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Use of the Relative Dose Response Assay to Determine the Vitamin A Status of Neonatal Calves: Effects of Season on Colostrum Quality and Immunological Parameters

Mary Jo Boner

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VITAMIN A STATUS OF NEONATAL CALVES: EFFECTS OF SEASON ON
COLOSTRUM QUALITY AND IMMUNOLOGICAL PARAMETERS.

BY

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This thesis is approved as a creditable and
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ABSTRACT

USE OF THE RELATIVE DOSE RESPONSE ASSAY TO DETERMINE THE VITAMIN A STATUS OF NEONATAL CALVES: EFFECTS OF SEASON ON COLOSTRUM QUALITY AND IMMUNOLOGICAL PARAMETERS.

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Plasma vitamin A is not a reliable indicator of vitamin A status. Therefore, the feasibility of using the relative dose response (RDR) assay as a method for determining vitamin A status of calves was examined. In addition, effects of colostrum quality and season on vitamin A status and health of calves were determined.

Blood was sampled from the jugular vein of 16 newborn calves to establish values over a 24 h period for immunoglobulin M (IgM) concentration, hematocrit, serum protein concentration, and leukocyte differentials. Liver biopsies were performed at birth, and successfully completed for 11 calves. Blood samples were obtained prior to feeding colostrum at 0, 12, and 24 h, with 0 h being the first feeding of colostrum. Blood samples also were obtained at 5, 6, 7, 8, 17, 18, 19, 20, and 22 h after the first colostrum feeding.

Blood plasma, liver, and colostrum samples were analyzed for vitamin A content using a spectrophotometric method. Based on peak vitamin A content, the RDR was calculated for 6, 8, and 20 h after the first feeding. Colostrum vitamin A was correlated with the 6 h RDR ($R=.49$, $P<.05$) and the 8 h RDR ($R=.51$, $P<.04$). Liver vitamin A concentrations were not correlated with the RDR assay at either time. Colostrum quality, as relates to both Ig and

vitamin A concentration, is extremely important for newborn calves and may help to alleviate the effects of season.

Serum protein concentrations were highly correlated ($R=.75$, $P<.0001$) with serum IgM concentrations for calves born in winter but the correlation was lower for calves born in the summer ($R=.48$, $P<.0001$). Serum IgM concentrations plateaued in summer at 12 h but continued to increase through 24 h for winter. Serum IgM concentrations were higher for calves born in winter than calves born in summer ($P<.03$). Hematocrit values decreased over time and cell differentials were similar to previously reported values for neonatal calves.

Calves are deficient in vitamin A at birth and throughout the first 24 h of life. Plasma vitamin A status of neonatal calves was affected by the amount of vitamin A present in the colostrum that they were fed. Also, indicators of immune status of neonatal calves, such as serum Ig and colostral Ig, were affected by season with both being greater in winter than in summer.

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LIST OF ABBREVIATIONS

CRABP = Cellular Retinoic Acid Binding Protein

CRBP = Cellular Retinol Binding Protein

CRBP I = Cellular Retinol Binding Protein type I

CRBP II = Cellular Retinol Binding Protein type II

ELISA = Enzyme Linked Immunosorbent Assay

Ig = Immunoglobulin

IRBP = Inter-photoreceptor Retinol Binding Protein

LPL = Lipoprotein Lipase

RBP = Retinol Binding Protein

RDR = Relative Dose Response

TTR = Transthryretin

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INTRODUCTION

Vitamin A plays several important metabolic roles within the body and is an important determinant in the health of neonatal calves. The importance of vitamin A has also been demonstrated by the protection afforded by the keratin-producing cells. Vitamin A controls epithelial and immune functions and regulates keratin secretion which could prevent several types of infections including diarrhea, upper respiratory infections, and genitourinary infections.

Concentrations of vitamin A need to be maintained above certain levels in the body. The adequate levels of vitamin A have been reported to be approximately 20 μg/dl of serum and 20 μg/g of liver for a newborn calf. Serum vitamin A concentrations, however, are generally maintained above 20 μg/dl in cattle until liver stores are practically depleted.

Because serum/plasma vitamin A concentrations are poor indicators of liver vitamin A status, the relative dose response assay was proposed. It determines the magnitude of increase in the serum retinol concentration after administering a dose of vitamin A. The change in serum retinol from time 0 to time 5 is divided by the time 5 serum level of retinol and expressed as a percent. It is based on the principle that if stores of liver retinol are high or adequate, plasma retinol levels are little affected by an oral dose of vitamin A but if liver stores are low, plasma retinol levels will increase after an oral dose.

The chief source of vitamin A for the newborn calf is colostrum. Colostrum is also an important source of other factors that are essential for neonatal calves.

Calves are born with very little ability to fight off a challenge to their immune system. As a result, it is imperative that they receive protection through the passive immunity capabilities of a good quality colostrum.

The degree of immunity that a calf is afforded by colostral dosing is directly related to the amount of immunoglobulin absorbed from colostrum. The higher the amount of immunoglobulin present in serum, the higher the amount of disease resistance.

Several studies have reported that there are a number of factors that affect serum immunoglobulin concentrations of calves. Some of these include: the age of the calf at the time of sampling, the parity of the dam, the degree of calving ease, the month of birth, and the temperature on the day of birth.

LITERATURE REVIEW

GENERAL BACKGROUND

Retinoids is a term used to encompass the following forms of vitamin A: retinol, retinaldehyde, retinoic acid, plus synthetic analogs that are related to retinol (52).

Retinol is the parent molecule of the vitamin A family (52). It is a 20 carbon unit compound with 5 double bonds and a single hydroxyl group (50). Retinol serves not only as the major transport form of vitamin A but also as the major storage form of vitamin A in the liver (as well as lower concentrations in other tissues).

Retinaldehyde, or retinal, is the major dehydrogenation product of retinol (39). It is also unique in the sense that it is the color pigment for the visual protein, rhodopsin (50, 52).

Retinoic acid is a lesser metabolite of retinol and ß-carotene (39). Retinoic acid belongs to the category of steroid and thyroid hormones that regulate metabolism, growth, and embryonic development. It has been shown to be important in the chemical reactions of living organisms for maintaining differentiation of cell types (50).

Retinoic acid, retinaldehyde, and retinol differ only in their chemical side groups (50). Of the retinoids, retinol, its esters, and retinaldehyde can all be changed from one form to the other and are of equal nutritional importance. The further oxidation of the aldehyde group (retinaldehyde) to the carboxyl group (retinoic acid) is irreversible.

Over the years, vitamin A has proven to be essential for the proper functioning of several biological processes.

Some of these processes include: growth (34, 45, 51, 56, 58, 70), cellular differentiation (34, 51, 56, 58), cell-to-cell and cell-to-substrate interactions (51), reproduction (34, 45, 58), vision (45, 56, 58), and immune function (45).

There are several physiologic factors that can increase the vitamin A requirement of animals. These include stress, disease, and high production (34). There are also several environmental factors that can increase the vitamin A requirement of animals. These include inclement weather, overcrowding, and uncomfortable boarding (26).

Vitamin A deficiency has been linked to several health problems. It has been reported to cause anemia in rats, even when there are sufficient stores of iron in the liver (25). Vitamin A deficiency has been linked to an increase in the risk of contracting cancer and it enhances the sensitivity of epithelial tissues to carcinogenesis by chemicals, irradiation, and viruses (48). Other research however, points to the carotenoids as preventing cancer by themselves or after their conversion to retinol (19).

Vitamin A is well known for its property as an anti-infective agent (6, 58, 69). A deficiency of vitamin A has been associated with an increase in vulnerability to microbial infections and seriousness of parasitic infestations (37, 45, 51, 58). An example of this is that vitamin A deficiency facilitates bacterial, viral, parasitic, and rickettsial infections (64).

