high concentrate diets. Hayes et al. (1966) found that the pantothenic acid concentration in ruminal fluid was 242 and 354 μg/100 mL for steers fed flaked and ground corn diets, respectively. Levels were intermediate when the corn diet was fed with ground hay, and lowest (65 and 77 μg/100 mL) for flaked and ground corn diets, respectively, when fed with long hay. Similarly, pantothenic acid concentration in rumen contents was nearly four times higher in sheep fed a diet containing 90% corn, 10% soybean meal, than in sheep fed prairie hay (30.1 vs 8.3 μg/g DM; Hollis et al., 1954).

As was observed for ascorbic acid, the lowest individual pantothenic acid levels were found in the dairy cows and stressed calves. Possibly in cattle, as in other species, ascorbic acid and pantothenic acid metabolism are interrelated (Fox, 1991). Low levels in dairy cows may partly reflect higher requirements due to pregancy and production, as milk contains 36 mg pantothenic acid/kg DM. Also, ruminal synthesis is lower with mixed diets than high concentrate diets; synthesis appears to be inversely correlated with ruminal pH. Dairy cow diets contain more forage than do feedlot diets; 40 to 60% roughage is typical.

The cows in this study were fed a total mixed diet which contained buffers.

The low levels of plasma pantothenic acid in stressed calves may be due to both decreased supply and increased demand. Reduced intake and impaired ruminal function in stressed calves would depress ruminal pantothenic acid synthesis. Furthermore, pantothenic acid is important in adrenal function. Corticosteroid production by adrenal glands in rats is reduced by a pantothenic acid deficiency and increased by intramuscular pantothenic acid injection (Fox, 1991). This suggests that requirements may be increased by stress, which increases corticosteroid production.

Vitamin B_6. The concentration of vitamin B_6, particularly pyridoxal phosphate (PLP), in plasma is one of the most commonly used indicators of vitamin B_6 status. Considerable interspecies variation occurs in the concentration and distribution of the B_6 vitamers in plasma (Coburn et al., 1984); there is little interspecies variation in vitamin B_6 requirements. Other techniques used in human nutrition are not appropriate for use in animal studies. They include urinary excretion of 4-pyridoxic acid, urinary excretion of certain tryptophan metabolites after a tryptophan load, and in vitro amino-transferase activity in red blood cells before and after the addition of PLP (Linkswiler, 1967; Reynolds and Leklem, 1981).

The presence of pyridoxine and elevated concentrations is PLP, PL and total B_6 in plasma of one stressed calf suggested that it had been injected recently with pyridoxine. Therefore, values for the other eight animals were averaged to obtain mean values for stressed calves. However, Coburn et.al. (1984) reported that mean plasma pyridoxine concentration was 50 ± 67 nM in 3 "normal" calves, so perhaps either pyridoxine is found naturally in cattle plasma or it is formed by interconversion from other B_6 vitamers during the analytical procedure.

Mean plasma PLP for the eight stressed calves was 97 nM; this is extremely low compared to a mean of 402 nM in 3 healthy, non-stressed calves previously found using the same HPLC technique in the same laboratory (Coburn et al., 1984). Pyridoxal, not detected in 5 out of 8 calves, averaged 2.9 nM, compared to 96 ± 11 previously reported. The B_6 excretory product, 4-pyridoxic acid, also was lower in the stressed calves, averaging 33.4 nM compared to 91 ± 68 nM reported. Total B_6 averaged 133.4 nM in the eight stressed calves, compared to 686 ± 311 nM reported by Coburn et al. (1984).

Our data suggest that vitamin B_6 status was severely compromised in the transport-stressed calf. More information on B_6 vitamer concentration and distribution in plasma for various classes of cattle, including stressed calves, is needed.

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CHAPTER V

EFFECTS OF B-VITAMIN INJECTIONS ON BOVINE HERPESVIRUS-1 INFECTION IN FEED-RESTRICTED WEANED BEEF CALVES

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ABSTRACT

Six-month-old beef steer calves were stressed to simulate the shipping/marketing cycle which may predispose calves to bovine respiratory disease. Stresses, which consisted of weaning, limit-feeding and fasting, were conducted in metabolism stalls. The calves lost 13.3% of initial weight between arrival and the end of a 3-day fast. At 20 days after arrival, the calves were inoculated intranasally with live attenuated bovine herpesvirus-1 (BHV-1), a pathogen implicated in bovine respiratory disease. The calves were injected with B-vitamins and vitamin C on alternate days every second day for 2 weeks prior to and 2 weeks after virus inoculation. The stress/BHV-1 model resulted in a mild respiratory infection in all calves. B-vitamin injections did not significantly affect infection and immunity as measured by virus and interferon titers in nasal secretions, serum neutralizing antibody titers, and ability of lymphocytes to undergo blastogenesis in vitro when exposed to pokeweed mitogen. However, B-vitamin treatment

increased serum IgG to BHV-1 ($P < .05$) at 14 days post-infection.

(Key Words: Bovine Herpesvirus-1, B-Vitamins, Calves, Immunity, Stress).

Introduction

The Bovine Respiratory Disease complex is responsible for approximately 75% of morbidity and mortality in feedlot cattle (Jensen et al., 1976), costing the cattle industry in the U.S. in excess of $500 million annually (McMillan, 1983). The incidence is highest during the first 3 weeks after arrival, probably due to the impact of stresses during the shipping/marketing process which can compromise both immunity and the nutritional status of the animal. Nutrition of cattle prior to and after arrival at the feedlot has been shown to affect morbidity (Hutcheson and Cole, 1986).

Requirements for some nutrients, particularly certain B-vitamins, are markedly affected by stress and disease (Mueller and Thomas, 1975). Specific B-vitamins (folic acid, pyridoxine, and pantothenic acid) are critical to the immune response, are not stored in the body, and can be rapidly depleted.

The purpose of this study was to measure the effects of B-vitamin injections on immunity in nutritionally-stressed steer calves inoculated with bovine herpesvirus-1 (BHV-1). Infection with BHV-1 was used as a model for the bovine

respiratory disease complex because it can cause a primary respiratory tract infection and predispose cattle to infection with Pasteurella (Yates, 1982). Further, BHV-1, producing only mild infections under laboratory conditions, is unlikely to cause mortality.

Materials and Methods

Experimental Procedure. During the first 20 days of the study, calves received a limited amount of feed and underwent a 3-day fast; this was an attempt to simulate the effects of shipping and handling on both nutritional status and ruminal function. The calves then were inoculated with BHV-1. All calves received B-vitamin or vehicle injections parentally every 48 hours for 2 weeks prior to and 2 weeks after BHV-1 inoculation.

Calves. Seventeen Hereford X Angus crossbred 6 to 8 month old calves from one herd which had been wintered on dormant native range were weaned on April 23, 1991 (day -21). These calves previously had been tested for presence of antibodies to BHV-1; all were seronegative. They were trucked 10 miles to the Veterinary Medicine building, weighed on arrival, and housed individually in a room isolated from other cattle. Twelve steer calves were placed in elevated metabolism crates, whereas five steer and heifer calves (spares) were kept in slatted floor pens but otherwise treated identically.

Diets. On arrival, each calf was fed .9 kg of native prairie grass hay (4% crude protein). From April 24 to May 5, and May 9 to May 16, calves were offered this hay once daily at 1% of body weight (Table 8). All calves were fasted May 6, 7, and 8, (days -8, -7, and -6). On May 17 (day 3), the feeding rate was adjusted to 1.5% of initial body weight so that calves would maintain their body weight. Because of the very low protein concentration (4% CP) in prairie hay, alfalfa hay (21% CP) was substituted for half of the prairie hay during the final week of the study.

Treatments. The calves were assigned randomly to two treatment groups (Control, +Vit) with 6 calves in metabolism stalls per treatment. Additionally, three of the spare calves received the control treatment and two received the +Vit treatment. Each control calf received sterile saline injections. Each +Vit calf received injections of B-vitamins and vitamin C (Table 9). These mixtures were custom formulated (American Veterinary Products, Fort Collins, Colorado). The injection treatments were imposed every 2nd day from 10 days after arrival (day -11) until the end of the experiment 31 days later. The parenteral route was selected for vitamin administration; had these vitamins been provided via either the water, the diet or a bolus, they would be subjected to either ruminal destruction and(or) variable intake.

Weight and Health. Body weight was recorded before feeding each week, and before and after the 3-day fast. Animals

Table 8. Daily feed intake (g/d)[a,b]

Treatment	Calf No.	Days -20	-19to-9	-8to-6	-5to2	3to14
Control						
	3	1710	2300(1900)[c]	0	1900	2700
	5	1600	1600	0	1600	2200
	6	1180	1800	0	1800	2450
	8	1400	1400	0	1400	2100
	10	1600	1600	0	1600	2300
	11	1400	1400	0	1400	2000
	13	1600	1600(1500)[c]	0	1500	2200
	14	1200	1200	0	1200	1600
	17	1400	1400	0	1400	2000
B-Vitamin						
	1	1400	1400	0	1400	2000
	2	1800	1800	0	1800	2500
	4	1600	1600	0	1600	2300
	7	1300	1300	0	1300	1800
	9	1800	1800	0	1600	2300
	12	1600	1600	0	1600	2300
	15	1070	1400	0	1400	1900
	16	1500	1500	0	1500	2100

[a]As-fed basis (90% DM).

[b]Prairie hay (4% CP) was fed from day -20 to day; 50% priairie hay plus 50% alfalfa hay was fed from day 7 to day 14.

[c]Feed intakes adjusted on day -12 based on corrected animal weights.

Table 9. B-vitamin requirements and levels supplied by injection

Vitamin	Estimated Requirement[a]	Stress Factor[b]	Dosage[c]
Thiamin	6.76 mg		13.5 mg
Riboflavin	16.91 mg		33.8 mg
Niacin	67.64 mg		135.0 mg
Folic acid	2.09 mg	15	60.0 mg
Pantothenic acid	54.1 mg	2	216.0 mg
Vitamin B_6	6.76 mg	8	108.0 mg
Vitamin B_{12}	67.64 ug	2	270.0 ug
Vitamin C	Unknown		1000.0 mg

[a]Daily B-vitamin requirements for a 190.5 kg calf were estimated on a metabolic body weight basis as equivalent to 3.56 times the requirements for a 34 kg pig (NRC, 1990).

[b]The estimated daily requirement was multiplied by factors of 2 for pantothenic acid and vitamin B_{12}, 15 for folic acid, and 8 for vitamin B_6 to account for the increased requirements of these specific B-vitamins during stress or "moderate" injury (Mueller and Thomas, 1975).

[c]Twice the daily estimated requirement was supplied by injection every 2 days, with one 3 ml dose containing the B-vitamins and one 4 ml dose containing the vitamin C. Controls received identical doses of sterile saline solution.

were observed daily; rectal temperature of calves were measured at 0700 every day before feeding.

Blood samples. All blood samples were collected by jugular venipuncture in 10 ml evacuated tubes (Vacutainers, Sherwood Medical, St. Louis, MO). One blood sample was collected from each calf for serum neutralizing antibody titers on days 0, 7, 14, and 28. Blood for antibody measurement was allowed to clot; serum was harvested and heat inactivated (56^{o}C for 30 minutes). Blood samples for lymphocyte blastogenesis were collected in Vacutainers with potassium EDTA shortly after arrival (day -15), and on days 0, 5, 7 and 14. Serum and plasma samples were stored at -20^{o}C until analyzed.

Nasal samples. Nasal secretions for interferon quantification were collected on days 0, 2, 4, 5, 7, and 9 by inserting tampons intranasally and removing them after 15 minutes as discussed by McKercher (1973). Nasal swabs for virus quantification were collected on these same days. The swabs were immediately expressed into vials containing 3 ml of transport medium (on ice), and stored at -70^{o}C until assayed (d'Offay and Rosenquist, 1988).

Viruses. The vaccine strain of BHV-1 (Nasalgen, Jensen-Salsbery Laboratories, Kansas City, Mo.) in its second serial passage in this laboratory was used for animal inoculations (Todd et al., 1972). The Indiana strain of vesicular stomatitis virus (obtained from B. Easterday, University of Wisconsin, Madison) was used in plaque

reduction assays for IFN. Viruses were propagated by inoculating confluent MDBK cells with virus at a low multiplicity of infection. Infected cells were harvested by scraping when cytopathology indicated that at least 90 to 100% of cells had been infected.

Virus Inoculation. On May 14 (day 0), the 12 test calves and one spare control calf were inoculated intranasally with 10^7 plaque-forming units (PFU) of BHV-1 (vaccine strain) in 1 ml of virus suspension, .5 ml/nostril, with a syringe.

Cell cultures. Madin Darby bovine kidney (MDBK) cell cultures were used for all assays, tests, and virus propagation. Growth medium for the MDBK cell cultures consisted of Eagle's minimal essential medium (MEM) in Earle's balanced salt solution supplemented with 5% fetal bovine serum (FBS), .075% $NaHCO_3$, 10 mM HEPES buffer and antibiotics (penicillin 200 µg/ml, streptomycin 200 µg/ml, fungizone .50 µg/ml). Maintenance medium was the same except that it also contained 2.5% FBS and 20 mM HEPES.

