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IN VIVO AND IN VITRO INFLUENCE OF VITAMIN A ON BOVINE LYMPHOCYTE BLASTOGENESIS

BY

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ABSTRACT

IN VIVO AND IN VITRO INFLUENCE OF VITAMIN A ON BOVINE LYMPHOCYTE BLASTOGENESIS

BY

Linda Terry Stern

Two trials, using 25 newborn calves, were conducted to determine the in vivo effect of dietary vitamin A on mitogen stimulated blastogenesis of neonatal calf lymphocytes. Lymphocytes from weekly blood samples were incubated for 48-72 hours in microtiter plates with concanavalin A.

In each trial the mortality rate and/or incidence of diarrhea was greatest among the vitamin A-deficient calves. Also, the lymphocyte transformation significantly increased ($P < 0.004$) between birth and 7 days of age correlating positively with dietary vitamin A supplementation.

The in vitro effect of retinyl palmitate on lymphocyte mitogen stimulated blastogenesis was also investigated, using 16 lactating Holsteins cows, by the addition of 0, 10, 100 and 1000 ng retinyl palmitate per culture well. In this study there was an inverse relationship between the degree of blastogenesis and level of retinyl palmitate in the absence of mitogen ($P < 0.0001$), and a positive correlation ($P < .01$) in the presence of mitogen and 10 and 100 ng retinyl palmitate.

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INTRODUCTION

High calf mortality rate in both the beef and dairy industry, estimated to be between 8 and 25%, represents a serious economic problem for Michigan as well as for U.S. agriculture as a whole.

While most of these calf deaths are ultimately due to infectious disease, especially E. coli and corona virus, suboptimal nutritional and environmental conditions increase susceptibility to these infectious agents. Despite new vaccines and bacterins, in utero vaccinations, antibiotics, herd health programs and considerable research effort to improve calf health, unacceptably high calf losses continue.

Recent advances in characterizing and understanding immune responses emphasize the role of nutrition, especially vitamin A, in both cellular and humoral immunity. Since the newborn calf is relatively deficient in vitamin A and dependent upon colostrum to meet its initial vitamin A requirements, the influence of vitamin A on calf mortality must be fully understood to optimize the resistance of calves to infectious disease.

The purpose of this research was to use mitogen-stimulated lymphocyte transformation studies, in conjunction with improved vitamin A analysis (high pressure liquid chromatography-technology), to examine:

(1) in vivo influences of vitamin A on cell-mediated immune mechanisms in calves and (2) in vitro influences of vitamin A on blastogenesis of lymphocytes from mature Holstein cows.

LITERATURE REVIEW

Calf Mortality Problem

A significant economic loss in dairy and beef cattle farms (Amstutz 1965, Amstutz et al. 1965, Oxender 1973) still exists because of premature deaths of calves. Calf losses range from 8-25% (Amstutz 1965, Amstutz et al. 1965, Bellows 1971, Grunsel 1956, Lassiter and Seath 1955), with a mean mortality rate at 13.5% observed in Michigan (Speicher and Hepp 1973). Using a 10% calf mortality rate, Amstutz (1965) estimated that economic losses approximate $50 million per year for the U.S. When adjustments for inflation were incorporated, Oxender et al. (1973) determined the loss due to calf mortality to be over $200 million per year. This estimate did not include extra labor and medical costs for the sick calves, nor the economic loss due to lower productivity of the survivors. Unfortunately there is no evidence in any regional area that the calf mortality problem is declining (National Academy of Science 1968, Martin 1974).

Most calf deaths occur during the first week postpartum, principally caused by diarrhea and pneumonia (Oxender et al. 1973), while a decline in mortality rate on after 2 months of age (Martin et al. 1975). The infectious etiologic agents include a variety of bacterial (*E. coli*, *Salmonella spp.* *Klebsiella spp.*) and viral

(infectious bovine rhinitis, pneumoenteritis) agents (Amstutz et al. 1965). These infectious agents are influenced by the nutrition of cow and calf, genetic make-up of the calves and environmental conditions including acute seasonal temperature variations (Martin et al. 1975).

Colostrum-deprived calves are still susceptible to septicemia despite antibiotic treatment (Fisher and De La Fuente 1971, Logan and Penhale 1971a). In fact, Loken et al. (1971) recognized that extensive use of antibiotics increases the problem through development of drug-resistant bacteria.