Vitamin A is critical for the development and functioning of T and B lymphocytes (6, 48, 70), hence,

vitamin A deficiency impairs the cell-mediated branch of immunity as well as the humoral branch (69, 70). Associated with this is a breakdown of the mucosa-associated lymphoid tissues whose primary role is to provide protection at all mucosal surfaces (58). One of the changes that takes place is that mucus-producing cells are replaced with keratin-producing cells (69). Examples of infections that could occur include: diarrhea, upper respiratory tract infections, genitourinary infections (58, 69), reproductive infections, and gastrointestinal infections (37).

A problem related to mucus-producing cells that plagues dairy farmers may be somewhat contributable to vitamin A deficiency. Cows that are low in vitamin A may show elevated levels of mastitis (17, 34, 37). The reasoning lies in the role of vitamin A to control epithelial and immune functions and in regulating keratin secretion of the teat canal and preventing the establishment of mastitis producing organisms.

Young calves commonly suffer from respiratory and enteric disease (34). During these diseases, there is an increased demand for cell renewal and in turn, an increased demand for vitamin A because of an increased utilization rate of the vitamin.

By administering supplemental vitamin A along with certain B vitamins, the occurrence of diarrhea and the rate of mortality has been shown to be reduced (47).

Other impairments of the immune system due to a vitamin A deficiency include: impaired antibody production (45), decreased contact sensitivity to antigen, depressed

natural killer cell activity, reduced lymphocyte proliferation induced by mitogens and antigens, and impaired delayed-type hypersensitivity (70).

Conversely, supplementation with vitamin A above normal levels has been shown to enhance the immune response (6,58). Retinoic acid supplementation increased the growth of granulocyte/macrophage precursor cells, increased phagocytic activity, and increased surface marker expression on immature monocyte/macrophage human cell lines (48). It was also noted that vitamin A was inhibitory towards the replication of the AIDS virus, in vitro (48).

Another facet of the capabilities of the retinoids is their ability to enhance IgM secretion from bovine lymphocytes in culture (45). Evidence has been found that all-trans-retinoic acid at 10^{-10} M and all-trans-retinol at 10^{-6} M increases Ig secretion by B cells that are stimulated by antigens or mitogens. This demonstrates the ability of changing tissue and plasma concentrations of retinoic acid and retinol to affect the immune system.

When 13-cis-retinoic acid was increased from 10^{-9} to 10^{-6} mol/L in culture, there was a decrease in the degree of enhancement of IgM secretion (44). This suggests that by supplementing retinoic acid at high dosages, mononuclear leukocyte function may be impaired.

Further research was performed using injections of the 13-cis-retinoic acid isomer which indicated that there was an increase of retinoic acid isomers in plasma but retinol and ß-carotene plasma concentrations were not affected (29). It was also suggested that the 13-cis-retinoic acid isomer provided the precursor pool from which all-trans-

retinoic acid (the active form of vitamin A at the molecular level) can be derived.

In a companion study, supplementation with 13-cis-retinoic acid at .4 mg/kg of body weight/day for 7 days had little effect on mononuclear leukocyte populations or serum Ig concentrations (28). It was noted however, that the degree of supplementation used may not have been high enough to illicit the expected immunological response.

METABOLISM OF VITAMIN A

The major natural sources of dietary vitamin A include long-chain retinyl esters found in animal tissues and carotenoid pigments that come from certain plants (32). An example of these pigments is ß-carotene.

Most of the conversion of ß-carotene into retinol takes place in the intestinal mucosa. There are two soluble enzymes, ß-carotene-15,15'-dioxygenase and retinaldehyde reductase, that are involved in this biosynthetic process.

The first enzyme catalyzes the cleavage of ß-carotene at the central double bond, which yields two molecules of retinal (8, 32, 65). Retinal is then reduced to retinol by retinaldehyde reductase.

The conversion of retinyl esters into retinol takes place in the intestinal lumen. Retinyl esters are hydrolyzed by pancreatic esterases and the resulting retinol is absorbed into the mucosal cell. Absorption takes place by facilitated diffusion when retinol is present in physiological concentrations or by passive diffusion when present in pharmacological amounts (8).

Retinol is re-esterified with long chain fatty acids while in the mucosal cell (32, 65). This reaction is brought about by the association of retinol with a fatty acyl-CoA ester and is catalyzed by an acyl-CoA:retinol acyltransferase enzyme (32).

Carrier proteins within the cell help to shuttle the retinol molecules between the plasma membrane and the endoplasmic reticulum of the enterocytes for esterification and then incorporation into the core of chylomicrons (8). This carrier protein has been termed cellular retinol-binding protein type II (CRBPII). It has a molecular weight of 14,600 daltons and carries a single molecule of retinol as its endogenous ligand (50).

After being processed in the Golgi apparatus, the chylomicrons are exocytosed from the basolateral surface of the enterocytes (8). They are transported through the mesenteric and then the thoracic duct lymph into general circulation.

During chylomicron catabolism a number of processes take place, which ultimately produce chylomicron remnants (8). One of these processes includes the hydrolysis of chylomicron triacylglycerols by lipoprotein lipase (LPL), an enzyme found on the luminal surface of capillary endothelial cells. During this process, other components of the chylomicron, such as phospholipids, apolipoproteins, unesterified and esterified cholesterol, carotenoids, and certain fat-soluble vitamins are transferred to other lipoproteins or cell membranes.

Most chylomicron remnants are rapidly and efficiently taken up by liver parenchymal cells (8). Uptake involves a

receptor-mediated process where apolipoprotein E is reported to be involved (32). After uptake into the liver, hydrolysis and reesterification of the retinyl esters takes place (7, 8, 32), retinol is secreted bound to RBP (8), and then is transported to the endoplasmic reticulum (7, 8) by CRBP I or II (8). Retinol binding protein is found in high concentrations in the endoplasmic reticulum of liver cells (7, 8) and when retinol binds to RBP, retinol-RBP moves to the Golgi complex, followed by secretion of retinol-RBP from the cells (7, 8).

Vitamin A status determines whether retinol is transferred to stellate cells for storage as retinyl esters or if it will escape this uptake process and make its way into general circulation (8). About 80-90% of the total retinol in the whole liver is contained in stellate cells.

Retinol binding protein is not only found in both parenchymal and stellate cells, but is synthesized in the former and presumably so in the latter (7, 8). Retinol is mobilized from liver bound to RBP and transported in plasma (8). While in the plasma, most of the protein circulates as the RBP complex (32).

Retinol-binding protein has a 3-dimensional structure which suggests that it should contain a hydrophobic pocket that is able to bind one molecule of retinol (7). Retinol binding protein has a molecular weight of approximately 20,000 daltons and plays a number of physiological roles (59).

The first of these roles is that it serves to solubilize the water-insoluble retinol molecule and serve as a vehicle for transportation of retinol from the liver

to peripheral tissues. Secondly, it serves to protect retinol from oxidative damage while in the plasma. Thirdly, RBP is important in the regulation of vitamin A mobilization from the liver. Finally, RBP may direct the delivery of retinol to specific sites at the surface of vitamin A requiring cells.

Most of the retinol-RBP that is found in the plasma is complexed with another protein, transthyretin (TTR) (7, 65). The normal level of RBP in plasma is approximately 40-50 μg/ml whereas that of transthyretin is about 200-300 μg/ml (32, 59). A review by Blomhoff et al. (8) states that "TTR seems to inhibit the binding of RBP indicating that retinol-RBP (rather than retinol-RBP-TTR) is the vehicle that delivers retinol to the receptor".

The mechanism for cellular uptake of plasma retinol is not fully understood (7). Retinol may partition into the plasma membrane of cells without the use of a cell surface receptor, as indicated by a small amount of free retinol that may be in equilibrium with retinol-RBP in plasma. Another possibility might be that retinol may enter cells as a result of fluid-phase endocytosis. There have also been indications that a cell surface receptor for RBP does exist (7, 8). It is not clear, however, whether retinol is delivered by RBP to tissues after receptor-mediated endocytosis or if the RBP receptor acts as a transporter for retinol (7).