Virus quantification. BHV-1 virus titers in nasal secretions were determined by plaque assay in 24-well tissue culture plates confluent with MDBK cells. Virus particles in different dilutions were quantitated in duplicate wells overlaid with 1% methylcellulose.

Interferon assay. Nasal secretions, diluted 1:5 in MEM, dialyzed at 4°C overnight in pH 2.0 buffer and then in PBS (pH 7.2) for an additional 24 hours, were centrifuged at 1500 X g for 20 minutes. Samples were screened at 1:20

dilution, and, when necessary, diluted further to determine end points. Interferon was measured by a plaque-reduction assay with vesicular stomatitis virus as the challenge-exposure virus using 24-well tissue cultures plates confluent with MDBK cells (Fulton, 1986). Titers (median plaque-reduction units/ml) were determined as described by d'Offay and Rosenquist (1986).

Neutralization tests. Neutralizing antibody titers in sera were determined by a microtiter serum neutralization test using 96-well tissue culture plates (Carbrey et al., 1972). Sera were diluted 2-fold and four wells were used per dilution. Approximately 100 PFU of BHV-1 were added to each well and the virus-serum mixture was incubated at 37^{o}C for one hour. Trypsinized MDBK cells (approximately 75,000 cells/well) then were added to each well. The plates were incubated at 37^{o}C in 5% CO_2 atmosphere for 4 days and results were read. Antibody titers were recorded as the reciprocal of the highest final sample dilution that completely protected at least 3 of 4 wells against virus infection.

ELISA Procedure. An ELISA was used to detect serum IgG antibody titers to BHV-1. Antigen for the ELISA consisted of lysates of BHV-1 infected and non-infected MDBK cells.

Round bottom 96-well polyvinyl plates (Dynatech Immulon II) were used for the assay. Wells were coated with 100 µl/well of either infected or control cell lysates and left overnight at 4^{o}C. The next morning, the coating buffer was

removed by inverting and blotting the plates. A blocking buffer consisting of 1% w/v bovine serum albumin in gelatin buffer (Gel-BSA) was added at 100 µl/well, and the plates were incubated at 37°C for 1 hour. Buffer was removed and various dilutions of the primary antibody (serum samples) diluted in Gel-BSA were added to wells coated with antigen and to wells coated with uninfected (control) cell lysates. After incubation for 1 hour on a rotator at room temperature, wells were washed 5 times with phosphate-buffered saline with Tween 20 (PBS-T). Then a secondary antibody consisting of rabbit anti-bovine IgG conjugated to peroxidase (Pel-Freez® Biologicals, Rogers, Arkansas) was added. After a 1 hour incubation period on the rotator at room temperature, wells were washed 5 times with PBS-T. A solution containing hydrogen peroxide and 0-phenylenediamine dihydrochloride (OPD) was added (100 µl/well), and color was allowed to develop for 20-30 minutes. The reaction was stopped with 50 µl 2M H_2SO_4 and absorbance was measured at 490 nm. Titers were expressed as the reciprocal of the highest dilution with an absorbancy index of at least 3. For a given dilution, the absorbancy index was the absorbancy (at 490 nm) in the antigen positive well divided by the absorbancy in the control well.

Lymphocyte Blastogenesis Assay. Procedures of Wyckoff and Confer (1990) were followed. Ten ml samples of blood containing K_2EDTA as an anticoagulant were kept at room temperature in the dark for up to one day before this assay

was performed. Peripheral blood mononuclear cells (PBMC) were obtained through density gradient centrifugation on a ficoll-sodium diatrozoate medium (Ficoll-Hypaque, Sigma Chemical Co., St. Louis, MO). The PBMC were removed with a pipette and washed three times with Hanks balanced salt solution (Gibco, Grand Island, NY). Lymphocytes were cultured in 96-well flat-bottomed microtiter plates (Corning, Cambridge, MA), with 5 X 10^5 cells/well in a total volume of .2 ml of Dulbecco's Modified Eagles medium (DMEM) containing 10% fetal bovine serum, 2 mM L-glutamine, and 50 ug/ml gentamycin. Stimulations were performed in quadruplicate with four concentrations (0, .25, .50, and 1.00 µg/ml) of pokeweed mitogen (PWM). The cultures were incubated in a humidified 10% CO_2 incubator for 5 days. On the final day, .025 ml of fresh DMEM containing 1.0 µCi [^{3}H] thymidine (ICN, Irvine, CA) was added to each well. After 4 hours, cultures were frozen at -70^{o}C. At the end of the study, cultures were harvested onto glass fiber filters and counted by scintillation spectroscopy (Model 300; Packard Instrument Co., Laguna Hills, CA). Lymphocyte transformation was expressed as a stimulation index (SI). The SI was calculated by dividing the mean cpm of sample wells by the mean cpm of non-stimulated control wells.

Statistical Analysis. Agglutination, viral replication, antibodies (neutralizing and ELISA), interferon, blastogenesis, weight and health data were analyzed by analysis of variance models using the main effects of

vitamin treatment and collection day. The effect of treatment was tested within each day using the animal within treatment mean square as the error term.

Results

Animal Observations. Weaning, handling, and experimental procedures did not clinically stress these calves. They readily adjusted to the metabolism crates, and within 1 to 2 days after arrival, they often remained at rest despite the presence of humans. Some were not even disturbed when rectal temperature was measured, preferring to remain resting instead of standing up during this procedure.

B-vitamin and saline injections appeared to cause little discomfort, although vitamin C injections may have caused temporary discomfort. The lack of discomfort from the B-vitamin injection, in contrast to some commercial B-vitamin injectable formulations, may be related to benzyl alcohol, an antibacterial preservative, present in commerical formulations but absent from our mixture.

During the fasting period, activity was reduced and the calves drank <10% of normal water intake. Water intake typically is correlated positively with feed intake (NRC, 1988). Removing water during the first day of the fast may not have been necessary to prevent water consumption because

when water was made available on the second day of the fast, the calves did not drink.

Feed Intake. Hay was fed once daily at 0800 except on fast days. Feed intake is shown in Table 8. Feed consumption was consistent throughout the study. All feed was consumed within 2 hours of feeding except on day -20, when 3 calves did not consume all their feed before the next day.

Calf Weight. On arrival, calf weight ranged from 121 to 192 kg, with a mean weight of 165.2 kg (Table 10). Average calf weight did not differ ($P > .05$) between treatments. During the first week after arrival, mean weight declined to 156.8 kg; body weight declined to 153.9 kg the following week immediately before the fast period. Following the 3-day fast, average weight was 143.2 kg; calves lost a mean of 10.7 kg (7.0%) during the fast. Four days later, weight was 152.4 kg; body weight was 152.9 and 153.9 kg the following weeks (on the maintenance feeding schedule). Total weight loss between day -21 and the end of the fast was 13.3% of initial weight; weight loss between day -21 and day 0 was 7.3% of initial weight.

Rectal Temperature. Body temperature as measured by rectal temperature (RT) appeared to fluctuate from day to day (Appendix Table A). The mean RT usually was about .25°C lower for the +Vit cattle than for the control cattle. This appeared to be due to individual differences in RT which were apparent from the beginning of the study before vitamin

Table 10. Effect of B-vitamin treatment on body weight of calves (kg)

Treatment	Calf No.	Day 4/23 d -21 In Wt	4/29 d -15 fast	5/6 before fast	5/9 end BHV-1[a]	5/13 d 0	5/20 d 7	5/27 d 14
Control								
	3	192.3	187.3	184.6	173.3	180.5	181.9	182.3
	5	161.9	152.0	148.8	137.0	143.3	145.6	147.4
	6	182.8	169.6	167.4	156.9	162.4	165.1	168.3
	8	147.4	145.2	142.0	132.9	139.3	142.0	144.2
	10	166.0	158.8	157.9	146.1	153.8	156.0	156.5
	11	145.2	137.0	132.9	124.3	130.2	136.5	134.7
	13	158.3	154.2	150.1	140.2	149.7	150.6	149.7
	14	121.1	114.3	108.9	102.1	107.0	107.5	107.5
	Mean	166.1	158.4	155.7	145.2	154.4	154.7	155.7
B-Vitamins								
	1	146.5	137.9	133.8	126.1	132.0	133.4	136.1
	2	188.2	176.9	172.4	162.8	165.1	169.2	170.1
	4	165.6	158.8	155.6	145.2	152.9	154.2	155.6
	7	130.6	122.9	122.5	108.9	117.0	122.9	124.7
	9	186.4	176.9	172.8	160.6	167.4	172.4	170.6
	12	167.8	156.5	155.1	142.9	150.1	154.2	154.7
	15	142.4	135.2	131.5	122.5	127.5	130.2	129.7
	16	152.9	151.5	144.2	132.4	137.9	141.1	139.7
	Mean	164.4	155.1	152.2	141.2	150.3	151.2	152.1
Overall	Mean	165.2	156.8	153.9	143.2	152.4	152.9	153.9
	S. E	8.5	8.1	8.0	7.9	7.7	7.5	7.3

[a]Calves 1 to 13 inoculated with 10^7 PFU BHV-1.

treatments were imposed. Therefore, the average of each animal's RT over the first 10 days of the study (before vitamin treatments began) was used as a covariate. B-vitamin treatment, when covaried by mean RT from day -20 to day -11, did not significantly affect RT ($P > .05$) on any given day.

Feed intake influenced RT. RT averaged 39°C during the first 3 days of the study. During the fasting period, RT declined consistently. On the day prior to the fast (d-9), RT averaged 38.7°C. During the 3 mornings of the fasting period, and the following morning before re-feeding, mean RT was 38.7, 38.4, 38.3, and 38.1°C, respectively. On the morning following re-feeding, temperature averaged 38.6°C. This indicates that RT is lower than normal in fasted calves; this may delay detection of infections.

No fever was expected to develop in BHV-1 infected cattle until at least day 4 post-inoculation. Fever can be influenced by the strain of virus given, its dosage, and the immune status of the cattle. Two calves, #5 (Control) and #12 (+VIT) exhibited a fever on day 4 (Appendix Table A). On day 5, only #12 had a fever, although #1,7,8, and 10 appeared to have slightly elevated RT (RT>39.4°C). On day 6, one calf, #8, had a fever, but no calves had a fever subsequently. Compared with previous rectal temperatures for each calf, temperatures post-infection were elevated in most calves for at least one day; however, no elevation in body temperature was detected in 3 of the calves on any day

after infection. Based on RT, this vaccine strain of BHV-1 caused only a mild infection.

Clinical Observations. The BHV-1 infection caused mild respiratory disease in all of the calves; this appeared to be limited to the upper respiratory tract. Clinical signs of respiratory disease were noted on days 4 to 6 post-infection. By day 4 and day 5, all calves developed a mild serous nasal discharge progressing to a mild mucopurulent discharge on day 5 in some of the calves. The nasal mucosa became inflamed with necrotic plaques observed in several calves.

Excretion of BHV-1 virus. Prior to BHV-1 infection, no BHV-1 virus was recovered from the nasal secretions of the calves. For 9 days after inoculation, all calves, with the exception of calf #4 on day 9, excreted BHV-1 in their nasal secretions. Calf #4 excreted BHV-1 for at least 7 days, with no virus present in nasal secretions on day 9. In both treatment groups, mean viral shedding after inocu-lation was highest on day 4, but higher on day 7 than on day 5. (Table 11). However, virus excretion in individual calves peaked at different times between day 2 and day 7. B-vitamin treatment did not affect ($P > .05$) mean daily virus excretion on any day.

Neutralizing antibody titers. No neutralizing antibodies were detected in sera on days 0 and 7 post-inoculation (Table 12). All calves seroconverted by day 28, with 11 out of 12 seroconverting by day 14. The exception

Table 11. Effect of B-vitamin treatment on BHV-1 titers[a] in infected calves

Treatment	Calf No.	Days 0[b]	2	4	5	7	9
Control							
	3	0[c]	3.5	5.3	5.4	5.4	4.0
	5	0	4.2	7.4	6.2	6.0	3.3
	6	0	4.5	6.6	5.5	6.0	3.3
	8	0	3.6	6.9	6.1	6.0	3.6
	10	0	6.1	5.7	5.6	6.0	2.3
	11	0	5.6	5.0	4.2	5.3	3.4
	Mean	0	4.58	6.15	5.50	5.78	3.32
	S.D.	0	1.06	0.96	0.72	0.34	0.56
B-Vitamins							
	1	0	4.2	5.6	5.4	5.6	4.7
	2	0	5.6	7.4	6.7	6.0	4.3
	4	0	5.1	5.6	4.0	4.1	0.0
	7	0	5.2	6.9	6.5	6.5	2.6
	9	0	4.0	5.3	4.4	5.9	4.4
	Mean	0	4.92	6.05	5.17	5.61	3.15
	S.D.	0	0.66	0.87	1.22	0.81	1.76
Overall	Mean	0	4.75	6.10	5.33	5.70	3.23
	S.E.	0	0.36	0.37	0.41	0.25	0.53

[a]Data expressed as BHV-1 titers ($\log_{10}$ PFU/ml).
[b]All calves were inoculated with 10^7 PFU BHV-1 on day 0.
[c]0 = No plaques in cultures inoculated with 0.5 ml of undiluted sample.