Bull et al. (1978) showed the effects of protein deprivation, energy deprivation, and mineral deprivation on immunoglobulin absorption and on calf mortality. Steward and McCallum (1938) revealed evidence that colostral vitamin A was involved in disease resistance. High vitamin A levels increased calf survival against infection only if the maximum level was received soon after parturition. No adequate replacements for good management practices exist (Oxender et al. 1973).

Vitamin A

Historical

Several thousand years ago a substance in liver was recognized as a curative for night blindness in humans. Eber's Papyrus, an Egyptian medical treatise dating to 1500 BC, as well as the Greek philosopher Hippocrates,

suggested liver supplementation for those suffering visual difficulties and thus recognized a relationship between diet and blindness (cited by Moore 1957). Isolation of this protective dietary substance was troublesome due to its instability in the presence of oxygen and during heat treatment (Sebrell and Harris 1967).

By the 1900's, night blindness and related problems such as xerosis were prevalent, (Blegvad 1924). Eye disorders were a problem among individuals in Japan on all grain diets, while those consuming fish had no eye disorder. Since administration of either cod liver oil or eel fat prevented eye disorders, a fat deficiency was initially determined to be the cause. Infectious eye diseases and ulcers on the corneas were also associated with fat-deficient diets (McCollum 1957). When isolation and identification of the curative agent were possible, this factor was found to be abundant in liver (Moore 1957). "Fat-soluble factor A" was named vitamin A in 1915 by Drummond (Sebrell and Harris 1967).

Histological studies began in the early 1900's on young rats (Freise et al. 1916) and continued studies revealed the detailed pathological manifestations occurring in the eye as a result of vitamin A deficiency (Mori 1922). Studies by other investigators established roles for vitamin A in epithelial structures (Wolbach and Howe 1925), bone growth (Mellanby 1939), nerve integrity (Sutton 1940), tooth enamel development (Wolbach and Howe 1925), and possibly in disease prevention (Green and Mellanby 1928).

Sources

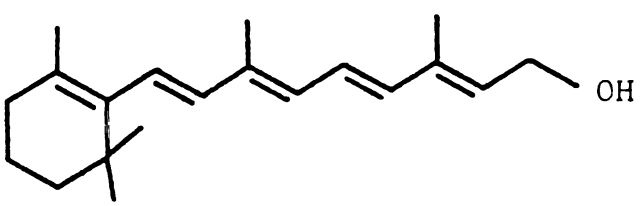
Natural preformed vitamin A is found only in animal tissues or fluids. The most abundant source of vitamin A is in the liver, the prime body storage area. Other locations of this vitamin include the kidney, blood plasma, egg yolk, colostrum and, to a lesser extent, milk. Variations in vitamin A levels are species dependent.

The principal vitamin A precursor, beta-carotene, found in most green or yellow vegetables is converted enzymatically in the intestinal wall to the active vitamin A form in most animals through oxidation at the central 15, 15' double bond. The efficiency for conversion is both age and species dependent. (Sebrell and Harris 1967).

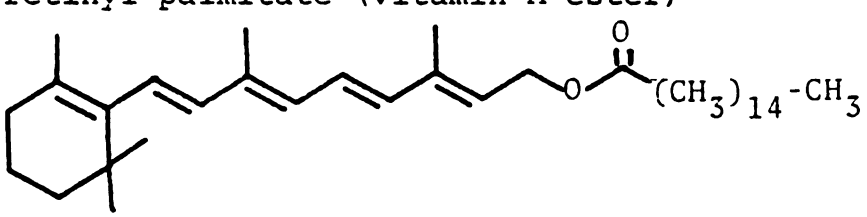
Forms

Vitamin A activity depends on the structural form of the vitamin. The major forms of vitamin A are illustrated as follows (Sebrell and Harris 1967).