There are a number of important retinol-binding proteins that are necessary for retinol uptake into their respective target tissues (7). There is an inter-photoreceptor retinol-binding protein (IRBP) that has been

identified between the retinal pigment epithelial cells and the photoreceptor cells (7, 9). The IRBP not only binds retinol, but also binds retinal, vitamin E, fatty acids, and cholesterol. Tear fluid contains retinol that is bound to a protein closely related to RBP (9). Conceptus RBP ensures that an optimal amount of retinol is delivered to the embryo. Epididymal-binding protein 1 and 2 are carrier proteins for retinoic acid in the epididymis. Intracellular retinol and retinoic acid binding proteins (CRBP and CRABP, respectively) are the predominant intracellular retinoid-binding proteins in most tissues (7).

Cellular retinol binding protein type I is concentrated mostly in the liver, lungs, kidneys, epididymis, and testis, whereas CRBP II is more restricted. Cellular retinol binding protein I limits or maintains the availability of unesterified retinol in the cytoplasm thereby serving an important buffering function (52). Cellular retinol binding protein II differs from CRBP I in that it is the product of a separate gene, is found abundantly in the intestinal mucosa, and has a different structure (50). Cellular retinol binding protein II may have a role in the uptake of dietary retinol and carotene. Both CRBP I and II are central to the direction of retinol metabolism in both the intestine and liver (52).

Cellular retinoic acid binding protein sequesters retinoic acid and limits its distribution and biological effects (52). It functions in the control of retinoic acid movement into the nucleus and regulation of the nuclear concentration of retinoic acid (50). Cellular retinoic

acid binding protein is important in establishing
equilibria between cytoplasmic and nuclear concentrations
and also between CRABP-bound and retinoic acid receptor-
bound retinoic acid in the nucleus. Cellular retinoic acid
binding protein is mostly found in the testis, skin, and
eyes (7).

After uptake of retinol by target cells, there are 3
main processes of intracellular metabolism (9). Temporary
storage of retinol may occur after its conversion to
retinyl esters, or retinol may be converted directly into
an active metabolite (such as retinoic acid), or retinol
may be catabolized to a form for excretion.

The process by which retinoic acid is formed from
retinol is not fully understood (9). One item which has
been determined is that CRBP I is the acceptor for retinol
after cellular uptake from the plasma (8, 9, 39). While
this is true, the assumption has been made that retinoic
acid is synthesized in two steps (9). The first step
involves the dehydrogenation of retinol to retinal by
alcohol dehydrogenases. The second step is the further
oxidation of retinal to retinoic acid. The first of the
two steps is reversible while the second is irreversible
(50).

The majority of retinol that leaves the plasma is
recycled (9). The average recycling time for normal
retinol reserves, in a rat, may take up to a week or more
(9). About 50% of plasma retinol turnover is to the
kidneys, 20% is to the liver, and 30% is to
extrahepatic/extrarenal tissues.

There are many questions about retinol recycling that cannot be answered at this point and time. It is an area that is sure to provide many research opportunities.

Excretion of vitamin A and carotenoids occurs as inactive metabolites in the urine and bile (65). They consist mostly of decarboxylated products. Excretion of the unabsorbed dietary fraction (especially ß-carotene), takes place in the feces.

DETERMINATION OF VITAMIN A STATUS

Liver contains over 90% of the total body vitamin A stores (3). Therefore, the best estimate of vitamin A status comes from liver biopsies (3, 15). Because performing liver biopsies isn't always feasible, other methods of determining vitamin A status have been developed.

In the past, serum or plasma vitamin A concentrations have been relied on heavily to determine the vitamin A status of an animal (42). There is considerable evidence, however, that serum or plasma vitamin A concentrations are not true indicators of vitamin A status (3, 12, 54, 55, 66).

Low body stores of vitamin A are detected reliably only when the plasma retinol concentration is less than 10 μg/dl (3). Normal concentrations of retinol may exist in plasma despite low concentrations of retinol obtained during liver biopsies. This is due to the fact that the liver will keep plasma retinol concentrations within a relatively narrow range until the liver has exhausted its store of vitamin A. Body stores of vitamin A in humans are

considered to be low when liver vitamin A concentrations
drop below 20 μg/g and are considered to be normal when
liver vitamin A concentrations are above 20 μg/g (3, 15).
In another study, body stores were considered to be
adequate, below average, or depleted when liver vitamin A
concentrations reach ≥58 μg/g, 30 μg/g, and 14 μg/g,
respectively (1). Because serum or plasma vitamin A
concentrations are poor indicators of vitamin A status, a
procedure known as the relative dose response (RDR) assay
has been proposed and to a lesser extent researched.

The RDR assay provides an indication of vitamin A
stores in the liver, unlike serum retinol concentrations,
and is regarded as a good indicator of vitamin A status
(49). Thus, it can be used to detect subclinical vitamin A
deficiency.

The RDR for rats and humans is defined as the
magnitude of increase in the serum vitamin A concentration
at 5 h after administering a dose of vitamin A compared to
the base-line (1, 3, 15, 27, 36, 42, 49, 53, 66-68). The
change in serum vitamin A from time 0 to time 5 is divided
by the time 5 serum level of vitamin A and expressed as a
percentage, RDR=$(A_5-A_0)/A_5*100$. The RDR is based on the
following principle: if stores of liver retinol are high,
plasma retinol levels are little affected when vitamin A is
orally administered but if liver reserves are low, plasma
retinol levels increase after an oral dose (1, 3, 36, 42,
49, 53).

The RDR assay results are reported as percentages.
The percent changes which indicate an adequate supply or
deficiency of vitamin A differ among studies. Increases of

14% to 40% may indicate vitamin A deficiency (1, 3, 15, 27, 36, 53) and an increase of less than 20% (15) or 10% (3) would indicate an adequate vitamin A supply. Another source reported that percent changes above 50% are indicative of marginal vitamin A status and that levels less than 40% indicate adequate storage (67).

Research has shown that a response in plasma vitamin A concentrations in cattle was seen 20 h after dosing with vitamin A (66, 67). This 20 h required in cattle to show the rise in plasma vitamin A is longer than for non-ruminants (67). Rats and humans require 5 h (40) and horses require 4 h (36). The longer response time for cattle for the RDR assay can be attributed to a slower rate of passage in ruminants.

Several factors can affect the RDR test results: dietary factors (36), absorption of vitamin A (36, 53, 66), RBP synthesis in the liver (36, 53, 66), mobilization of RBP (36, 53, 66), vitamin A status itself (36), rumen microbial population (67), and liver uptake of vitamin A (53, 66).

Additionally, there are several factors that can affect the uptake of vitamin A and have a direct impact on the results of a RDR test. Protein-energy deficiency (27, 53, 66, 68), liver dysfunction (53, 66), and vitamin E, zinc, and selenium content of the diet (66) can all affect uptake of vitamin A.

The validity of the RDR is questioned in specific cases such as malnourished children (49). Even in such a specific case as this, others have reported that the RDR test can be effectively applied under field conditions to

detect children who are at risk of vitamin A deficiency (27). Another study reported that the RDR test is invalid when used among sick patients (53). According to this study, the RDR test would only work when used on healthy individuals.

More papers than not validate the use of the RDR test. They have considered it to be a reliable, sensitive, and useful indicator of vitamin A status in rats (68), adults (1, 15), children (3, 15), horses (33), and possibly cattle (67).

THE IMPORTANCE OF VITAMIN A IN CALVES

Previous research has shown that there are little or no vitamin A reserves present in newly born calves (11, 60). It is also known that if there is a lack of vitamin A or it's precursor, ß-carotene in a pregnant cow's diet, the resulting calf may be stillborn, blind, or weak (18). This indicates that the prepartum diet does influence the vitamin A content of the fetal liver, although there have been other studies that refute this (11, 60).

Fetal calves require a minimal level of vitamin A for normal growth (11). Herdt and Stowe (34) have reported the adequate ranges of vitamin A to be approximately 20 μg/dl of serum and at least 20 μg/g of liver for a newborn calf.

An important contribution to early health and survival may come from stores of vitamin A which are present at birth. Successful attempts have been made to improve vitamin A concentrations of newborn calves by using dietary supplements in the diet of the dam prepartum (11, 23, 71). Supplementation with vitamin A resulted in decreased ß-

carotene and increased vitamin A present in blood plasma, and greater liver storage of vitamin A in calves (23).