Table 12. Effect of B-vitamin treatment on serum neutralizing antibody titers in calves infected with bovine herpesvirus-I[a]

Treatment	Calf No.	Days 0[b]	7	14	28
Control					
	3	0[c]	0	4	64
	5	0	0	8	8
	6	0	0	4	8
	8	0	0	8	32
	10	0	0	16	16
	11	0	0	8	8
	Mean	0	0	8.0	22.7
	S.D.			4.4	22.3
B-Vitamins					
	1	0	0	16	32
	2	0	0	16	16
	4	0	0	0	32
	7	0	0	8	64
	9	0	0	4	4
	12	0	0	16	16
	Mean	0	0	10.0	27.3
	S.D.			7.0	20.9
Overall	Mean	0	0	9.0	25.0
	S.E.	0	0	2.4	8.8

[a]Data expressed as the reciprocal of the serum dilution protecting at least 3 out of 4 wells from 100 PFU BHV-1.

[b]All calves were inoculated with 10^7 PFU BHV-1.

[c]Titer < 4 (not detectable).

was calf #4; it developed a high titer by day 28. Mean neutralizing antibody titers were somewhat higher for +Vit treatment compared to controls on days 14 and 28, but this difference was not significant ($P > .50$).

Interferon in nasal secretions. Interferon was detected in nasal secretions of 2 calves on day 2, and of all 13 calves on day 4 (Table 13). Interferon titers peaked on day 7, and dropped to very low levels on day 9. Peak IFN titers for individual calves averaged 5640 for controls, and 6750 for +Vit calves. IFN titers tended to be lower for +Vit calves on days 2 and 4, but higher on days 5 and 7. However, these treatment differences were not significant ($P > .15$) on any day.

Serum IgG titer to IBR virus. IgG titers to BHV-1 are shown in Table 14. The first IgG antibodies to BHV-1 were detected on day 14 and titers increased by day 28. IgG titers were 100 times higher on average than neutralizing antibody titers. However, for individual calves, the IgG and neutralizing antibody titers were highly correlated ($r^2=.90$; $P < .05$) on days 14 and 28. The mean IgG titer to BHV-1 was higher ($P < .08$) in +Vit calves than in control calves on day 14 (1120 versus 530). IgG titers were similar ($P = 0.30$) on day 28, with means of 2400 and 1870 for +Vit and control calves, respectively. Averaged across day 14 and day 28, mean IgG titer for +Vit calves was 1760 compared to 1210 in controls ($P = .13$).

Table 13. Effect of B-vitamin treatment on IFN titers[a] in nasal secretions in calves infected with bovine herpesvirus-I

Treatment	Calf No.	Days 0[b]	2	4	5	7	9
Control							
	3	0[c]	0	740	0	0	0
	5	0	0	810	6200	5300	0
	6	0	0	10200	2700	9500	530
	8	0	0	3100	550	5100	210
	10	0	140	1900	4100	5100	250
	11	0	0	4500	1800	6500	0
	Mean	0	23	3540	2540	5250	165
	S.D.	0	57	3560	2320	3070	210
B-Vitamins							
	1	0	0	3500	620	830	50
	2	0	0	210	7700	20800	320
	4	0	0	1400	5500	4500	0
	7	0	110	1300	3300	8900	0
	9	0	0	150	560	1400	0
	12	0	0	400	0	200	460
	Mean	0	18	1160	2950	6100	140
	S.D.	0	45	1270	3100	7900	200
Overall	Mean	0	20.8	2350	2740	5680	152
	S.E.	0	2.9	1090	1125	2440	84

[a]Data expressed as interferon titers (Plaque Reduction$_{50}$ units/mL).

[b]All calves were inoculated with 10^7 PFU BHV-1 on day 0.

[c]0 = Titer < 20.

Table 14. Effect of B-vitamin treatment on serum IgG titers[a] in calves infected with bovine herpesvirus-1

Treatment	Calf No.	Days 0[b]	7	14	28
Control					
	3	0[c]	0	400	3200
	5	0	0	800	3200
	6	0	0	100	800
	8	0	0	400	1600
	10	0	0	800	1600
	11	0	0	800	800
	13	0	0	400	1600
	Trt Mean	0	0	530[d]	1830
	S.D.			255	930
B-Vitamins					
	1	0	0	1600	1600
	2	0	0	1600	3200
	4	0	0	100	3200
	7	0	0	1600	1600
	9	0	0	200	1600
	12	0	0	1600	3200
	Trt Mean	0	0	1120[e]	2400
	S.D.			680	800
	Overall Mean	0	0	830	2130
	S.E.			230	400

[a]IgG titer expressed as reciprocal of the highest dilution with absorbancy index ≥ 3.

[b]All calves were inoculated with 10^7 PFU BHV-1 on day 0.

[c]0 = Titer < 50.

[d,e]Means in the same column with different superscripts differ ($P < .08$).

Blast transformation of lymphocytes to pokeweed mitogen. The mean stimulation indices (SI), calculated as the mean index for the low, medium, and high levels of mitogen, are presented in Table 15. Variation within animals and sample periods was extremely high for the lymphocyte blastogenesis test; no clear trends were evident. Generally, stimulation indices were similar for all three levels of mitogen, although the highest stimulation indices often were found with the highest mitogen level (Appendix table B).

The SI was higher ($P < .05$) for the control calves in the initial samples taken 5 days after arrival (day -15), and on day 5 postinfection (440 and 1640 vs 210 and 250). Because the initial samples were taken on day -15, and B-vitamin treatments did not begin until day -11, the initial difference cannot be ascribed to vitamin injection. We attempted to adjust the model using initial SI as a covariate; this was not useful because the SI for individual calves varied drastically from period to period.

Discussion

The restricted feeding/fasting regimen resulted in an average shrink of 13.3% over the 16 day period from arrival to the end of the fast. Shrink normally refers to weight lost during transit. Length of transport has been correlated with total shrink. Shrink by shipping-stressed calves has been correlated with morbidity during the receiving phase (Camp et al., 1983; Griffin, 1983).

Table 15. Effect of B-vitamin treatment on lymphocyte blastogenesis. Mean stimulation indices

Treatment	Calf No.	Days -15	0[a]	5	7	14
Control						
	3	346.7	25.7	1057.3	27.0	27.0
	5	464.0	172.0	1680.0	na	839.0
	6	767.7	40.0	3816.7	212.7	882.3
	8	271.7	103.7	348.3	19.3	55.7
	10	530.0	70.0	1108.0	558.3	403.7
	11	264.7	678.0	1838.0	316.3	815.7
	Mean	440.8[b]	181.6	1641.4[b]	226.7	503.9
	S.D.	191.7	248.8	1189.1	224.2	397.8
B-Vitamin						
	1	50.7	na	55.3	937.0	517.0
	2	117.3	na	128.7	29.0	29.0
	4	269.3	na	251.3	27.7	70.7
	7	100.3	129.0	171.3	603.3	187.0
	9	405.7	425.7	520.0	492.3	91.0
	12	328.0	600.0	386.3	1229.0	549.3
	Mean	211.9[c]	384.9	252.2[c]	553.1	240.7
	S.D.	142.6	238.1	173.4	481.9	232.6
Overall Mean		326.0	249.3	946.8	404.7	372.3
	S.E.	69.0	117.4	346.9	158.8	133.0

[a]All calves were inoculated with 10^7 PFU BHV-1.

[b,c]Treatment means in the same column with different superscripts differ ($P < .05$).

na = Little or no blastogenic response; data omitted from statistical analysis.

Transport causes weight loss in addition to that caused by the feed and water deprivation; both gastrointestinal tract contents and muscle tissue are lost. Digestive capacity is lost due to reduced ruminal volume, altered microbial populations, and impaired ruminal function (Koers et al., 1974; Damron et al., 1979; Hamlett et al., 1983). As a result, appetite and intake recover slowly. Many calves do not eat for 5 or more days after being transported.

The stress model used in this study, with a relatively long period of feed restriction and a 3-day fast after weaning, approximates some of the conditions undergone by shipping-stressed calves. Although total shrink over the 20 days before virus inoculation (7.3%) was similar to that achieved in many cattle during a single haul, it was less severe than a shrink of more than 11% observed in highly stressed cattle (Griffin, 1983).

In general, compared with stresses of commercial cattle, the stresses we imposed certainly were less acute (no long-distance trucking), fewer in number, and not imposed simultaneously. Cattle undergo additional stresses during the shipping/marketing process which are reimposed each time the cattle are sold or transported. These include psychological stresses (exposure to novel surroundings, feedstuffs, and cattle), as well as sorting, handling, loading, transport, crowding and physical discomfort due to fatigue, hunger, and thirst. As in humans, psychological stimuli (e.g., novel environments) are as effective as

physical stimuli in elevating stress hormones in animals (Dantzer and Mormède, 1985).

Another important difference between our stress model and the shipping/marketing cycle involves duration of stress. In a traceback study of cattle from Kentucky, most animals were from different sources and most had been in transit for 8 to 9 weeks (D. G. Gill, Personal Communication, 1992). It is likely that many of those cattle had been resold throughout this period to buyers speculating on cattle and or attempting to assemble loads uniform in weight for sale to feedyards. Consequently, our prolonged period of nutritional restriction (20 days before BHV-1 inoculation) probably was shorter than typical.

Fasting and reduced intake during the marketing cycle and after arrival presumably would affect the postruminal B-vitamin supply, because the supply is positively correlated with feed intake (Zinn et al., 1987). However, if stress increases requirements and rate of depletion in stressed calves, our model may not have been adequate to reflect responses to injected B-vitamins in stressed calves.

Clinical signs. The degree of fever and serous nasal discharges after viral inoculation were mild and similar to signs reported by others using the same strain of virus (Todd et al., 1972; d'Offay and Rosenquist, 1988).

Rectal temperature. The observed day to day variation in rectal temperature of individual animals probably was normal because cattle do not control their body temperature

closely but allow it to fluctuate diurnally in response to environmental temperatures. However, because our calves were kept indoors under fairly constant temperature conditions, environmental temperature changes should not have altered body temperature.

To minimize variation and simplify detection of elevated temperatures in sick calves, all temperatures were recorded early in the morning before any other activities began. Previous research has shown that temperature measurements are most accurate when taken in the early morning and when activity of the cattle is limited (Gill and Richey, 1982).

We defined fever arbitrarily as an early morning rectal temperature above 40^{o}C (104^{o}F) (Dunbar et al., 1980; Janzen and McManus, 1980; Gill and Richey, 1982; Axe et al., 1984). This cut-off point has been found to be useful under feedlot conditions where animals must be moved and handled; movement and excitement tends to increase body temperature of cattle. Others have suggested that a fever could be defined as a rectal temperature over 39.4^{o}C (103^{o}F) (Lofgreen et al., 1975; Lofgreen et al., 1981); this might be more appropriate under the conditions of our study.

Using the criterion of 40^{o}C, animal #10 had a fever (105.2F) on day 0, the day that this animal was infected with BHV-1. We decided not to eliminate #10 from statistical analysis in this study, because his temperature was normal on day 1 and other clinical signs of illness were

absent. His temporarily elevated RT is not unusual; normal body temperature in cattle can range from 100 to 108 ^{o}F depending on activity, environmental temperature and diurnal variation (Hicks, 1985).

RT may be an indication of the severity of BHV-1 infection. Mean temperature increased gradually due to BHV-1 infection, peaking 5 days after inoculation. On day 0, (before BHV-1 inoculation), mean RT was 38.5^{o}C. For the first 6 days following inoculation, RT averaged 38.6, 38.8, 38.7, 39.1, 39.2, and 38.8^{o}C, respectively. This corroborates the clinical observation that the infection was most severe on day 5. In previous studies, BHV-1 infection appeared to peak day 4 or 5 as judged by rectal temperature, clinical signs, and BHV-1 titers in nasal secretions (d'Offay and Rosenquist, 1986, 1988).

Defense strategies versus virus growth. In inoculated animals, antibody and interferon production are associated with the growth cycle of BHV-1. Both are produced in direct response to the presence of BHV-1. The effectiveness of individual defense mechanisms depends in part on the method of spread of an individual virus, i.e., whether it spreads intra- or extracellularly. Intracellular spread can limit the effectiveness of antibodies which do not penetrate cells. Some defense strategies develop at an early stage following viral infection; these are important for limiting the extent of infection. Others are late to develop but may

be important either in recovery to infections or in maintaining immunity to subsequent infections.