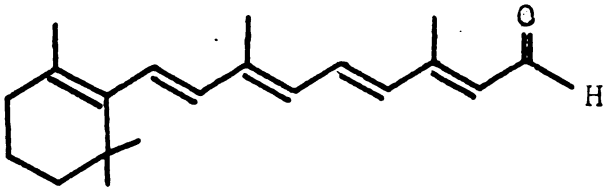
a. retinol (vitamin A alcohol)



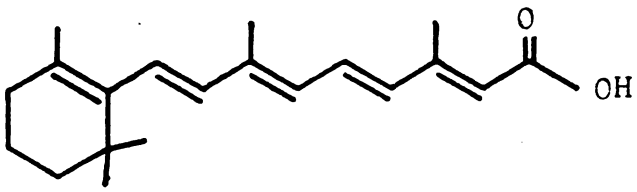
b. retinyl palmitate (vitamin A ester)



c. retinal



d. retinoic acid (vitamin A acid)



Vitamin A activity is described in International Units (IU). One IU is equivalent to 0.55 ug all-trans retinyl palmitate or 0.30ug all-trans retinol. The provitamin A activity of 0.60ug all-trans-beta carotene equals one IU of vitamin A.

The storage form, primarily found in liver, is vitamin A palmitate (retinyl palmitate). Retinol is considered the most biologically active form. It is also the major form found circulating the vasculature and the only form that functions in vision. Retinoic acid, another active vitamin A derivative, is the only one that cannot be converted to retinol (Sebrell and Harris 1967).

Function

The most well known function of vitamin A is its role in the visual process. Retinal, protein, and opsin are components of rhodopsin, the photosensitive pigment of rod cells. Rods are important elements in the eye for providing vision in semi-darkness (low intensity light).

Without functional rods, night blindness will occur (Wald 1938, 1960).

The basal cells of epithelium require vitamin A to differentiate into mucus-secreting cells (Fell 1957). The principal component of mucus is glycoprotein, and vitamin A stimulates its production. In the absence of vitamin A, cells produce keratin (De Luca and Wolf 1969). Differentiation into mucus-secreting cells is dependent upon the vitamin A action threshold for that particular tissue (Parnell and Sherman 1962). If vitamin A is insufficient for these cells, gastro-intestinal disturbances such as diarrhea (Keener et al. 1942), and respiratory disturbances, eg. pneumonia develop with the disruption in epithelial cell integrity (Sutton et al. 1940).

The influence of vitamin A on lipoprotein membranes is primarily mediated through its action on lysosomes. The precise mode of action is unclear. Vitamin A stimulates organelles to release their lysosomal enzymes, resulting in the activation of cell division. Mitotic division is impaired in deficiency states (Dingle and Lucy 1965).

Vitamin A is also involved in bone formation by controlling the number and activity of osteoblasts and osteoclasts. Growth becomes disproportional during insufficiency of the vitamin (Mellanby 1939). Odontoblasts are also defective in vitamin A deficiency, thus, there can be failure of enamel formation on teeth

(Wolbach and Howe 1925). Although hypovitaminosis A has been associated with nerve degeneration, the neuropathy is recognized as a secondary effect resulting from impaired bone development (Sutton 1940). The role of vitamin A in the immune response is reviewed in the section on immunity.

Requirements

During fetal development there is very little storage of vitamin A creating a dependency on the high vitamin A level found in colostrum. Fetal liver vitamin A levels remain low even if the cows are receiving high levels in their diet (Eden and Sellers 1949, Guilbert and Hart 1934). Absorbance of vitamin A through the intestinal mucosa is dependent upon dietary factors and integrity of the tract, as well as environmental conditions. There appears to be increased vitamin A requirements during cold weather (Keener et al. 1942). Deficiency symptoms have been observed when blood levels of vitamin A in the diet were less than 1-8ug/dl (Boyer et al. 1942). To avoid severe diarrhea in newborn calves a minumum of 220 IU/kg body weight are required (Hansen et al. 1946).
Growing heifers and bull calves fed milk only require 1,100-2,100 IU vitamin A/day while mature lactating cows require 27,000-60,000 IU vitamin A/day (NRC 1978).

Assay

Quantitation of pure vitamin A was first made possible by Carr and Price (1926) with antimony trichloride in chloroform, commonly referred to as the Carr-Price test. It is a color reaction specific for vitamin A. Shortcomings of this assay procedure include the instability of the color, interference by some sterols and cartenoids and sensitivity of the reagents to moisture (Freed 1966, DeRitter 1967, Maynard and Loosli 1969).