Studies (11, 23, 71) have shown that the level of supplementation needed is high (45,000 - 1,000,000 international units of vitamin A), especially when considering the modest elevation that is achieved in vitamin A stores. The key component in establishing a satisfactory vitamin A status in newborn calves is milk (11). Colostrum initiates this process of supplementation.

It is well known that colostrum is a rich source of nutrients and is reported to be highly digested by the newborn calf (46). Vitamin A content has been reported to be as high as 735 μg/dl and as little as 124 μg/dl (4). Values reported by Johnston and Chew (37) ranged from 470 μg/dl to 270 μg/dl. The average for each of these two studies was about 370 μg/dl. Detectable vitamin A absorption from colostrum has been reported to average between 81 to 95% (46).

COLOSTRAL TRANSFER OF IMMUNOGLOBULINS

Passive immunity that neonatal calves depend on for survival relies on the absorption of immunoglobulins (Ig) present in colostrum (13). Absorption in this case is defined as the movement of substances from the lumen of the intestine to the blood (14). Immunoglobulin is transferred from the intestinal lumen to the blood through a cell membrane to cell membrane passageway for a limited time following birth. Closure, which is when macromolecule absorption from the gut to the blood stops (38), takes place automatically over time after 12 h of age with an

average closure time of roughly 24 h (14). Devery et al. (20) showed that calves were incapable of absorbing colostral Ig after about 36 hours of age, but did have the capability for the endogenous production of small amounts of IgG during the first three weeks of life.

A study by Stott et al. (62) indicated that by delaying the first feeding of colostrum, the estimated time of closure is also delayed. They also found that if closure was delayed, the length of time that the calf was able to absorb Ig was reduced from 21 hours to 8 hours.

In a companion study, Stott et al. (63) reported that the rate of absorption of Ig depends on the amount of colostrum fed as well as how soon after birth ingestion occurs. By feeding two liters of colostrum verses .5 - 1 liter within the first four hours of life, optimal Ig absorption was obtained. When calves were fed another two liters 12 hours later, there was no response. This indicates that two liters of colostrum was sufficient to satisfy needs of the calves.

SEASONAL INFLUENCES ON SERUM IG CONCENTRATIONS

There have been conflicting reports of the effects that season has on serum protein/Ig concentrations. A study performed by Donovan et al. (21) in Florida, determined that there was an increase in serum protein/serum Ig concentration in winter (5.7 g/dl) and a decrease in the summer (5.1 g/dl). Serum protein was affected by the month of birth and the temperature on the day of birth. With higher temperatures the serum protein was lower.

Another study conducted in Glasgow, Scotland by McEwan et al. (41) found that serum Ig was higher during the summer and lowest during the winter. A study by Boyd (10) in Cheshire England also found that serum Ig was higher in summer and lower in winter and that there was a significant correlation between total serum protein and serum Ig.

In studies that were performed in Washington state, Gay et al. (30, 31) also found that IgG_1 concentrations were higher in summer and lower in winter. One explanation offered for this difference was the influence that season had on the Ig concentration present in colostrum.

INFLUENCES OF VITAMIN A ON VARIOUS CELL TYPES

Vitamin A has been reported to have various effects on several different functioning cell types of the immune system. Differences in the response of neutrophils and macrophages to vitamin supplementation vary with individual cells, functions under observation, vitamin supplementation, and also with the age of the animal in question (24).

Eicher et al. (24) showed that by supplementing with both vitamins A and E, increased neutrophil phagocytic activity in three week old calves could be seen. They also showed that neutrophil bactericidal activity responded favorably to vitamin A supplementation at three and six weeks of age.

Cell-mediated cytotoxicity has been reported to be enhanced in mice by supplementation with retinoic acid (16). Retinoic acid was also stimulatory to the activity of natural killer cells in humans (16). It has been

proposed that the increase in natural killer cell activity may be due to the alteration in cell surface structure and increase in cell-surface receptor expression (5).

It has also been suggested that vitamin A may act via the stimulation of interleukin-2 secretions (16). If this is true, vitamin A can be expected to enhance T helper cell and natural killer cell activities since these cell types need interleukin-2 for proliferation and action.

As can be seen, there is cause to be concerned with the body stores of vitamin A present throughout the life of an individual and also with a reliable way of determining these body stores. This trial focused on neonatal calves due to the fact that they are born with little or no reserves of vitamin A and therefore, are at high risk for contracting several diseases. By having a reliable way to detect vitamin A deficiency in its early stages, there is a good chance that any further health complications can be avoided.

MATERIALS AND METHODS

Comparison of micro- and macrosamples of liver

Liver from a dead calf was obtained from South Dakota State University Veterinary Diagnostic Laboratory to use for comparison of microsamples of liver with macrosamples for vitamin A content. Several macro-size samples ranging from 4.03 to 7.67 g were taken along with micro-size samples ranging from .0108 to .0420 g.

In a procedure similar to that of Amedee-Manesme et al. (2), liver samples were ground with 1.5 g anhydrous sodium sulfate and 12 ml methylene chloride in a mortar and pestle, filtered through a 60 ml Pyrex funnel with a fritted disc, and mixed with .5 mg butylated hydroxytoluene and .1 ml ethanol before performing further analyses.

Animal Procedures

Sixteen Holstein calves were used to assess the RDR for neonatal calves. Procedures were approved by the Institutional Animal Care and Use Committee on the South Dakota State University campus. At birth, calves were separated from their dam and placed in observation pens. All calves were healthy at birth and displayed no signs of distress during the sampling period.

At birth, calves were prepared for liver biopsy by shaving a four inch square area approximately 13 cm down from the spine and covering the 10th to 13th ribs on the right side of their body. Alcohol was applied to the shaved area. Lidocaine, (1/2 to 1 cc) was injected as a local anesthetic. Liver samples were obtained by making a small incision approximately 13 cm down from the spine and between the 11th and 12th ribs. A 14 mm Bauer One-handed

biopsy needle (Products Group International, Inc., Boulder, CO) was then inserted into the incision and 5.5 - 29.3 mg samples of liver were obtained. Eleven livers were successfully sampled. To prevent infection, an iodine solution was applied to the incision site.

Blood was obtained by jugular venipuncture into evacuated plasma or serum separation tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ). The first 3 calves were used to determine at which hour(s) vitamin A peaked in the blood. Blood samples were obtained at 0, 2, 4, 5, 6, 8, 10, 12, 14, 16, and 24 h, with 0 h being the first feeding of colostrum. From this, it was determined that vitamin A peaked in plasma between 6 and 8 h. Therefore, blood samples were obtained at 0, 5, 6, 7, 8, 12, 17, 18, 19, 20, 22, and 24 h for calves 4 through 16. Colostrum was the soul source of vitamin A which was fed after bleeding at 0, 12, and 24 h.

Colostrum samples were taken for laboratory analysis. Calves were fed approximately 2.3 kg of colostrum from their respective dams after performing liver biopsies and blood sampling at 0 h and blood sampling at 12 and 24 h. Immediately after all samples were taken, samples were placed on ice in the dark so as to avoid destruction of the vitamin A by ultra-violet light.

Laboratory Procedures

Whole blood was used to prepare slides for cell differentials and to determine hematocrit values using a micro-hematocrit centrifuge (Adams Readacrit, Clay Adams, Parsippany, NJ). Blood was centrifuged at approximately 650 x g for 20 minutes (Jouan CR4-12 centrifuge,

Winchester, VA) to separate plasma and serum from other blood components. Plasma, liver, and colostrum samples were frozen at -20°C until vitamin A analysis could be performed. Serum was harvested and serum protein was determined using a hand refractometer (American Optical Corporation, Buffalo, NY). Remaining serum was frozen until Ig concentration could be determined.

Extraction of vitamin A from liver samples was as described for liver micro- and macrosample comparisons (2).

Liver, plasma, and colostrum vitamin A analyses were performed by the triflouroacetic acid method of Dugan et al. (22). All chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Five ml of blood plasma was used for vitamin A extraction where possible, but in some cases, a smaller amount had to be used due to limited sample availability (22).