BHV-1 replicates in the nucleus of infected cells. The virus starts to appear in the nucleus four to six hours post-infection, and then enters the cytoplasm. The infective virus particle is formed by addition of a virus-modified lipoprotein coat from the cytoplasmic membrane of the infected cell (Curtis et al., 1966). Virus antigens on membranes of BHV-1 infected MDBK cells appeared on some cells by 4 hours PI, and on all cells by 7 hours PI. Infective viruses can be isolated from infected cells at 9 to 10 hours PI, but are not released extracellularly until 2 to 3 hours later (Babiuk et al., 1975). Herpesviruses can spread either intracellularly (type II spread) or extracellularly (type I spread). In bovine cell cultures, BHV-1 initially spreads only by the intracellular route, but two hours later extracellular viral spread occurs also (Rouse et al., 1976). Lysis of infected cells is more important for the spread of BHV-1, which is unusually cytopathic compared to other herpesviruses.

Virus excretion. In our study, virus excretion was at high levels from day 2 to day 9 PI for all animals except for one calf which had completely eliminated the virus by day 9. Virus excretion was not measured on day 1. The peak mean virus titer occurred on day 4; lower titers were observed on days 5 and 7 (6.10, 5.33, and 5.70, respectively). The lower virus titer on day 5 compared to day 4

and day 7 titers may reflect the increased nasal discharge at this time. The nasal discharge was evident by day 4, and most profuse on day 5. Alternately, the decrease on day 5 may be due to differences in sample handling because BHV-1 is sensitive to drying and temperature.

Virus was first detected in the NS of calves 9 hours after intranasal inoculation with 10^9 PFU of BHV-1 (Cooper strain), and 14 hours after inoculation with 10^6 PFU (Lupton and Reed, 1980). Similarly, inoculation with 2 X 10^6 $TCID_{50}$ (50% total cell infection dose) of the vaccine strain resulted in high BHV-1 titers at one day PI (d'Offay and Rosenquist, 1988).

Experimentally, BHV-1 excretion in infected calves is limited to the first 7 to 11 days after infection (d'Offay and Rosenquist, 1988). However, the duration and extent of virus excretion may be affected by the initial virus dose, virulence of the virus, genetics, and disease and nutritional history of the calves.

Antibodies. In this study, we measured the serum antibody response to BHV-1 using two different analytical methods out of many possible procedures. Procedures for measuring antibodies in body fluids can be categorized as primary and secondary binding assays. The primary assays are the most sensitive and directly measure the binding of antibody to antigen. They include direct and indirect immunofluorescence assays, ELISA, and other enzyme immunoassays. Secondary binding tests tend to be easier to

perform, but they are less sensitive. They measure the outcome of antibody-antigen binding such as precipitation, neutralization, hemagglutination, and complement fixation or activation. They can measure from 10^{-3} to 10^{-7} mg of protein per mL of fluid compared to as little as 10^{-8} mg protein per mL using primary tests (Hietala, 1989).

In addition to the absolute quantity of specific antibody, any analytical method for an antibody is affected by the subclass and properties of the specific and nonspecific antibodies in serum; these change over time after an antigenic challenge. In respiratory tract infections, antibody titers in serum and nasal secretions can differ. The implication is that no single antibody method gives a comprehensive picture of the antibody response. Also, the analytical method and fluid selected for analysis is important. Typically in cattle exposed to a new antigen, the IgM class develops early, followed by the IgG class of antibodies. The IgM and the early IgG have relatively low affinity compared to the later IgG. However, IgA predominates in the seromucous secretions of the respiratory tract.

The **neutralizing antibody** is one of the various types of antibody produced (others include precipitating, complement-fixing, and hemagglutination inhibition), but it has the most specific antibody reaction against viruses. Thus, the neutralizing antibody test is one of the most sensitive, specific and clinically significant serological

tests (Peters and Bellanti, 1980). Agglutination methods readily detect IgM and are useful to detect early infection (Hietala, 1989).

Neutralization refers to the loss of infectivity of a microorganism such as a virus after its interaction with specific antibody. Viruses have antigens located on their outer surface which are important for attachment to host cells. Neutralizing antibodies in body fluids or an mucosal surfaces can prevent viral attachment (Osburn and Stott, 1989).

The serum neutralization test is widely used for measurement of antibodies to many human and animal viruses. Neutralizing antibodies can persist in serum for long periods and therefore can be used serologically to determine prior exposure to viruses; paired sera showing either a four-fold increase in titer or seroconversion are necessary to diagnose a current infection.

In our study, the neutralizing antibody response after virus inoculation was not different between treatments. Negative titers on day 0 indicated that the calves had not previously been exposed to BHV-1. Antibodies were not detected until day 14, similar to findings of Todd et al. (1972) and others, although very low levels of neutralizing antibodies were reported to occur as early as day 7 after vaccination with BHV-1 (Rouse and Babiuk, 1974). While one calf had not seroconverted by day 14, all calves had

neutralizing antibody titers on day 28 greater than or equal to titers on day 14.

Rossi and Kiesel (1976) found that calves produced neutralizing antibodies to BHV-1 in the IgM class followed by the IgG class in response to an i.m. injection of an attenuated strain of BHV-1. Also, during the first month PI, this antibody was primarily complement-requiring neutralizing antibody (CRNAb). No antibody was detected on either day 0 or day 4 PI. On day 9, CRNAb titers in the IgM class ranged from 32 to 512, and between 4 and 16 in the IgG class. In comparison, the titers without complement were much lower, between 1 and 16 for IgM and between 1 and 4 for IgG. On day 32 PI, antibody was primarily IgG and still predominatedly complement-requiring. Similarly, House and Baker (1970) observed a two to four-fold increase in titer when complement was used, equivalent to a .6 log increase in titer. They considered this of questionable significance compared to an error level in the test of .5 logs. In addition, they noted that the constant-serum varying-virus method of measuring neutralizing antibodies was 30 to 50,000 times more sensitive than the routinely used constant-virus varying-serum version which we used. Compared to neutralizing titers against some other viruses, bovine titers to BHV-1 tend to be relatively low, making it harder to detect treatment differences.

Our procedure for neutralizing antibodies would have measured only the non-CRNAb, because the sera were heat-

treated to inactivate complement as recommended by Carbrey et al. (1972). However, bovine serum is a poor enhancer of CRNAb; rabbit and guinea pig sera were equally good enhancers (Rossi and Kiesel, 1976).

Neutralizing antibody titers to BHV-1 are first detected after peak virus titers and reach maximal concentrations late after infection at about 19 days (Rouse and Babiuk, 1974), whereas the virus usually is cleared between day 7 and day 11 PI. Therefore, neutralizing antibodies may not be important in recovery, although perhaps they are important in preventing infection from subsequent challenges. Nonetheless, neutralizing antibody was successful in reducing virus dissemination in vitro more than 50-fold when rabbit complement was added every 2 hours (Babiuk, Wardley and Rouse, 1975).

Serum IgG antibodies to BHV-1 were measured by the enzyme-linked immunosorbent assay (**ELISA**). In the area of infectious diseases, the ELISA is widely used to measure antibodies both for individual diagnosis of viral, bacterial, and parasitic diseases, and for epidemiological assessment of viral diseases.

The basis of the ELISA is the use of an antibody or antigen which is linked to an enzyme, so that the complex retains both immunological and enzymatic activity. Thus, the technique utilizes the extraordinary ability of antibodies to discriminate among very closely related

antigens, while the high enzyme activity allows amplification of the reaction by up to 10^5 times.

The ELISA can be used to quantify any antibody if the appropriate antigen can be immobilized on a solid phase. Advantages of the technique include low cost, reagent stability, safety, sensitivity, and ease of procedure. However, for optimum reproducibility, ELISA tests must be rigidly controlled (Voller et al., 1980; Kurstak et al, 1984).

IgG titers could not be detected at day 0 or day 7 PI. Generally, the IgG response to an antigen appears 4 to 7 days and peaks between 1 and 3 weeks post-exposure (Hietala, 1989). On day 14, titers ranged from 100 to 800 in controls (mean 530), and from 100 to 1600 in +Vit calves. Four +Vit calves had titers of 1600, and the mean for +Vit calves was 1120. The treatment difference approached significance ($P = .115$). If the titer (400) for control calf #13 had been included in the analysis, the difference would have been significant at $P < .08$. Titers at 28 days were similar for controls and +Vit treatments (1870 vs 2400; $P > .05$). However, when 14 and 28 day titers for each animal were averaged, titers in +Vit calves were higher than titers in control calves (1760 vs 1180, $P < .09$). Vitamin treatment appeared to influence the early IgG response; this could be biologically more significant than the later response. It would have been interesting to have found at what time IgG

titers were detectable, and if there was a time difference due to treatment.

The correlation observed between neutralizing antibody titers and ELISA IgG titers is not surprising. Both measure late antibodies, specifically IgG for the ELISA and predominately (at 14 and 28 days) IgG for the neutralizing antibody test. Collins et al. (1985) observed a linear correlation between neutralizing antibody titers (range <1/2-128) and ELISA titers (range 1/20-20,240), demonstrating a 10 to 640-fold increase in sensitivity with the ELISA. While our data indicated that ELISA titers were about 100 times higher than neutralizing antibody titers, the ELISA was not necessarily more sensitive. However, it did detect IgG in the one animal which had no measurable neutralizing antibody titer at day 14.

The ELISA may be more useful in detecting treatment differences than the neutralizing antibody test, due to its higher sensitivity. Depending on the specific antibovine-Ig utilized, it can be adapted to measure either IgG, IgM, or IgA in serum or nasal secretions. Initial differences in immunoglobulin production, particularly in local IgA at the site of viral challenge, may be important in limiting viral spread and in assisting recovery. Therefore, antibody titers early in the infection prior to viral clearance would be more meaningful as an assessment of treatment effects than levels at 14 days or later post-infection.

Interferon. Interferon is an important early nonspecific immune response closely correlated with onset of recovery from BHV-1 infection. Interferons (IFN) are vertebrate proteins which are produced and released by cells in response to stimuli such as virus infection. IFN are biological regulators of cell function, with many diverse functions mediated through interaction with specific receptors on cell walls (Staeheli, 1990). They are extremely potent; the binding of one or a few molecules of IFN per cell is adequate to induce the many IFN-related effects (McMahon and Kerr, 1983).

IFN suppresses the growth of many DNA and RNA viruses through the action of numerous IFN-induced proteins acting in concert and synergistically at several stages of virus growth (Staeheli, 1990). IFN converts susceptible, uninfected cells into cells resistant to virus challenge. The antiviral state takes several hours to develop in response to IFN, and normally lasts for two to three days. BHV-1 induced IFN in nasal secretions has protected cattle from subsequent challenge with BHV-1 (Todd et al., 1972; Le Jan and Asso, 1980) and infection with rhinovirus and parainfluenza-3 virus (Cummins and Rosenquist, 1980; Cummins and Rosenquist, 1982).

In addition to their antiviral functions, IFN regulate natural and adapted immunity in many ways. They can enhance the cytotoxicity of immune T cells, NK cells and macrophages, stimulate lymphokine production, and alter

lymphocyte responses. IFN modulate antibody production, and affect differentiation of B lymphocytes. Many changes in plasma membranes and cytoskeleton of cells in response to IFN have been identified, many of which can affect immunity.

In our study, IFN was detected in nasal secretions of only 2 animals on day 2 PI. All animals had detectable IFN titers on day 4, with peak titers for both vitamin treatments occurring on day 7 PI. The relatively slow IFN response in this study is unusual; it may reflect the mildness of the viral infection due to use of a vaccine strain of BHV-1 and administering it by pipette rather than aerosol. In contrast, Cummins and Rosenquist (1977) noted the highest serum IFN titers occurred on day 1 after BHV-1 was inoculated intravenously at $10^{7.8}$ median tissue culture dose of virus/kg body weight. Intranasal infection with avirulent BHV-1 induced low IFN titers in NS at 40 hours PI, and maximal titers at 4 to 6 days PI in one study (Todd et al., 1972) and, in another study, measurable levels in 3 of 16 calves on day 1 PI, IFN titers in all calves by day 2, and maximal titers on day 7 PI (d'Offay and Rosenquist, 1988).

IFN titers tended to be lower on day 4 and higher on days 5 and 7 in the +Vit calves, but these differences were not significant ($P < .05$). The IFN concentration in early infection may be most important in limiting the severity of the infection, although IFN may be important in recovery after peak virus titers were reached on day 4 in both

treatment groups. However, interferons have numerous functions in regulating the immune response other than through their antiviral activity; these may be equally important in limiting infection and assisting in recovery. Also, many other nonspecific factors including numerous cytokines also may be important.

Lymphocyte blastogenesis. The lymphocyte blastogenesis test (LBT) often is performed in survey experiments as an indicator of cell-mediated immunity (CMI). The temporal appearance of reactivity based on in vitro tests of CMI has been correlated with the onset of recovery to herpesviruses in domestic animals (Rouse and Babiuk, 1978). Further, recrudescence of BHV-1 in cattle treated with corticosteroids has been associated with depressed CMI, as measured by the LBT (Davies and Carmichael, 1973).