A more sensitive fluorescence procedure for identifying and quantitating vitamin A was designed by Sobotka (1943). Dennison and Kirk (1977) refined the assay for vitamin A using high pressure liquid chromatography in conjunction with a fluorescence detector. A modification of the Dennison and Kirk (1977) procedure (Stowe and Goelling 1981) was used for the vitamin A analyses in this research.

Immunology

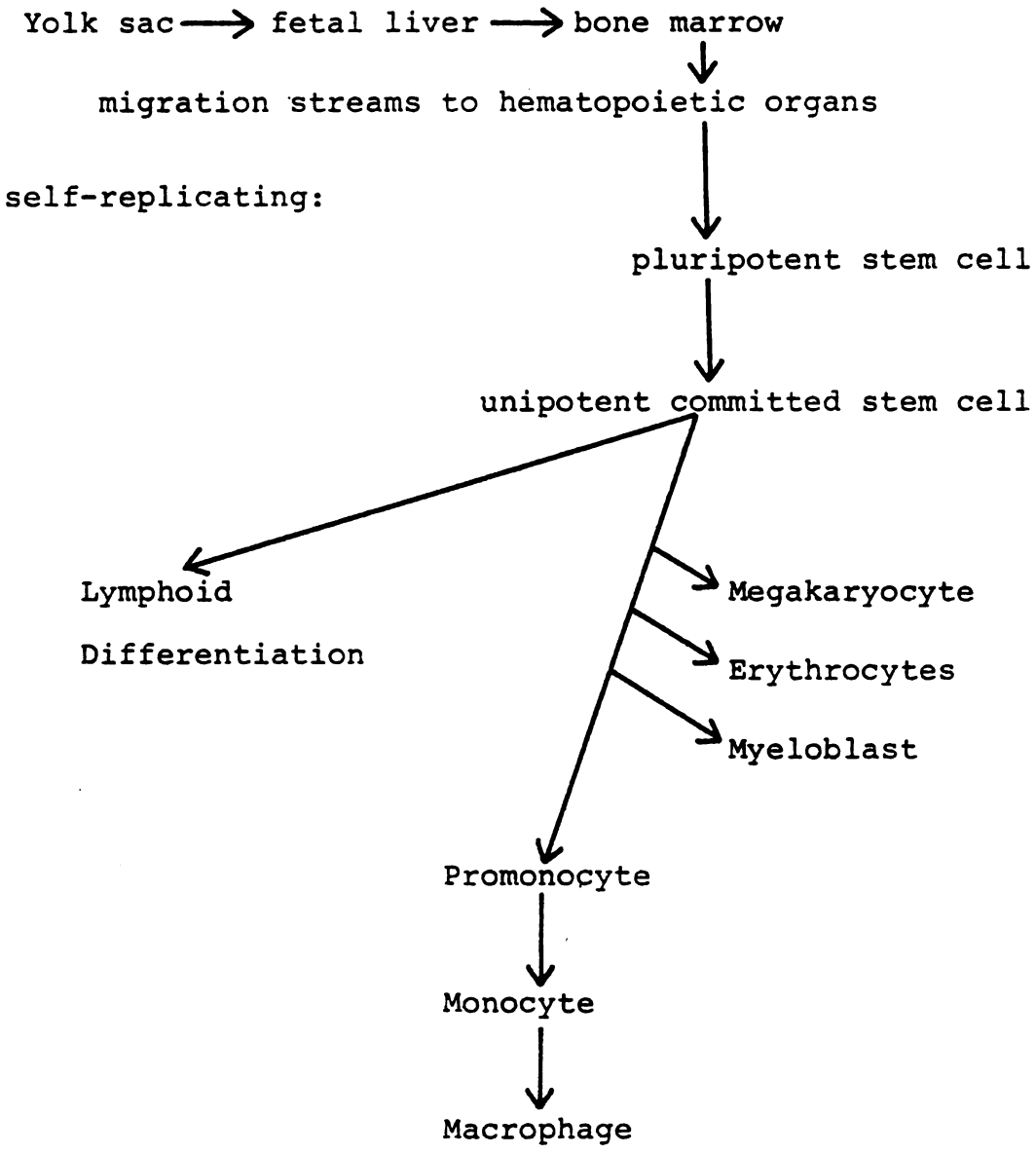
Historical

Resistance to infection has been noted since the early writings of mankind. Preexposed individuals who recovered from a disease were less likely to be reinfected, and, if reinfected, suffered a less severe form of disease. This type of resistance was observed by Thucydides 500 BC during epidemics in Athens (Eisen 1974). Physicians in the Alexandria school (Elves 1972), as early