Retinol was released and plasma proteins were precipitated by adding denatured ethanol to the samples (22). Retinol was then extracted into petroleum ether and centrifuged to separate the ether layer. The petroleum ether layer was then transferred to a centrifuge tube where it was evaporated to dryness in a heated centrifuge (43°C) (Savant Speed Vac Plus SC 110A, Farmingdale, NY). The resulting residue was resuspended in chloroform followed by addition of trifluoroacetic acid for color development. The solution was mixed quickly, transferred to a cuvette, and read on a spectrophotometer within 10 seconds (Beckman DU-50, Fullerton, CA) at 616 nm visible light.

Vitamin A content was calculated by the formula: K*A*D*1/plasma sample volume*100=μg vitamin A/ dl, where K

= the constant for vitamin A standard which was 6.94, A = absorbance, and D = the dilution factor which was the ml of ether added divided by the ml of ether harvested.

The procedure for colostral vitamin A extraction differed from the plasma procedure only in the amount of sample used. One ml of colostrum, 1 ml of denatured ethanol, and 5 ml of petroleum ether were used for extraction of vitamin A from colostrum.

Immunoglobulin quantification for blood serum IgM and colostral IgM was performed by the enzyme linked immunosorbent assay (ELISA) of Franklin et al. (28). Colostrum was prepared for the ELISA procedure by centrifuging at 650 x g for 10 minutes to obtain a more pure sample of the immunoglobulin fraction. Both the colostrum and the serum samples were diluted with antigen sample buffer (.015 M Na_2CO_3, .035 M $NaHCO_3$ at pH 9.6) to 1:400,000 for loading into Corning Disposable Sterile 96 Well, Flat Bottom ELISA Plates (Corning Glass Works, Corning, NY). After loading, plates were incubated for 1 h at 39°C and then stored for one month at 4°C.

After a minimum incubation time of 18 h, plates were washed with a Nunc-Immuno Wash 12 (Inter Med, Denmark) in ELISA wash solution (.145 M NaCl, .002 M $NaH_2PO_4H_2O$, .008 M Na_2HPO_4, 8 ml Tween 80) and tapped dry on paper towels. A blocking solution of 1% phosphate-buffered saline gel was added to the wells and incubated at 39°C for 30 minutes.

Plates were washed and dried again and diluted anti-IgM antibody (1:5000) (Sigma) was added to each well. Plates were incubated at 39°C for 1 h.

Plates were washed and dried a third time after which, a 1:1500 dilution of anti-mouse biotinylated Ig (Amersham, Arlington Heights, IL) was added to the wells. Plates were incubated at 39°C for 2 h.

Plates were once again washed and dried. Strepavidin-biotinylated peroxidase conjugate (Amersham) was diluted to 1:1600 and added to the wells. Plates were incubated at 39°C for 30 minutes.

Plates were washed and dried for the final time. A substrate solution of 2,2'-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid, ABTS) (Sigma), (.05 M citric acid buffer and .04 M ABTS at pH 4.0) was added to the wells. After a 10 minute incubation at room temperature, plates were read on a microplate reader at 405 nm (Bio-Tek Instruments, Winooski, VT).

Measurements on vitamin A, IgM, and colostrum samples were analyzed by the General Linear Models procedure of SAS (57). Data with repeated measurements were analyzed by the Mixed Model procedure of SAS. Significance was determined at $P < 0.05$ unless otherwise noted. Analysis was conducted for correlations of colostral or liver vitamin A with the 6 h, 8 h, and 20 h RDR values.

RESULTS AND DISCUSSION

Analysis of micro- and macrosamples of liver revealed that samples taken from the same lobe of the liver had similar vitamin A values for all but one microsample (Table 1). These results agree with those obtained by Amedee-Manesme et al. (2) in a comparative study. The authors determined that micro samples (7 to 70 mg) agreed well with macro samples (1 to 6 g) with little sacrifice in precision. It was therefore determined that the micro-sampling technique was adequate for determination of vitamin A stores in neonatal liver of calves.

Liver sample weights varied among calves with a range of 5.5 mg to 29.3 mg. Vitamin A concentration ranged from 3.0 to 52.9 μg/g of liver with an average of 11.68 μg vitamin A/g of liver (Table 2). The liver sample containing 52.9 μg vitamin A /g of liver was unseemingly high and would be more plausible if attributed to experimental error rather than actual liver vitamin A content. The average liver vitamin A concentration with the 52.9 μg/g sample removed is 7.66 μg/g, thus utilizing 10 liver samples. This is closer to the average obtained by Branstetter et al. (11), which was 7.3 μg/g of liver. The missing values were because of difficulty in obtaining liver samples in some of the calves. Inexperience in performing liver biopsies and an unwillingness to stress the calves led to problems in obtaining liver samples or obtaining enough sample to run analyses in duplicate.

Vitamin A concentrations in plasma of calves were averaged for each time point (Figure 1). In general, there was an increase over time in the plasma vitamin A

TABLE 1. Comparison of vitamin A concentrations in macro- and microsamples of calf livers.

Sample #[1]	Liver wt (g)	Vitamin A (ug/g)
Macro:		
1a	4.03	120
1b		120
2a	4.85	101
2b		101
Micro:		
1a	.042	99
1b		90
2a	.031	230
2b		107

[1]a and b represent duplicate vitamin A analysis of samples.

TABLE 2. Vitamin A concentrations in colostrum, liver, and plasma (0, 6, 8, and 20h) with corresponding relative dose response (RDR) values for individual neonatal calves.

Calf	Colostral vit. A (µg/dl)	Liver vit.A (µg/g)	Plasma 0h vit. A (µg/dl)	Plasma 6h vit. A (µg/dl)	RDR 6h (%)	Plasma 8h vit. A (µg/dl)	RDR 8h (%)	Plasma 20h vit. A (µg/dl)	RDR 20h (%)
1	170.6	.	4.1	4.4	5.8	5.3	22.6	.	.
2	134.3	.	10.5	6.8	-55.5	9.9	-6.7	.	.
3	152.6	.	16.9	19.0	11.3	15.0	-12.1	.	.
4	183.0	4.9	5.6	5.9	5.3	6.9	10.7	9.8	42.9
5	326.4	.	4.6	6.9	33.8	9.9	43.5	15.1	69.5
6	125.8	5.6	5.0	6.0	16.3	7.5	34.5	.	.
7	87.3	.	9.8	7.9	-24.6	7.9	-8.8	8.9	-10.5
8	85.4	4.7	9.2	7.1	-30.1	.	-19.5	.	.
9	190.3	52.9	8.1	9.0	10.2	10.9	18.0	14.1	42.6
10	242.7	3.0	9.7	8.9	-10.1	13.4	15.8	7.2	-34.7
11	434.1	13.0	5.0	8.7	42.7	8.7	48.2	16.2	69.1
12	248.3	21.2	6.1	5.5	-10.0	9.7	-17.6	12.4	50.8
13	102.9	6.9	4.7	4.9	4.2	6.1	-4.1	6.5	27.7
14	393.9	4.0	6.3	6.4	2.0	6.4	3.0	7.9	20.9
15	303.9	5.5	8.4	7.8	-7.9	10.9	3.6	15.2	45.1
16	312.2	7.8	9.3	8.9	-3.9	16.6	26.9	.	54.2
x̄ ± s.d.	218.4 ± 109	11.8 ± 14.6	7.7 ± 3.3	6.7 ± 3.3		9.7 ± 3.3		11.3 ± 3.7	
Colostrum[1]					R^2=.516		R=.839		R=.466
					P^3=.041		P=.031		P=.148
Liver					R= .211		R=.070		R=.279
					P=.533		P=.839		P=.466