LBT results are susceptible to large day to day fluctuations for individuals, nonspecific responses due to cross-reactivity or impurities of the test antigen, and the difficulties of maintaining cell viability in long-term cell cultures, especially microassay systems. Procedures and methods for reporting results suffer from the lack of standardization (Bellanti et al., 1980; Schultz, 1981). The presence of adherent macrophages is essential for maximal stimulation in this assay; failure to control macrophage numbers may be an additional source of variation.

Either mitogens or antigens may be used to stimulate the lymphocyte blastogenesis. Mitogens are non-specific

activators; within any system they may stimulate 80-90% of cells. Common mitogens include phytohemagglutinin (PHA), concanavalin A (Con A), pokeweed mitogen (PWM) and bacterial lipopolysaccharides (LPS). Based on work with other species, PHA and Con A are believed to induce primarily a T-cell response, PWM induces both a B and T cell response, and LPS induces a B cell response. However, various factors may influence the nature of the lymphocyte blastogenesis response (Schultz, 1981).

Some workers have used antigens rather than mitogens in order to generate a response specific to a virus or bacteria. For example, lymphocytes from cattle previously vaccinated or exposed to BHV-1, undergo blastogenesis in vitro when cultured with inactivated BHV-1. Lymphocyte blastogenesis was detected about 5 days after animals were intranasally infected with BHV-1, and peaked between 7 and 10 days PI (Davies and Carmichael, 1973; Rouse and Babiuk, 1974). Most antigens are T cell-dependent; therefore, the response is presumed to be a T cell response. But even if this is true, proliferation of T cells in vitro when exposed to an antigen may not correlate with CMI if the cells are primarily T helper cells, T suppressor cells, and(or) T memory cells, rather than T effector cells (Schultz, 1981). The LBT is often misused; it is not synonymous with CMI. It may have a relationship to CMI only if it measures a response by T effector cells.

In this study, variation within animals and sample periods was extremely high for the LBT; no clear trends were evident (Table 15). The stimulation index was significantly higher in the control calves in the initial sample taken 5 days after arrival (day -15) and on day 7 postinfection. Because the LBT was higher for controls initially, even before vitamin treatments began on day -11, it is difficult to attribute the significant difference in LBT on day 7 to vitamin administration.

The LBT procedure used involved the culture of the lymphocytes for 5 days in Dulbecco's Modified Eagle's Medium (DMEM) with fetal calf serum. DMEM contains various nutrients, including pyridoxine, while fetal calf serum contains variable nutrient levels depending on its source. Therefore, one major criticism of this assay for nutrition studies is that the provision of nutrients by the medium and serum could standardize the in vitro response. The in vitro response would be unrelated to the in vivo response if nutrition affected cell blastogenesis directly through availability of nutrients for replication rather than by affecting cell numbers, populations or other factors prior to stimulus. Only long-term deficiencies of some nutrients have been shown to affect lymphocyte populations or numbers in animals.

The effect of nutritional status on the LBT has been investigated for selenium in livestock. Pooled serum from selenium-supplemented lambs enhanced blastogenesis of

lymphocytes from both selenium-deficient and selenium-supplemented lambs (Turner et al., 1985). Similarly, the LBT was markedly depressed when cells from vitamin E/selenium deficient pigs were incubated with autologous serum, but blastogenesis was improved when fetal bovine serum was used instead (Lessard et al., 1991). Studies with cattle indicated that the selenium concentration in the culture as well as the ratio of cell numbers to selenium may be critical to the interpretation of in vitro assays of the immunomodulatory effects of selenium (Stabel et al., 1990).

In vitro assays, particularly the LBT which is unique in involving extremely rapid growth and division of lymphocytes, may amplify nutritional requirements of the lymphocyte, and thereby may be inappropriate and completely unrelated to in vivo effects. This would be of greatest concern in nutritional studies involving short term changes in nutritional status. (Previous research indicating depressed CMI using the LBT in certain nutrient deficiencies have involved chronic nutrient deficiencies and depression of lymphocyte populations. In short-term deficiencies, the lymphocyte numbers may not be altered, even though proliferation in vivo may be nutrient-related). To a lesser extent, this concern may apply to other immunoassays involving cell culture.

The LBT could be useful in nutritional studies if it could be adapted to assess how variation of nutrient concentrations in serum might impact on the ability of

lymphocytes or specific cell populations to undergo blastogenesis. One possible method would be to use an adapted medium lacking the nutrient(s) of interest, but supplemented with autologous serum from each animal tested. The lymphocytes used could also be autologous, or could be from a single animal. In order for this to work, normal serum from healthy animals would have to supply enough of the limiting nutrient to generate a proliferative response. This approach would allow one to investigate effects of limiting nutrients on cell proliferation, and determine which nutrients can alter proliferation.

In addition to the LBT, the other two major in vitro tests of CMI to viruses are the macrophage inhibition factor (MIF) assay, and lymphocytotoxicity. All three of these possible parameters of CMI are positive earlier than humoral immunity in cattle infected with BHV-1, and their appearance tends to coincide with recovery (Rouse and Babiuk, 1978).

The inhibition of macrophages (MIF) in vitro is correlated quite well with DTH to many antigens, but it is used infrequently to study CMI to viruses. The procedure depends on sensitized lymphocytes exposed to viral antigen releasing a soluble factor which inhibits macrophage or leukocyte migration. The MIF assay is extremely sensitive, but technical difficulties in performing the assay and nonspecificity due to qualitative differences in antigen preparations are drawbacks.

In vitro lymphocyte-mediated cytotoxicity tests have been developed for numerous viral agents including BHV-1. These tests involve the interaction of specifically sensitized lymphocytes with viral antigens on the surface of an infected tissue culture line, leading to the lysis of the infected target cells. This can be measured in several ways, such as by the release of ^{51}Cr from the cells (Bellanti et al., 1980).

As with the LBT, MIF and cytotoxicity assays may be inappropriate to measure in vivo responses to nutritional status. More appropriate in vivo tests would be desirable. In vivo tests of CMI include allograft rejection, delayed hypersensitivity skin test, and lymphocyte transfer. Some of the these techniques are useful in studies with laboratory animals using highly inbred lines; this has been impossible with ruminants. Delayed hypersensitivity skin tests have been used in some nutritional studies with cattle. Studies of CMI in ruminants would make the most rapid progress if a convenient bovine disease model could be identified in which CMI is shown clearly to affect infection or recovery. However, no clear CMI model for ruminants is available, unlike for mice in which CMI protects against ectromelia virus infection (Schultz, 1981).

Many consider CMI important in recovery from virus infections including BHV-1 while humoral immunity is thought most important in preventing reinfection (Rouse and Babiuk, 1978). Nonspecific aspects of immunity, both cellular and

humoral, may be critical in preventing initial infection and throughout the infection. For example, many cytokines including interferon are produced extremely early after infection and function to regulate the immune response, protect susceptible cells, and enhance cytotoxicity (Babiuk et al., 1991). Macrophages may be important in limiting viral dissemination (Roúse and Babiuk, 1978). Cytototoxic T cells sensitized to viral antigens reach peak activity about day 7 PI (Jakab, 1982), about the time virus titers begin to drop. Antibodies are detected at only low levels during virus recovery, peaking after the virus is eliminated. However, while low antibody concentrations are ineffective in neutralizing viruses, they may assist in recovery through antibody dependent cytotoxicity of antibody-complement cells lysis (Babiuk et al., 1975; Rouse et al., 1976a,b).

Conclusions

The BHV-1 infection in this study appeared to be very mild based on clinical signs. The virus successfully replicated in all calves, inducing an effective immune response in these calves. Previous research at O.S.U. has shown that effects of nutrition or management in stressed receiving calves are measurable only in batches of calves with a high incidence of morbidity to BRD. Similarly, it is possible that the mild infection in this study did not provide a rigorous test of treatment effects.

Further, the viral-induced suppression of the immune system is crucial to the pathogenesis of BRD in stressed

calves. Viral infections enhance adherence and colonization of bacteria in the respiratory tract, impair macrophage and neutrophil activity,and destroy the pneumocytes that secrete surfactants (Jakab, 1972; Babiuk, 1984; Yates, 1982). The effects of nutrition on this immune suppression are completely unknown. It also should be noted that the respiratory system is compartmentalized immunologically into upper, middle and lower areas with functional differences. Therefore, immune responses in the upper respiratory tract during BHV-1 infection may not relate to immune responses in the lung.

One limitation of this study was that the stress model used may have caused insufficient stress. Stress itself may greatly affect B-vitamin requirements in the shipping-stressed calf. Also, high plasma cortisol in stressed calves mediates immunosuppression through interaction with glucocorticoid receptors on leukocytes (Roth, 1985). High plasma cortisol levels cause altered function of neutrophils, macrophages and monocytes, and may affect concentrations of serum antibodies and various soluble immunoregulatory factors.

One role for vitamin B_6 is modulating the cortisol-induced effects of stress; vitamin B_6 may block cortisol the same way it affects other steroid hormones. Steroid hormones affect expression of specific target genes through interaction with specific receptor proteins to form a complex. This complex localizes to the nucleus, associating

there with specific DNA sequences to modulate expression of certain genes (Allgood et al, 1990). Pyridoxal phosphate affects biochemical properties of steroid hormone receptors, subcellular localization and DNA binding capacity of these receptors. Animal studies indicate that translocation of steroid receptor complexes from the cytoplasm to the nucleus is enhanced by a vitamin B_6 deficiency (Symes et al., 1984; Bunce and Vessal, 1987) and decreased by elevated vitamin concentrations (Holley et al., 1983).

Different immune mechanisms appear to function and vary in importance at different times in the disease cycle of BHV-1 and other respiratory infections. Numerous specific and nonspecific mechanisms exist and the importance of many have not been elucidated for BHV-1 infections. The effects of nutrition, particularly B-vitamins, on these mechanisms in cattle is unknown. Our study focussed on specific immune mechanisms except for interferon during a primary virus infection.

The implications of short-term versus long-term nutrient deficiencies should be considered. Although in this study we injected B-vitamins over a prolonged period, under practical conditions short-term nutrient defiencies (e.g. in the first 1 to 2 weeks after calves enter feedlots) appear most critical in affecting morbidity. B-vitamin dietary supplementation reduced the number of animals clinically diagnose as sick during the first 10 days of a trial (Zinn et al., 1987); similar results were reported

earlier by Cole et al. (1982). Perhaps investigations of nutrient interactions with immunity should focus on aspects of immunity which relate to the initial susceptibility to infection. Relatively late responses, such as neutralizing antibodies in this study, may be of lesser importance.

In summary, infection and immunity were not affected by B-vitamin treatment as measured by BHV-1 titers and interferon in nasal secretions, neutralizing antibodies in serum, and clinical signs including rectal temperature. However, IgG titers on days 14 and 28 were higher ($P < .09$) in the +Vit calves than in control calves, indicating that the humoral immune response was enhanced by B-vitamin treatment. The high animal to animal variation for each assay suggests that a greater number of animals should be used per group in order to better detect treatment differences. Due to the complexity of the immune response, it is difficult to determine which of the many assays available should be used to assess nutritional effects on immunity. In order to survey effects of individual nutrients on immunity in cattle, studies may need to be far more comprehensive in scope. In particular, it may be important to investigate various components of innate immunity, and functions of both nonspecific and specific phagocytic cells, in addition to humoral immunity.

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CHAPTER VI

EFFECTS OF B-VITAMIN INJECTIONS ON PLASMA VITAMIN LEVELS IN FEED-RESTRICTED WEANED BEEF CALVES BEFORE AND AFTER INOCULATION WITH BOVINE HERPESVIRUS-1

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ABSTRACT

Six- to eight-month-old beef steer calves, limit-fed and fasted for 3 days during a 20-day period after weaning, were inoculated intranasally with live attenuated bovine herpesvirus-1. A mild respiratory infection developed in all calves 4 to 5 days after inoculation. The calves were injected with a B-vitamin mixture and ascorbic acid on alternate days every second day for 2 weeks prior to and 2 weeks after inoculation. Vitamin injections increased plasma concentrations of ascorbic acid, folic acid, vitamin B_{12}, pantothenic acid, and vitamin B_6 ($P < .002$). Plasma concentrations of ascorbic acid, vitamin B_{12}, pantothenic acid, and vitamin B_{12}, but not folic acid, were markedly reduced at the time the BHV-1 infection peaked, 5 days after BHV-1 inoculation.

Introduction

Stress is an important factor in the development of infectious disease such as the bovine respiratory disease complex which causes major economic losses to the cattle industry. Cattle are particularly vulnerable to disease during the first weeks after entry into feedlots, partly because stress during the shipping/marketing process can compromise the immune response. Stresses such as shipping can elevate serum cortisol levels for 4 to 7 days (Von Tungeln, 1986). Cortisol and other glucocorticoids have been shown to suppress the immune response in cattle and other species (Roth, 1985; Golub and Gershwin, 1985).

Cortisol mediates immunosuppression through interaction with glucocorticoid receptors on leukocytes (Roth, 1985). It impairs the function of neutrophils, macrophages and monocytes, inhibits cell mediated immunity, and can alter the concentration of serum antibodies and soluble immunoregulatory factors.