as 300-200 BC, revealed that the lymphatics were involved. By the Middle Ages an unrefined procedure was initiated for innoculating individuals against smallpox using skin scrapings from infected individuals (Eisen 1974). Eventually the lymphatic system was associated with the blood system and immunity, while other cellular components were identified following the discovery of the microscope. Current concepts of the immune system began evolving even in the late 1800's with improved microscopes, development of the phase contrast microscope and advancements in histochemical staining techniques (Elves 1972).

Embryological Origin

Pluripotent stem or progenitor cells originate during embryonic development from the fetal yolk sac mesoderm. These cells give rise to leucocytes and other hematopoietic cell lines (Moore and Owen 1967, Moore and Metcalf 1970, Wu et al. 1968). The progenitor cells migrate to colonize the hematopoietic organs: liver, spleen, bone marrow and thymic rudiment (Fowler et al. 1967, Metcalf 1968, Trenton 1971). These cells then give rise to the unipotent committed cells including erythrocytes, granulocytes, megakaryocytes, and lymphocytes (Wu et al. 1968). Route of commitment is influenced by the microenvironment of the developing cells. This process is summarized as follows (Fowler et al. 1967, Metcalf 1968, Trentin 1971):



Immune System

The body's defense system is mediated through cells in the lymphoreticular system when there is foreign antigenic intrusion (Beisel et al. 1967, Craddock et al. 1977). These specialized cells eliminate, neutralize or sequester endogenously-produced (mutations) or exogenously acquired pathogens through a complexity of cell interactions and specialized products (Craddock et al.

1977). Included are: opsonins (which depend on serum protein factors), antibodies, complement and other cellular components necessary for functioning (Saba and Di Luzio 1968).

When leukocytes are mobilized, there is an increase in production, chemotaxis and migration of microphages and macrophages (Humphrey and White 1970). Within the cell, metabolic changes occur (Sbarra and Karnovsky 1959, Selvary and Sbarra 1966) with the engulfment of foreign cells in preparation for their destruction. Oxygen consumption, aerobic and anaerobic glycolysis and glycogenolysis, flow of glucose through the hexose monophospate pathway (HMP), lipid turnover and fumarate oxidation are all increased (Skeel et al. 1969). Glycolytic activity of polymorphic mononuclear cells provides energy for particle uptake. Stimulation of lysosomal enzymes and HMP provides bactericidal activity (Skeel et al. 1969).

Lymphocytes are the major cells involved in the immune system. They circulate inside and outside the vascular and lymphatic channels (Craddock et al. 1977, Olson 1969, Woelfel et al. 1970) distinguishing self from nonself antigens, reacting against foreign antigen and previously encountered (memory) antigen. Antigenic material is captured in the germinal centers (Ada et al. 1964, Nossal et al. 1972) of lymphoreticular tissue and

presented to immunocompetent T and B lymphocytes that pass through. Interactions with these cells, with assistance from auxilliary circulating and cellular factors, initiate a complex series of antigen-specific and nonspecific cell interactions in the elaboration of humoral and cell mediated immunity (Beisel 1980). Cells involved in humoral and cellular immune responses are distinguished by their organ source (Gershun and Kondo 1970). Respectively, bone marrow-derived cells are B lymphocytes and thymus-derived cells are T lymphocytes (Bach 1976, Greaves et al. 1974). Either humoral or cell-mediated immune responsiveness can occur depending on local tissue environment, cell types present and concentration of antigens as examples (Cline 1974).

Humoral Immune Response

The humoral system is comprised of B lymphocytes (named after similar cells found in the bursa of Fabricius of chickens) which migrate to specific areas of lymphoid organs from the bone marrow. These B cells include short-lived plasma cells, large pyroninophilic lymphocytes and small lymphocytes. B lymphocytes function in the production of antibodies (Greaves et al. 1974). Antibodies are divided into classes known as immunoglobulins (Ig) (Cline 1975, Gahmberg et al. 1976). They contain characteristic polypeptide chains identifying

them morphologically, immunogenically or chemically (Fahey and McKelvey 1965).