[1]Correlation analyses for colostrum and liver with 6h RDR, 8h RDR, and 20h RDR values.
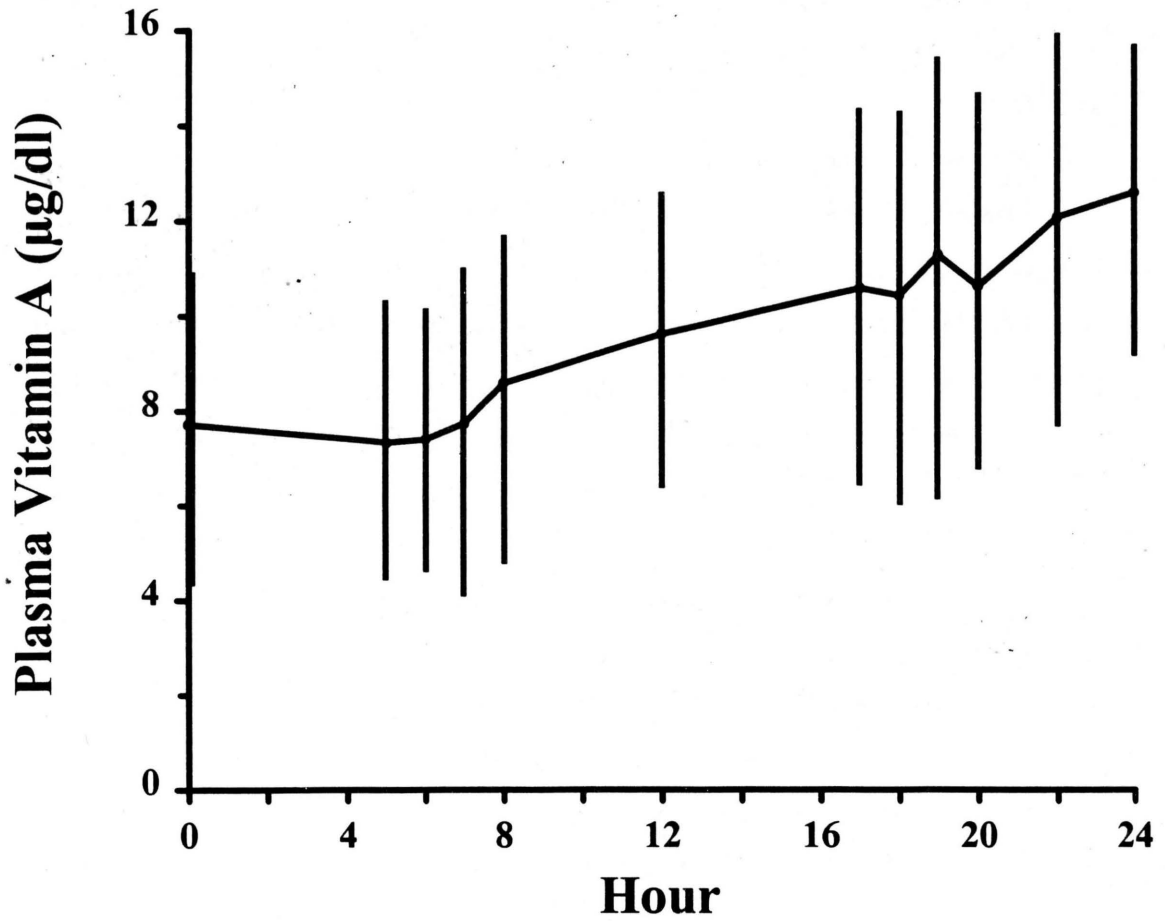[2]R=Pearson correlation coefficients.
[3]P=Probability > R.

Figure 1. Mean plasma vitamin A concentrations ± s.d. of newborn calves during the 24 h period after the initial feeding of colostrum.

concentrations, however, the increase was maintained within a fairly narrow range. A sufficient level of vitamin A in plasma has been reported to be approximately 20 μg/dl (3, 15). All calves, with the exception of calf #16 at the 19 h sampling time, were vitamin A deficient at birth and throughout the first 24 h of their lives. The average vitamin A concentration at birth was 6.66 μg/dl of plasma and the average 24 h range was 4.52 to 20.31 μg/dl. This agrees with data obtained by Spielman et al. (60) where the mean vitamin A concentration of newborn calves was low at 9.3 $\pm$ 2.2 μg/dl of plasma.

There was an overall decrease in the plasma vitamin A concentration at 5 h after the first feeding. This trend was not seen at 5 h after the second feeding (12 h). One explanation for these results might be that there is little RBP present at birth. With little or no RBP to mobilize vitamin A from the liver, there would be a decrease in circulating vitamin A because of utilization. By the fifth hour after the second feeding, the body would have had time to build up its store of RBP and hence more effectively transport vitamin A out into the body for use.

Comparison of the liver vitamin A concentrations to the 6 and 8 h RDR values, revealed no significant correlation between them (P>.05) (Table 2). We had expected to see a correlation between liver vitamin A concentrations and RDR values at these times because neonatal calves typically are deficient in vitamin A and should respond to a large dose of vitamin A in a manner similar to vitamin A deficient rats and humans. Calculations were also made for the 20 h RDR since previous

research on older cattle had shown the RDR to be at 20 h post-dosing (66, 67). The values looked promising but there was still no significant correlation (P>.05).

Colostral vitamin A concentrations ranged from 85.4 - 434.1 μg/dl of colostrum (Table 2) and were comparable to those reported by Spielman et al. (61) which ranged from 124 to 735 μg/dl. In general, as colostrum vitamin A increased so did the plasma vitamin A at both 6 and 8 h.

Even though a significant correlation was not observed between the liver and 6 and 8 h RDR values, there was a significant correlation between the colostrum values and the 6 (P=.04) and 8 (P=.03) h RDR values, but not at the 20 h RDR (P>.05) (Table 2). This may have been a result of direct absorption of colostral vitamin A into the blood stream and utilization before storage in the liver could take place.

By splitting the RDR values into 2 categories, a difference could be seen in the outcome (Table 3). In general those calves fed colostrum containing more than 155 μg/dl of vitamin A had positive RDR values and those fed colostrum containing less than 155 μg/dl of vitamin A had negative values. Within each group there was one outlier identified that did not follow the trend. Calves with positive RDR values may not have received adequate vitamin A to improve their status in the first 12 h of life. They may have utilized retinol from the plasma at a greater rate than retinol was absorbed from colostrum.

Other factors that may have affected our results are the vitamin A status of the dam or the presence or absence of RBP in the calf at birth. The vitamin A status of the

TABLE 3. Mean relative dose response (RDR) values of calves grouped by colostral vitamin A concentration.

Vitamin A in Colostrum (ug/dl)	Mean Vitamin A concentration in Colostrum (ug/dl)	Mean Vitamin A concentration in Liver (ug/g)	6h RDR (%)	8h RDR (%)
Above 155[1]	260.3	6.4	9	21
s.d.	±78	±2.9	±17	±15
Below 155[2]	99.5	5.9	-19	-10
s.d.	±22	±1.0	±24	±5
Outliers				
calf 6	109.9	5.6	16.3	34.5
calf 12	211.7	21.2	-10	-17.6

[1]Colostral samples containing greater than 155 ug/dl of vitamin A (n=9).
[2]Colostral samples containing less than 155 ug/dl of vitamin A (n=5).

dam, which was not determined in this study, may affect the vitamin A status of the calf (23, 60). Also, when the calf was born, there may not have been enough RBP present to carry the vitamin A out into the blood stream. It may take the calf hours to make enough RBP to efficiently transport the vitamin to tissues where it can be utilized. These are areas for further research.

In addition to vitamin A status, several parameters relating to the immunological status of the calves at birth were measured, which included serum protein, serum IgM, hematocrit %, and colostral IgM. Data also were analyzed to determine whether season affected immunological status of newborn calves. In general, mean serum protein concentrations gradually increased throughout the sampling period ranging from 4.6 g/dl at 0 h to 6.0 g/dl at 20 h. Serum protein concentrations peaked at 20 h and maintained a plateau through 24 h. Jain (35) has reported the normal range of serum protein present in a newborn calf to be 5.0 ± .8 g/dl. The present study results were similar as the over all mean for serum protein as determined by a refractometer was 5.55 ± .62 g/dl. There was no significant effect of season on serum protein.