Requirements for some water soluble vitamins, particularly pyridoxine, folic acid, pantothenic acid, and ascorbic acid, are believed to be increased by stress or disease (Mueller and Thomas, 1975). For example, the RDA Committee (1980) officially recognizes acute emotional and environmental stresses as conditions that increase the ascorbic acid requirement to maintain normal plasma levels. Requirements for vitamin B_6 also may be increased by stress, as it may modulate the effects of cortisol through binding

to cortisol receptors (Maksymowych et al., 1990; Allgood et al., 1991).

Both ascorbic acid supplementation and vitamin B_6 supplementation have helped to overcome cortisol-induced immunosuppression in man and experimental animals. Ascorbic acid improved the antibody response to SRBC in heat stressed or cortisol-treated chickens (Thaxton and Pardue, 1984; Pardue and Thaxton, 1984), reduced circulating glucocorticoids in chickens, guinea pigs and man (Nockels et al., 1973; Degwitz, 1987; Komindr et al., 1987), diminished some effects of dexamethasone on neutrophil function in cattle (Roth and Kaeberle, 1985), and potently stimulated in vitro lymphocyte blastogenesis at physiological concentrations (Panush and Dalafuente, 1979).

Plasma levels of vitamin B_6, folic acid, vitamin B_{12}, pantothenic acid and ascorbic acid were measured in cattle to investigate the effects of vitamin injections, restricted feeding and fasting, and BHV-1 infection.

Materials and Methods

Experimental Procedure. The experimental procedure was previously described (op cit Chapter 5).

Blood Samples. All blood samples were collected by jugular venipuncture in 10 ml evacuated glass tubes (Vacutainers, Sherwood Medical, MO). Five blood samples (3 with potassium EDTA as an anticoagulant and 2 with sodium heparin as an anticoagulant) were collected for B-vitamin analysis one

month before weaning and on days -15, -8 (before fast), -5 (end of fast), 0 (prior to BHV-1 inoculation), 7 (peak BHV-1 infection, and 14 (recovery from BHV-1). B-vitamin injections were discontinued and additional blood samples were collected on days 16 and 18 from +Vit calves only. When blood samples were taken for B-vitamin analysis on the same days that B-vitamins were injected, blood samples were obtained before injection. Cattle sometimes were sampled at 1 day after injection, and sometimes 2 days after injection (Table 16).

Vitamin Assays. Multiple samples of plasma were frozen at -20°C in appropriate aliquots for subsequent analysis of the water soluble vitamins. Samples were processed under very subdued lighting to minimize photodegradation. Heparinized plasma for vitamin C analysis was added 1:1 to 10% (w/v) metaphosphoric acid prepared immediately before use. These samples were vortexed and frozen immediately.

Vitamin C in heparinized plasma was measured using a colorimetric method involving 2,4-dinitrophenylhydrazine (McCormick, 1988).

Folate and vitamin B_{12} plasma concentrations were measured in K_2EDTA plasma using a radioassay kit for human samples (Quantaphase Folate and Vitamin B_{12} Radioassay, Bio-Rad Clinical Division, Hercules, California).

Pantothenic acid in heparinized plasma was measured using a sensitive indirect ELISA assay (Song et al., 1990).

Table 16. Sampling date for B-vitamins versus number of injections and time since last injection

Experimental day	Number of B-vitamin injections	Days since last injection
-15 (initial)	0	
-8 (before fast)	2	2
-5 (before fast	4	1
0 (BHV-1)	6	2
5 (peak BHV-1)	9	1
14 (end injections)	13	2
16 (after injections)	13	4
18 (after injections)	13	6

Each sample was assayed by two technicians, and results were averaged.

The B_6 vitamers were analyzed in heparinized plasma using ion-exchange reverse-phase chromatography with post-column derivitization (Coburn and Mahuren, 1983).

Statistical Analysis. Plasma vitamin data were analyzed by analysis of variance models using the main effects of vitamin treatment and collection day. The effect of treatment was tested within each sample period using the animal within treatment mean square as the error term.

Results and Discussion

Ascorbic Acid. Plasma ascorbic acid concentrations were similar for the two treatment groups on day -15, before B-vitamin injections began (Table 17). The decrease ($P < .39$) in plasma ascorbic acid in control calves between day -15 and day -8 may be related to the negative energy balance in these calves. However, plasma ascorbate did not change in control calves due to fasting. Possibly the initial ascorbic acid levels were temporarily elevated due to stress of sampling so that values did not reflect true plasma levels.

Stress due to handling and blood sampling is a common source of error in blood ascorbic acid levels for livestock species. Blood samples were taken after haltering the cattle and restraining their heads in order to take samples from the jugular vein. This procedure appears to be more stressful than sampling from a cannula which can be done

Table 17. Effect of experiment day and B-vitamin treatment on plasma ascorbic acid

Experiment Day	Treatment Mean (mg/dL)[a] Control	+Vit	SE	Probability
-15	.97	.89	.05	.26
-8	.70	.70	.03	.94
-5	.70	.82	.04	.09
0	.74	1.84	.10	.001
5	.38	.57	.06	.07
14	.65	.74	.07	.31
16	na[b]	.53		
18	na[b]	.51		
Contrast				
B-vitamins (d-8 vs d-15)	-.27	-.19	.06	.39
Fasting (d-5 vs d-8)	0.00	+.12	.05	.11
Refeeding (d0 vs d-5)	.05	1.03	.08	.001
BHV-1 (d5 vs d0)	-.36	-1.26	.09	.001
Recovery from BHV-1 (d14 vs d5)	.26	.14	.06	.14

[a] n = 6.
[b] na = Not analyzed.

without restraining the animal. Stressful procedures may elevate blood ascorbate due to release from the adrenal glands. After animals become accustomed to the handling, ascorbic acid levels do not respond.

In humans, the major excretory route for ascorbic acid is the urine. When plasma concentration exceeds about 1.4 mg/dL, substantial amounts of ascorbic acid appear in the urine (Jaffe, 1980). Plasma levels in man can be maintained as high as 4.0 mg/dL depending on the extent and frequency of ascorbic acid intake. The ascorbate body pool size in healthy adult human males is greater than 1400 mg; the range is from 900 to 17,000 mg. Daily turnover rate is about 60 mg/day (Moser and Bendich, 1991).

Plasma ascorbic acid in +Vit calves was not immediately increased by the injection of 1000 mg ascorbic acid every 2 days. Ascorbic acid is carried nonspecifically in the blood and is taken up by organs using specific transport mechanisms to accumulate ascorbate. On day -8, the +Vit calves had received 2 ascorbic acid injections, the most recent being 2 days earlier, but plasma concentrations were the same as in control calves (0.70 mg/dL). This could be explained by repletion of ascorbic acid in some tissues. Ascorbic acid levels are several hundred times higher in the pituitary, adrenal, and thymus and 10 to 100 times higher in small intestinal mucosa, lymph glands, lung, liver, spleen, white blood cells and various other tissues than in plasma.

At the end of the fast period (day -5), plasma ascorbic acid tended ($P < .09$) to be higher in the +Vit calves than in the controls. At this time the calves had been injected 4 times, most recently being one day earlier.

On day 0, plasma ascorbic acid was much higher ($P < .001$) in +Vit calves compared to controls, 1.84 vs 0.74 mg/dL, respectively. The magnitude of difference is surprising because calves previously had been injected 2 days earlier, and one would expect plasma levels to peak before 2 days post-injection (PI) and then diminish due to renal clearance and(or) catabolism. Again, tissue release due to stress may partly explain the treatment effect. On day 0 and day -1 (practice day), nasal swab and tampon samples were first obtained. The cattle were haltered and restrained for these procedures, and then restrained again for blood sampling. Control calves may have had low ascorbic acid concentrations in the adrenals, whereas +Vit calves may have had much greater concentrations. Therefore stress due to the restraint may have markedly increased plasma ascorbic acid levels only in the +Vit calves.

In both groups of calves, plasma ascorbic acid was reduced ($P < .001$) at the time of peak BHV-1 infection compared to on day 0, although levels tended to be higher in the +Vit calves ($P = .07$). The depression in plasma ascorbate due to BHV-1 has not previously been studied in cattle. However, in other species including man, ascorbic acid content of plasma and leukocytes has been reduced by

disease, reflecting increased requirements during the immune response.

Leukocytes contain very high levels of ascorbic acid compared to most tissues and plasma. Leukocytes can actively take up ascorbic acid during viral infection. Viral infections such as the common cold rapidly deplete ascorbic acid content of leukocytes (Wilson and Loh, 1973; Hume and Weyers, 1973). Ascorbic acid stimulates natural resistance. In vitro, ascorbic acid has been shown to enhance macrophage hexose monophosphate shunt activity, directed and random migration, and phagocytosis, and protect against the injurious effects of the toxic metabolites generated from macrophage activation (Ganguly et al., 1984). Ascorbic acid also enhances production of interferon, a complement component, serum immunoglobulins and antibodies in some animal and human models (Prinz et al., 1979: Siegel and Leibovitz, 1982).

Recovery from BHV-1 had occurred by day 14; indeed, clinical symptoms were evident only for several days (day 4 to 6) in most calves. Recovery from BHV-1 resulted in increased plasma ascorbic acid concentration in both groups of calves ($P < .001$). However, levels remained lower in controls than at any time prior to BHV-1 infection, and in +Vit calves compared to levels on days -5 and day 0. This suggests that either requirements continued to be elevated or that repletion of tissues or leukocytes was continuing at the expense of plasma.

After cessation of ascorbate injections, plasma ascorbic acid in +Vit calves dropped to levels lower than previously observed in control calves. In humans, increased ascorbic acid catabolism has been demonstrated for some time after discontinuation of macro-dosages of ascorbic acid. It has resulted in scurvy in humans receiving dietary ascorbate in normal amounts. Therefore, long-term administration of ascorbic acid at high levels should be used with caution. However, normal endogenous production of ascorbic acid by animals may greatly exceed normal human dietary requirements.

Folic acid concentrations are shown in Table 18. B-vitamin injections increased (P = .002) mean plasma folic acid concentration on day -8, and throughout the remaining part of the study. The folic acid dose was equivalent to 60 mg/day; this is estimated to be 15 times the requirement. In comparison, Girarde et al. (1989) injected 2 week old heifers with 2.5, 5.0, 10 and 20 mg folic acid, and successfully increased serum folate to 14.8 ng/mL, the level found in 4-month old heifers. Similar injections of 4-month-old heifers did not affect serum folate, possibly due to the low dosage used.

Girard et al. (1989) concluded that rapid renal clearance of excess folates maintained a constant serum folate of approximately 14 ng/mL in dairy heifers. The role of folate-binding proteins is not clear. Folate-binding proteins are found in serum at higher concentrations in

Table 18. Effect of experiment day and B-vitamin treatment on plasma folic acid

Experiment Day	Treatment Mean (ng/mL)[a] Control	+Vit	SE	Probability
-15	11.8	11.8	1.3	.97
-8	13.0	28.3	2.4	.002
-5	13.1	32.8	2.0	.001
0	10.1	410.8	64.3	.002
5	10.9	29.4	2.1	.001
14	12.2	28.2	2.6	.002
16	na[b]	38.0		
18	na[b]	24.7		
Contrast				
B-vitamins (d-8 vs d-15)	2.5	16.5	2.3	.003
Fasting (d-5 vs d-8)	-.1	4.5	3.1	.32
Refeeding (d0 vs d-5)	-3.0	378.0	65.0	.002
BHV-1 (d5 vs d0)	.8	-381.5	65.8	.002
Recovery from BHV-1 (d14 vs d5)	1.2	-1.2	2.2	.45

[a] n = 6.
[b] na = Not analyzed.

folate-deficient patients than in normal people; this may affect folate metabolism (Brody, 1991). Data from this study indicates that higher folic acid dosages than used by Girard et al. (1989) will increase plasma folic acid. However, the minimum dosage of injectable folic acid that will elevate plasma folic acid is not known. Perhaps folic acid was elevated only because of the extremely high dosages used. However, similarly high concentrations (26.0 ng/mL) were observed in feedlot cattle (Table 4).

In the control calves, plasma folic acid did not seem to be sensitive to feed intake, at least in the short term. Mean plasma folate was 11.8 ng/mL on day -15, after 5 days of restricted feeding. After an additional week of restricted feeding, it was 13.0 ng/mL. Following the 3-day fast, it was 13.1 ng/mL. Five days later, it was 10.1 ng/mL. Plasma folic acid did not appear to be affected by fasting, nor did it decrease gradually as a result of the continued restricted feeding.

BHV-1 infection did not decrease mean plasma folic acid in the control calves, which was 10.1, 10.9, and 12.2 ng/mL, respectively, for day 0, day 5 (peak BHV-1), and day 14.