Immunoglobulin G (IgG) is the prototype for distinguishing the other classes. Each class of immunoglobulin has a specialized location and function in the body described as follows (Bernier 1978, McGregor 1971):

1. IgG is found in vascular and extravascular spaces and is involved in complement fixation.
2. IgM is located only in vascular spaces and is involved in complement fixation, early response to antigenic stimulation and prenatal immune response.
3. IgA is found in vascular spaces and is the first line of defense against organisms on mucosal surfaces.
4. IgE is found intravascularly and is involved in sensitization and mediates allergic reactions.
5. IgD is found intravascularly and its involvement in the immune response is not yet understood.

Antibody production is a complex process requiring macrophages for antigen processing and the help of T lymphocytes (Diener et al. 1971). Foreign antigens are attached to surface receptors (SmIg) of the B cell (Nossel et al. 1968) and specific antibodies of that antigen are produced (Diener et al. 1971 Nossel 1972) when the B lymphocyte proliferates into plasma cells, the active antibody producers. Some bacterial antigens can

trigger the response of B lymphocytes without the cooperation of other cell types (Diener et al. 1971).

Cell-Mediated Immune Response

Lymphocytes specifically involved in the cell-mediated immune response, first migrate to the thymus to be processed into specialized thymus-derived T lymphocytes (Cline 1975) in the cortical region. Migration to thymic-dependent areas of lymph nodes, spleen, tonsils and Peyers patches then follows (Weissman 1967a).

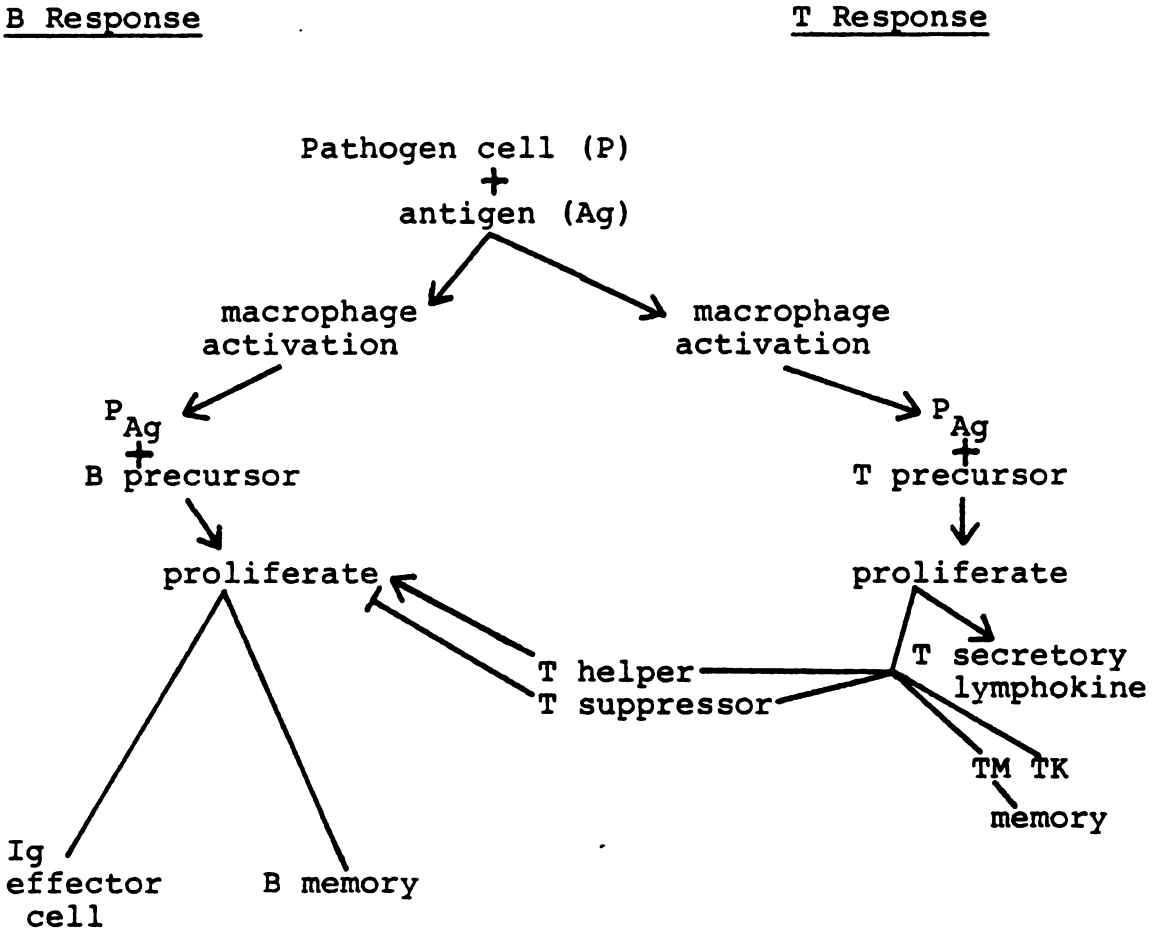
T cells are the major circulating lymphocytes migrating between the blood and lymph vasculature of lymphoid organs and other tissues. Though the main involvement of T cells is in cellular immune reactions, they can participate in antigen recognition. T lymphocytes function (1) in delayed hypersensitivity reactions (Cline 1975) against intracellular facultative microorganisms, viruses (Allison 1974), and protozoa (McGregor 1971), (2) in transplantation immunity (allograft reactions) (Hellstrom and Hellstrom 1969), and (3) in tumor immunity (Cerottini and Brunner 1974). The immune response occurs with the attachment of foreign antigen on surface receptors. This stimulates the T cells to proliferate and release effector factors identified as lymphokines. Lymphokine production is characteristic of T lymphocyte response and is responsible for its beneficial actions (Billingham et al. 1954, Cline 1975). The major lymphokines and their respective actions are described as