One explanation for the occurrence of the plateau and it's being maintained at a constant level, can be attributed to the act of intestinal closure. The ability of colostral Ig to pass through the intestinal cell wall declines at a progressively increasing rate as the calf ages with the hours immediately following birth (62). Stott et al. (63) reported that colostral immunoglobulins are absorbed at a faster rate during the first 4 hours

after the first feeding than at any other time. Closure takes place automatically over time after 12 hours of age with an average closure time of roughly 24 hours (14).

Serum IgM concentrations also increased over time (Figure 2). Values for winter were greater than for summer at 8, 22, and 24 h (P=.0017). Serum IgM concentration plateaued in summer at 12 h but continued to increase through 24 h in winter.

There was a significant correlation between serum protein concentrations and serum IgM concentrations (P=.0001, R=.54) (Figure 3). The correlation was greater in winter (R=.75) than summer (R=.44).

The results obtained in the present study more closely resemble the results of Donovan et al. (21) who determined that serum Ig was higher in winter and lower in summer. Because that study was performed in a more subtropical climate, and temperature has been noted to affect serum protein/serum Ig concentrations, results from the present study should have more closely resembled Gay's et al. (10), McEwan's et al. (41), and Boyd's (30) results due to the likeness in climates. Gay et al. (10), McEwan et al. (41), and Boyd (30) have all reported results that are contradictory to Donovan et al. (21). The difference seen here might be attributable to such gross extremes between the lows and highs in daily temperatures.

Colostral IgM was greater in winter (10.71 ± 4.87 mg/ml) than in summer (6.25 ± 2.74 mg/ml) (P=.03) (Table 4). This may partly explain why the serum IgM values were higher in winter than in summer. Nocek et al. (43) reported that calves fed colostrum with a higher amount of
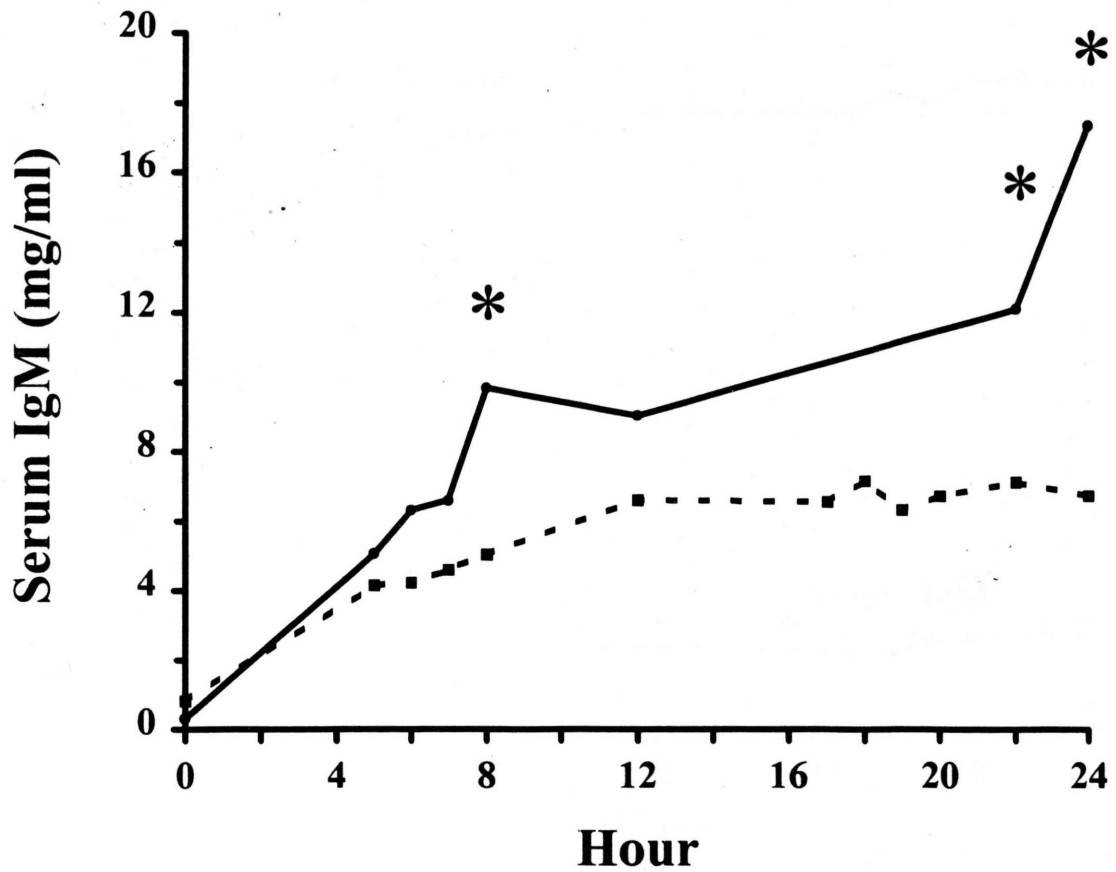
Figure 2. Comparisons of winter (—●—) and summer (-■-) serum immunoglobulin (Ig) M concentrations (mg/ml) in calves during the 24 h period after the initial feeding of colostrum. Asterisks indicate differences (P < 0.05) between winter (n=5) and summer (n=11) values.
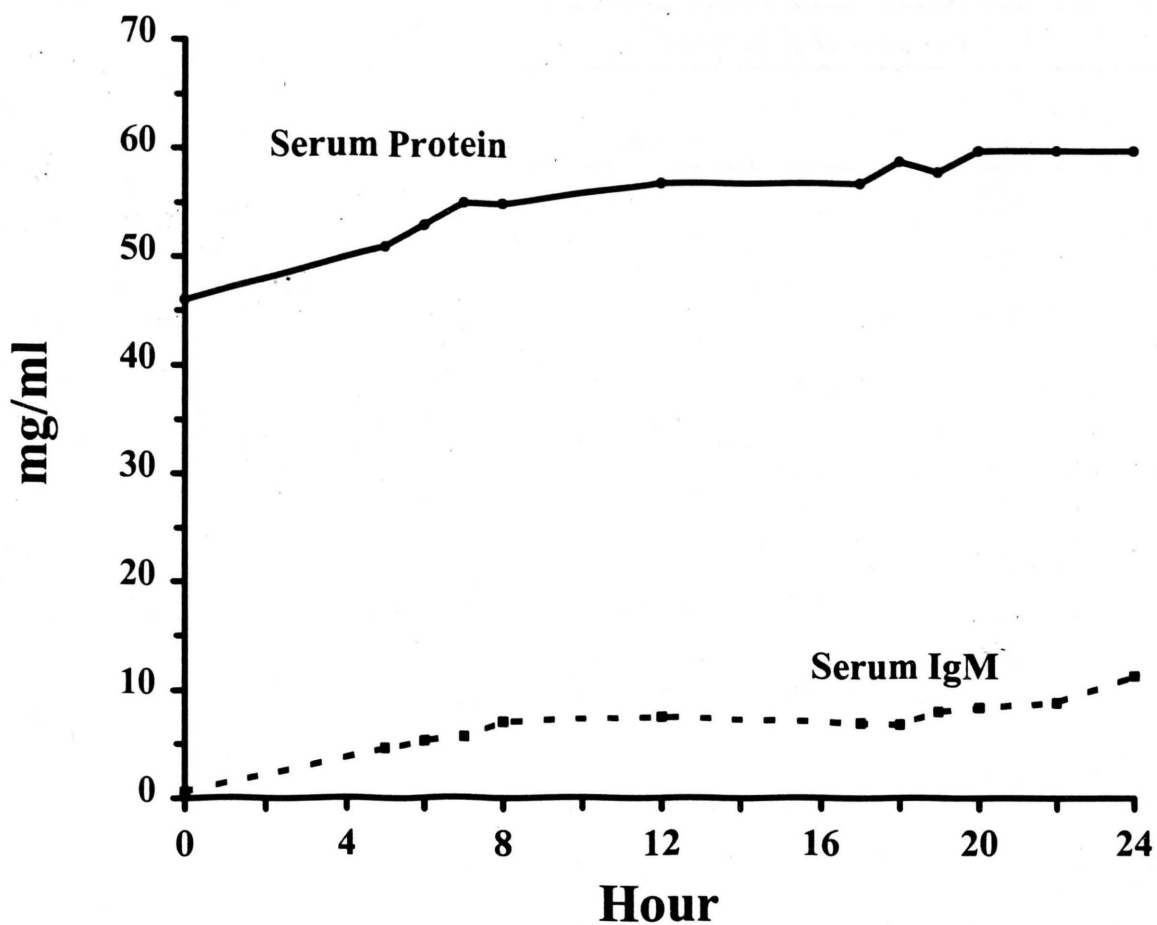
Figure 3. Comparison of mean serum immunoglobulin (Ig) M and mean serum protein concentrations from calves during the 24 h period after the initial feeding of colostrum.