The extremely high plasma folic acid concentration (410.8 ng/mL) in +Vit calves on day 0 is difficult to explain. These samples were taken 2 days after the previous B-vitamin injection. If plasma levels were increased more at 2 days instead of 1 day after intramuscular injection, similarly high concentrations also would be expected on day

14, but this did not occur. Samples were analyzed in several different runs, and at different dilutions, ruling out experimental error. The extremely high folate levels may be an anomaly related to a combination of factors such as high tissue levels, effects of stress during sampling, and(or) delay in processing of the blood samples. The folate concentration in erythrocytes of normal humans is at least 25 times higher than the serum folate concentration. Hemolysis must be avoided when measuring plasma or serum levels. If folate injections dramatically elevate erythrocyte folate concentrations, even minor hemolysis or leakage of folate from erythrocytes could affect plasma levels.

Because of the unusually high plasma folic acid for +Vit calves at day 0, the drop at day 5 may not have been caused by BHV-1 infection. The contrast for BHV-1 was signficant (P=.002) as folate increased in controls but decreased markedly in +Vit calves. If BHV-1 had increased folic acid requirements, plasma folic acid should have decreased in controls as well. Also, the mean day 5 plasma folic acid concentration was very similar to that for days -8, -5, and 14 (29.4 vs 28.3, 32.8, and 28.2 ng/mL, respectively). Therefore, it appears that plasma folic acid may not be affected by a mild BHV-1 infection.

Relatively high plasma folic acid concentrations (6.4 to 14.5 ng/mL) in stessed calves in the earlier study (Table 4) also suggest that folic acid status may not be seriously

depressed by stress and reduced intake. Nonetheless, it would be interesting to check folic acid status in sick feedlot cattle which have developed either an acute viral infection or bacterial pneumonia following a primary viral infection.

Vitamin B_{12}. Plasma vitamin B_{12} results are shown in Table 19. Short term changes in plasma B_{12} have not been investigated except in response to injection. The plasma B_{12} observations for the control calves are interesting. While the mean plasma B_{12} concentration in suckling beef calves was 229 pg/mL, the initial B_{12} concentration at day -15 following 6 days of restricted feeding was 298 pg/mL. Plasma B_{12} further increased on days -8, -5, and reached the maximum concentration on day 0 (301, 373, and 408 pg/mL, respectively). This can be explained by the observation that starvation tends to increase B_{12} concentration in liver and kidney (Andrews and Hart, 1962). Plasma concentrations therefore also should be increased by starvation because plasma, liver and kidney concentrations are positively correlated. Tissue vitamin B_{12} stores in cattle, similar to humans, may be a thousand times greater than daily requirements. Liver and other organs contain high concentrations; the volume of gut tissues, including liver, is decreased when cattle move to a lower plane of nutrition, and vice versa.

While weight loss increases plasma B_{12}, increased requirements would work in the opposite direction. This was

Table 19. Effect of experiment day and B-vitamin treatment on plasma vitamin B_{12}

Experiment Day	Treatment Mean (pg/mL)[a] Control	+Vit	SE	Probability
-15	298	290	32	.86
-8	301	442	49	.07
-5	373	713	49	.001
0	408	2165	202	.001
5	281	618	32	.001
14	376	733	40	.001
16	na[b]	665		
18	na[b]	566		
Contrast				
B-vitamins (d-8 vs d-15)	15	152	45	.06
Fasting (d-5 vs d-8)	57	270	23	.001
Refeeding (d0 vs d-5)	36	1452	158	.001
BHV-1 (d5 vs d0)	-127	-1547	193	.001
Recovery from BHV-1 (d14 vs d5)	94	115	23	.54

[a] n = 6.
[b] na = Not analyzed.

observed on Day 5, when plasma B_{12} dropped 31% in controls from 408 to 281 pg/mL. Plasma B_{12} concentration had recovered to 376 pg/mL at day 14, reflecting both decreased requirements and an increased supply, as feed intake was increased to maintenance levels from day 3 to day 14.

The role of vitamin B_{12} status in resistance to disease is not clear. Research in humans has centered on the autoimmune phenomena in pernicious anemia. Animal studies are limited due to difficulties in producing an absolute deficiency in experimental animals. However, B_{12} deficiencies in ruminants can occur within months of feeding a cobalt-deficient diet, whereas it can take several years of a vegetarian diet to produce a vitamin B_{12} deficiency in man. Consequently, the ruminant could be a useful model for B_{12} metabolism.

The reason for the fall in plasma B_{12} due to BHV-1 is not known. B_{12} requirements are interrelated with folate, methionine and choline requirements (Nauss and Newberne, 1981).

In +Vit calves, plasma B_{12} tended to be increased over that in controls at day -8 ($P = .07$) and plasma B_{12} was elevated ($P < .01$) in +Vit calves compared to controls on subsequent days. As occurred for plasma folate, B_{12} levels were extremely high on day 0 (2165 vs 408 pg/mL in +Vit vs control calves, respectively). Hemolysis does not appear to be a problem with plasma vitamin B_{12} analysis (Bio-Rad, 1989). The peak blood vitamin levels on day 0 in injected

calves may have occurred naturally due to the gradually increasing vitamin status. Once tissues were saturated, plasma levels can increase at a very high rate. If so, then the decrease in plasma B_{12} (and similarly, other B-vitamins) on day 5 indicates a drastic change in vitamin metabolism or distribution due to BHV-1. The plasma B_{12} concentration was higher (733 pg/mL) on day 14 than on day 5 (618 pg/mL), and similar to the means for day -5, day 0 and day 14 (373, 408, and 376 pg/mL, respectively). If there is a rapid-turnover B_{12} pool which is depleted during infection, plasma B_{12} levels in +Vit calves would be expected gradually to attain levels as high as were observed on day 0. Unfortunately, injections were terminated on day 14 so this could not be tested.

Pantothenic Acid. Plasma pantothenic acid concentrations appeared to be sensitive to restricted intake and fasting, and BHV-1 infection, but responded slowly to B-vitamin injection (Table 20).

Mean plasma pantothenic acid was relatively high (.190 nM) on day -15 in both control and +Vit calves, compared to means of .138 and .095 previously observed in suckling and stressed calves, respectively. In both groups, day 5 plasma pantothenate was lower than at any other time. In both groups, plasma pantothenate rebounded on day 14, possibly due partly to increased feed intake levels as well as recovery from BHV-1.

Table 20. Effect of experiment day and B-vitamin treatment on plasma pantothenic acid

Experiment Day	Treatment Mean (nM/mL)[a] Control	+Vit	SE	Probability
-15	.196	.183	.017	.60
-8	.137	.143	.015	.79
-5	.122	.156	.013	.11
0	.159	.771	.079	.001
5	.100	.113	.037	.75
14	.164	.179	.013	.42
16	na[b]	.19		
18	na[b]	.20		
Contrast				
B-vitamins (d-8 vs d-15)	-.059	-.040	.021	.53
Fasting (d-5 vs d-8)	-.014	.013	.014	.20
Refeeding (d0 vs d-5)	.030	.611	.074	.001
BHV-1 (d5 vs d0)	-.029	-.676	.090	.001
Recovery from BHV-1 (d14 vs- d5)	.033	.066	.043	.60

[a]n = 6.
[b]na = Not analyzed.

Vitamin B_6. B_6 vitamers were measured in plasma samples taken on days -15, 0, and 5 only (Table 21). Total B_6 was similar in both treatment groups before B-vitamin treatments began (day -15). However, pyridoxal phosphate (PLP) was higher and pyridoxal (PL) lower in control compared to +Vit calves. This may be due to variable hydrolysis of PLP to PL during a prolonged storage period (approximately one year). Stability of B_6 vitamers was a major concern due to lack of data on stability in bovine plasma. Freezing rat plasma at -20°C for 55 weeks did not affect B_6 content; however, these workers hydrolyzed the phosphate esters of the B_6 vitamers using a potato acid phosphatase before chromatography, so breakdown of PLP to PL was not assessed (Hefferan et al., 1986).

Dr. Coburn suggested that total B_6 as well as PLP may be depressed in the samples due to degradation in storage. Using a similar HPLC method, Coburn et al. (1984) measured plasma PLP concentrations of 402 ± 131 nM in 3 older calves, or 308 ± 67 nM measured enzymatically. PLP was the primary B_6 vitamer in calf plasma. Based on this limited evidence, our PLP and total B_6 concentrations appear very low.

Compared to samples from stressed calves analyzed simultaneously (Table 5), the day -15, 0 and 5 samples all are higher in total B_6, lower in PLP, and higher in PL. The stressed calf samples were stored only half as long. These data suggest that longer storage did cause hydrolysis of PLP to PL, but may not affect total B_6. Total B_6 was lower in

Table 21. Effect of experiment day and B-vitamin treatment plasma B_6 vitamers

Day	B_6 Vitamer	Treatment Mean(nM)[a] Control	+Vits	SE	P <
-15[b]	Pyridoxal PO_4	65.3	35.2	10.6	.07
	Pyridoxal	124.7	133.2	20.4	.78
	Pyridoxine	0.0	0.0	0.0	1.00
	4-Pyridoxic acid	25.0	24.8	2.2	.96
	Total B_6	215.0	193.2	23.5	.53
0[c]	Pyridoxal PO_4	56.8	55.7	10.7	.94
	Pyridoxal	93.2	645.2	42.4	.001
	Pyridoxine	0.0	1822.3	305.4	.002
	4-Pyridoxic acid	32.7	2060.0	133.5	.001
	Total B_6	182.7	4583.2	321.3	.001
5[d]	Pyridoxal PO_4	68.8	91.5	22.2	.49
	Pyridoxal	63.0	95.0	4.9	.001
	Pridoxine	0.0	9.7	6.8	.34
	4-Pyridoxic acid	23.2	51.2	10.7	.10
	Total B_6	155.0	247.3	26.4	.04

Contrasts and Interactions	PLP	PL	PA	PN	B_6
Day					
-15 vs 0 and 5[e]	.18	.001	.001	.006	.001
0 vs 5[f]	.13	.001	.001	.001	.001
BHV-1 x B-vitamin Interaction	.35	.001	.001	.003	.001

[a] n=6

[b] Before B-vitamin injections began.

[c] Calves inoculated intranasally with 10^7 PFU BHV-1 on day 0 after blood samples taken.

[d] Day 5 = Peak BHV-1 injection.

[e] Contrast compares B_6 vitamers before B-vitamin treatment (day-15) and after B-vitamin treatment began (days 0 and 5).

[f] Contrast compares B_6 levels before BHV-1 injection (day 0) and at peak BHV-1 injection.

stressed calves than in the day -15 calves (133 vs 204 nM). Evidently, severe stress incurred during the shipping and marketing process may depress vitamin B_6 status in calves.

The effect of B-vitamin injection was analyzed using the contrast day -15 versus day 0 plus day 5. B-vitamin treatment affected PL, 4-pyridoxic acid (PA), total B_6 ($P < .001$), and pyridoxine (PN; $P < .006$). The interaction of the effects of BHV-1 and B-vitamin treatment also was significant for these vitamers, so effects of B-vitamin treatment will be discussed separately for day 0 and day 5.

On day 0, PL, PN, PA and total B_6 all were higher ($P < .002$) in plasma from +Vit than control calves. PLP was not different ($P = .94$). Plasma from injected calves contained high levels of pyridoxine because pyridoxine hydrochloride was the B_6 source injected. It is converted into the other B_6 vitamers. Plasma from +Vit calves also contained extremely high amounts of 4-pyridoxic acid, a major B_6 metabolite in many species that is excreted in the urine.

Compared to day -15, on day 0 total B_6 was lower in control calves but higher in +Vit calves (interaction $P < .001$). Total B_6 may have fallen in controls due to diminished supply with restricted feeding because B_6 supply is correlated with intake (Zinn et al., 1987). Conversely, vitamin B_6 was supplied at eight times the estimated requirements by injection for +Vit calves. Because these estimates were designed to meet elevated requirements due to

stress and disease, they would be excessive for the relatively unstressed animals in this study.

Plasma and tissue concentrations of B_6 vitamers may be highly sensitive to changes in supply. Blood and tissue levels were reduced 10 to 90% in rats by feeding a B_6 deficient diet for only 2 weeks (Sampson and Connor, 1989). In contrast, fasting healthy male dogs for 40 hours decreased plasma PLP by 15%, and increased PL by 20%; baseline levels again were achieved by 48 hours after refeeding (Barnard et al., 1986).

Various studies indicate that plasma PLP is derived primarily from recent dietary vitamin B_6 intake, particularly pyridoxine, and little PL is recycled through the liver into PLP in plasma (Coburn et al., 1992). The similar PLP concentrations in the two treatment groups on day 0, and higher levels of PL in +Vit calves (645 vs 93 nM, $P < .001$) provide evidence that PLP in plasma was degraded to PL during sample storage.

Total B_6 and all vitamers were lower in both control and +Vit calves on day 5 than on day 0. The most striking observation was the almost complete absence of PN from plasma of +Vit calves; only one calf had a detectable content of PN (57 nM). Similarly, both 4-pyridoxic acid and total B_6 were extremely depressed by BHV-1 infection in +Vit calves compared to control calves. PL was depressed in both treatment groups as well.