TABLE 4. Colostral immunoglobulin (Ig) M concentrations by season.

Calf Number	Colostral IgM (mg/ml)	
	Winter	Summer
1		3.9
2		3.2
3		3.7
4	18.5	
5	11.2	
6	10.4	
7	8.0	
8	5.6	
9		9.6
10		6.6
11		10.5
12		9.1
13		8.0
14		3.3
15		6.6
16		4.2
Mean (P=.03)	10.7 ± 4.9	6.2 ± 2.7

immunoglobulin present will have higher serum protein concentrations. Serum IgM and colostral IgM were correlated at 8 h (P=.02, R=.70). This time point was used because we wanted to remove the effects of 0 h where there would be no absorption and 5, 6, and 7 h where there would be less absorption and were fewer samples.

Hematocrit values decreased over time (P<.001) with a starting mean of 27% and ending mean of 21.9% (Figure 4). The average for all calves over all sampling periods was 24.65%. This is somewhat lower than values reported by Jain (35) for newborn calves which should be approximately 34.5 ± 7.7%. There was no effect of season but this may have been due to such few observations between seasons.

Hematocrit values should not change significantly over time. The decrease in values seen here may be due to the fact that the calves were bled so often. Cells may have been removed too rapidly for the calves to replenish their supply before the next bleeding. However, subsequent unpublished data have shown the same trend even when the calves were bled only 3 times in 24 h versus 12 times in 24 h. A second explanation may be that ingestion of colostrum caused increases in the fluid portion of the blood.

Microscope slides made from whole blood stains were used for cell differentials. Granulocytes were split into three cell types: neutrophils, basophils, and eosinophils. The mononuclear leukocytes (lymphocytes and monocytes) are not easily differentiated in cattle and were counted together as one cell type. The mean leukocyte differential values fell within the range of values reported by Jain (35) for newborn calves (Table 5).
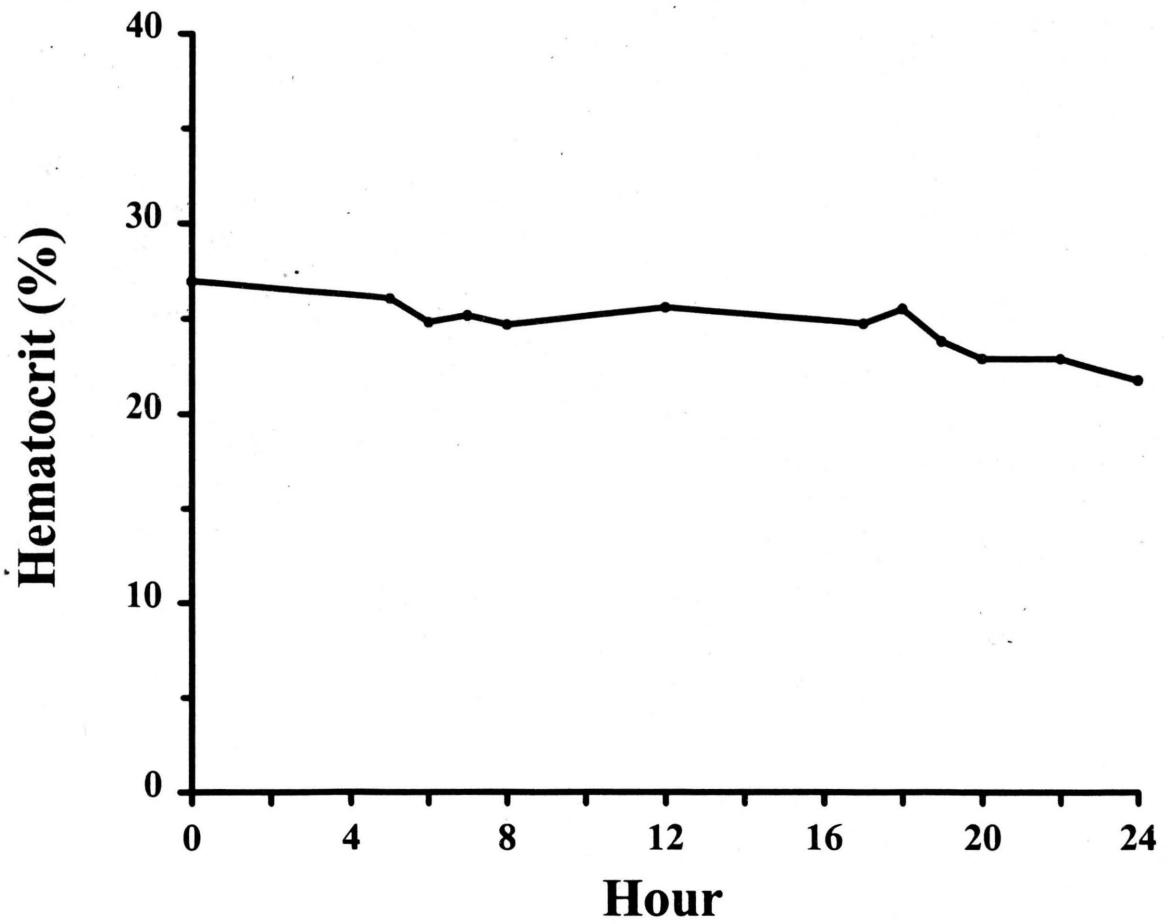
Figure 4. Mean hematocrit percentages for whole blood from calves during the 24 h period after the initial feeding of colostrum.

TABLE 5. Mean cell differentials of neonatal calves sampled over a 24 hour period.

Cell Type	Mean ± s.d.
Granulocytes:	%
Neutrophils	51.9 ± 21.2
Basophils	0.5 ± 0.8
Eosinophils	0.2 ± 0.4
Mononuclear Leukocytes[1]	47.5 ± 30.8

[1]Consisting of lymphocytes and monocytes.

CONCLUSIONS

Calves sampled for the study were vitamin A deficient at birth and throughout the first 24 h of life. It appears that the plasma vitamin A concentration of newborn calves is not just a function of innate vitamin A present in the liver at birth. Instead, it is a combination of several factors, such as the vitamin A status of the dam, vitamin A content of the colostrum, and possibly the presence or absence of RBP at birth of the animal.

There was no correlation between liver vitamin A content and the RDR at 6, 8, or 20 h, but there was a correlation between colostral vitamin A content and the 6 and 8 h RDR values while not at the 20 h RDR. Based on these methods and results, the RDR assay does not work for neonatal calves. It is plausible, however, that by sampling blood at different times and for longer than 24 h, a positive result might be seen.

Serum protein values increased through 20 h and then maintained a plateau through 24 h. There was no significant effect of season on serum protein values. Serum IgM concentrations also increased over time with values greater in winter than in summer. Colostral IgM was also higher in winter than in summer. Serum protein and serum IgM were significantly correlated, while colostral IgM and serum Igm were correlated at 8 h.

Hematocrit percentages decreased over time and were some what lower than expected. There was no effect of season on hematocrit values, however, it was noted that

this may have been due to the low number of observations between seasons.

Season had no effect on cell differential values, as, on the average, these remained constant throughout the study.

Further research is needed to determine the amount of RBP present in the liver of calves at birth and the length of time it takes the calf to provide enough RBP for efficient transport of vitamin A.

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