These observations indicate that B_6 metabolism is markedly affected by infection. Plasma concentrations were reduced, B_6 excretion appeared to be reduced, and B_6 may be taken up by tissues (leukocytes) at the expense of plasma levels. This may indicate an adaptive process rather than a deficiency (Wilson and Davies, 1983). Alternatively, changes in vitamin status or metabolism due to a viral infection may contribute to the animal's subsequent susceptibility to other infections.

In conclusion, a very mild BHV-1 infection markedly depressed plasma concentrations of ascorbic acid, vitamin B_{12}, pantothenic acid, and vitamin B_6 but not folic acid, at 5 days post-infection. Restricted intake and a 3-day fast decreased plasma levels of vitamin B_6 and pantothenate, and increased plasma B_{12}, but did not appear to affect blood plasma folic acid and ascorbic acid concentrations.

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APPENDIXES

Appendix A. Effect of B-vitamin treatment on rectal temperature of calves before and after inoculation with BHV-1 on day 0

Treatment	Calf No.	Days								
		-20	-19	-18	-17	-16	-15	-14	-13	-12
Control										
	3	38.9	39.1	39.1	39.1	38.9	38.6	38.8	38.9	38.9
	5	38.9	38.9	38.9	39.1	39.0	38.7	38.8	38.7	38.7
	6	39.1	39.1	38.9	38.9	38.8	38.8	38.9	38.6	38.8
	8	38.9	39.0	39.0	39.0	38.8	38.6	38.4	38.8	39.0
	10	39.3	39.4	39.1	39.2	39.1	38.9	38.8	38.9	38.8
	11	38.7	38.9	38.8	38.9	38.8	38.7	39.1	38.7	39.0
L.S. Mean		38.90	39.02	38.93	38.87	38.88	38.6	38.80	38.64	38.79
B-Vitamin										
	1	38.9	39.1	38.9	38.6	38.6	38.6	38.6	38.4	38.8
	2	39.2	39.0	38.8	38.8	38.9	38.6	38.5	38.5	38.9
	4	39.1	38.9	38.8	39.1	38.3	38.8	38.6	38.7	39.1
	7	38.9	38.9	38.7	38.2	38.9	38.4	38.6	38.1	38.4
	9	38.7	38.9	38.7	39.0	38.7	38.6	38.7	38.5	38.8
	12	39.0	39.0	38.7	39.1	38.9	39.1	38.7	38.8	39.1
L.S. Mean		39.05	39.02	38.77	38.94	38.73	38.78	38.63	38.63	38.92
Mean		38.98	39.02	38.85	38.91	38.81	38.69	38.71	38.63	38.86
S.E.		0.08	0.06	0.04	0.11	0.08	0.08	0.07	0.09	0.08

Appendix A. Effect of B-vitamin treatment on rectal temperature of calves before and after inoculation with BHV-1 on day 0 (continued)

Treatment	Calf No.	Days								
		-11	-10	-9	-8	-7	-6	-5	-4	-3
Control										
	3	38.7	39.4	38.7	38.6	38.4	38.3	38.7	38.7	38.9
	5	38.9	38.7	38.6	38.2	38.2	38.1	38.3	38.4	38.5
	6	38.8	39.7	38.7	38.4	38.6	38.4	38.8	38.7	38.7
	8	38.8	39.4	38.9	38.6	38.6	38.1	38.8	38.9	38.7
	10	39.2	39.1	38.8	38.8	38.6	39.4	39.2	39.1	39.5
	11	38.9	39.1	38.5	38.5	38.6	38.2	38.8	38.6	38.8
L.S. Mean		38.79	39.20	38.63	38.44	38.53	38.29	38.65	38.60	38.72
B-Vitamin										
	1	38.4	39.3	38.7	38.2	38.6	38.4	38.5	38.3	38.3
	2	38.7	39.3	38.6	38.5	38.3	38.3	38.4	38.3	38.7
	4	38.7	38.6	38.8	38.3	38.2	38.2	38.4	38.6	38.2
	7	38.3	38.7	38.6	38.2	38.4	38.0	38.0	38.0	38.4
	9	38.6	39.3	38.6	38.2	38.3	37.8	38.5	38.6	39.0
	12	38.9	39.3	38.8	38.3	38.1	37.9	38.7	38.7	39.0
L.S. Mean		38.71	39.12	38.72	38.33	38.28	38.24	38.56	38.54	38.76
Mean		38.75	39.16	38.68	38.39	38.40	38.26	38.61	38.57	38.74
S.E.		0.08	0.12	0.05	0.07	0.07	0.16	0.11	0.10	0.14

Appendix A. Effect of B-vitamin treatment on rectal temperature of calves before and after inoculation with BHV-1 on day 0 (continued)

Treatment	Calf No.	Days								
		-2	-1	-0	1	2	3	4	5	6
Control										
	3	39.0	38.5	38.5	38.4	38.8	38.4	38.6	38.7	38.7
	5	38.7	38.4	38.7	38.6	38.8	38.7	40.2	39.1	38.5
	6	38.6	38.8	38.3	38.6	38.9	38.7	38.7	39.3	38.9
	8	39.1	38.7	38.6	38.8	38.9	38.7	39.1	39.8	40.1
	10	39.4	40.7	39.3	39.5	39.2	39.3	39.3	39.7	39.1
	11	39.1	38.7	38.2	38.9	39.2	38.8	38.5	38.7	38.6
L.S. Mean		38.88	38.68	38.46	38.65	38.92	38.71	38.88	39.15	38.93
B-Vitamin										
	1	38.5	38.3	38.3	38.3	38.4	38.5	38.8	39.9	38.4
	2	38.7	38.5	38.4	38.3	38.6	38.3	39.4	38.4	38.8
	4	38.9	38.4	38.6	38.7	38.8	39.1	38.3	38.7	38.4
	7	38.7	38.1	38.2	38.2	38.7	38.6	38.9	39.4	38.7
	9	38.9	38.6	38.5	38.4	38.9	38.8	38.8	38.5	38.2
	12	38.9	38.8	38.5	38.7	38.8	38.6	40.4	40.1	39.1
L.S. Mean		38.87	38.74	38.54	38.60	38.74	38.73	39.30	39.23	38.63
	Mean	38.88	38.71	38.50	38.62	38.83	38.72	39.09	39.19	38.78
	S.E.	0.10	0.25	0.12	0.13	0.07	0.12	0.27	0.25	0.19

Appendix B. Effect of B-vitamin treatment on rectal temperature of calves before and after inoculation with BHV-1 on day 0 (continued)

Treatment	Calf No.	Days 7	8	9	10	11	12	13	14
Control									
	3	39.0	38.6	38.5	38.7	38.9	38.6	38.3	N/A
	5	38.4	38.3	38.7	38.7	38.8	38.4	38.2	N/A
	6	39.2	38.6	38.8	38.9	39.2	38.6	38.4	N/A
	8	38.9	38.2	39.0	38.7	38.9	38.6	38.6	N/A
	10	39.2	38.3	39.1	38.9	39.0	38.5	38.6	N/A
	11	39.1	38.8	39.3	38.8	39.2	38.6	38.7	N/A
L.S. Mean		38.87	38.51	38.83	38.69	38.98	38.49	38.44	N/A
B-Vitamin									
	1	38.6	38.1	38.1	38.4	38.5	38.3	38.3	N/A
	2	38.6	38.3	38.5	38.5	38.5	38.2	38.4	N/A
	4	38.8	38.4	38.4	38.6	38.8	38.5	38.3	N/A
	7	38.5	38.2	38.6	38.2	38.9	38.3	38.4	N/A
	9	38.2	38.3	38.6	38.3	38.9	38.4	38.3	N/A
	12	38.9	37.9	38.9	38.8	39.1	38.7	38.6	N/A
L. S. Mean		38.66	38.19	38.56	38.58	38.81	38.46	38.42	N/A
	Mean	38.76	38.35	38.69	38.63	38.89	38.47	38.43	N/A
	S.E.	0.11	0.09	0.12	0.07	0.08	0.06	0.07	N/A

Appendix B. Effect of period and B-vitamin treatment on lymphocyte blastogenesis.

Day	Treat	Calf No.	Lymphocyte Blastogenesis Data: Blank (cpm)	Low S.I.	Medium S.I.	High S.I.
-15	Control	3	613.3	349.5	327.7	362.4
Initial		5	415.8	470.6	436.7	484.5
Sample		6	208.8	632.9	726.5	944.3
Before		8	161.0	215.7	278.1	321.0
Vitamin		10	336.2	532.7	515.6	541.4
Treatment		11	894.2	249.5	243.1	301.8
		Mean		408.7[a]	421.3[a]	492.3
	B-Vitamin	1	2079.5	51.8	48.3	52.1
		2	124.5	114.3	112.2	125.7
		4	182.2	221.8	262.0	323.6
		7	126.2	80.4	113.3	108.0
		9	261.8	355.4	346.7	515.0
		12	284.0	262.5	300.5	420.3
		Mean		181.0[b]	197.2[b]	257.5
		Mean		294.8	309.2	374.9
		S.E.		58.6	62.9	88.3
0	Control	3	70.5	15.7	21.8	39.2
Before		5	68.5	128.8	173.6	212.5
BHV-1		6	86.0	17.5	38.5	65.3
Inoculation		8	218.7	78.5	105.5	127.3
		10	157.7	41.9	60.0	107.5
		11	214.0	630.0	656.9	747.3
		Mean		152.0	176.2	216.5
	B-Vitamin	1[c]	80.5	2.18	1.47	2.93
		2[c]	63.0	1.57	0.97	1.83
		4[c]	109.7	5.90	6.37	8.39
		7	192.5	126.1	108.0	152.5
		9	114.7	201.0	301.1	774.7
		12	324.0	557.5	591.0	652.4
		Mean		294.7	333.3	526.7
		Mean		199.6	228.6	319.9
		S.E.		96.3	98.9	116.8

[a,b]Means in the same column with different superscripts differ ($P < .05$).

[c]Data omitted in statistical analysis due to very low stimulation indices for this animal.

Appendix B. Effect of period and B-vitamin treatment on lymphocyte blastogenesis (continued)

Day	Treat	Calf No.	Lymphocyte Blastogenesis Data: Blank (cpm)	Low S.I.	Medium S.I.	High S.I.
5	Control	3	195.7	929.8	1007.9	1234.1
After		5	107.0	1417.0	1584.5	2039.4
BHV-1		6	51.7	3154.8	3806.5	4487.6
Inoculation		8	176.2	323.6	267.3	454.2
		10	156.5	1006.4	1097.1	1220.9
		11	96.2	1628.2	1737.1	2149.3
		Mean		1410.0[a]	1583.3[a]	1930.8[a]
	B-Vitamins	1	183.5	42.9	58.5	64.6
		2	129.2	100.9	137.4	147.7
		4	119.0	224.4	266.2	263.5
		7	154.8	163.9	159.7	189.8
		9	450.7	495.4	505.5	558.8
		12	235.5	297.2	424.3	437.9
		Mean		220.7[b]	258.5[b]	277.3[b]
		Mean		815.3	920.9	1104.1
		S.E		282.7	351.7	407.1
7	Control	3	98.2	16.1	23.7	41.3
After		5	155.0	2.7	6.0	5.8
BHV-1		6	85.2	85.0	149.7	402.7
Inoculation		8	299.8	13.5	18.1	27.1
		10	146.2	383.5	506.4	784.8
		11	497.0	211.5	324.3	412.9
		Mean		142.0	204.4	333.8
	B-Vitamins	1	156.0	969.3	901.3	940.9
		2	109.0	17.4	25.5	44.8
		4	102.8	17.9	25.2	40.1
		7	100.5	571.8	509.3	728.9
		9	391.0	492.4	497.8	487.0
		12	81.2	578.2	1179.4	1930.0
		Mean		441.0	522.8	695.3
		Mean		305.1	378.1	531.0
		S.E.		119.8	151.9	230.8

[a,b]Means in the same column with different superscripts differ ($P < .05$).

Appendix B. Effect of period and B-vitamin treatment on lymphocyte blastogenesis (continued)

			Lymphocyte Blastogenesis Data			
Day	Treat	Calf No.	Blank (cpm)	Low S.I.	Medium S.I.	High S.I.
14 After BHV-1 Inoculation	Control	3	227.8	10.2	15.5	55.3
		5	95.2	506.5	746.0	1265.1
		6	76.0	370.0	590.7	1686.4
		8	190.3	57.3	61.6	48.2
		10	206.5	363.0	467.3	380.6
		11	147.5	1045.3	760.9	641.4
		Mean		391.8	440.5	679.3
	B-Vitamins	1	118.2	443.7	447.3	669.8
		2	153.5	26.4	30.3	30.9
		4	78.2	62.8	74.7	74.4
		7	136.8	226.6	185.0	148.8
		9	372.5	88.6	86.2	98.0
		12	145.2	705.6	598.1	344.5
		Mean		257.5	236.8	227.7
		Mean		324.7	338.7	453.5
		S.E.		132.4	116.3	205.3

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