follows (modified version from Williams et al. 1977:

Lymphokine	Major Putative Action
Transfer Factor (TF)	Induction of immunocompetent uncommitted T cell into Ag-specific clonal proliferation.
Lymphotoxin (LT)	Direct killing effect on target cells.
Macrophage Migration Inhibition factor (MIF)	Localization of mobile phagocytes, amplification of immunocyte induction.
Macrophage-Activating Factor (MAF)	Increased function of macrophages, nonspecific cytocidal action.

T lymphocytes also interact with other cellular components of the immune system, including B lymphocytes, in most if not all disease states (Craddock et al. 1977).

The B and T immune response is summarized in the following illustration (modified version from Williams et al. 1977).



Lymphocyte Ultrastructural Description and Metabolic Function

There are no apparent morphological differences found between B and T cells (Zucker-Franklin 1969), however, they differ in their origin, function, membrane and physical properties and reactivity to various mitogens (Bach 1976, Greaves et al. 1974). The cytoplasm contains small or moderate numbers of mitochondria and occasional

vesicles (Renau-Piqueres et al. 1980, Zucker-Franklin 1969). The endoplasmic reticulum and golgi bodies are not well defined, and only individual ribosomes are observed. The nucleus often has a slight invagination and a small nucleolus (Matter et al. 1972). The cells are somewhat motile without noticeable specific tropisms (Harris 1954), except toward mitosing cells entering telophase (Humble et al. 1956). The lymphocyte has a minimal capacity for respiration and protein synthesis (Hedeskov 1968).

Lysosomal activity in the cell has an important role in the digestion and the breakdown of both endogenous and exogenous materials in lymphocytes (Weissman 1967b). Lysosomal rearrangement and release of lysosomal contents into cytoplasm also trigger the initiation of cell division (Allison 1974). Evidence also indicates lysosomal participation in phagocytosis, antigen processing (Weissman 1967) and possibly in delayed-hypersensitivity reactions (Turk et al. 1966).

Lymphocyte Mitogen Stimulation Assay

Lymphocyte stimulation is initiated when the mitogen (antigen) binds to surface receptors on the plasma membrane, increasing membrane permeability (Freedman and Gelfand 1976). The cell enlarges and appears immature. DNA replication and RNA transcription formulates the blast or transformation stage of the lymphocyte, (Williams et al. 1977), and can be measured with methyl ^{3}H-thymidine uptake during replication. The methyl ^{3}H-thymidine uptake

is measured with a beta-scintillation detector (Bloom et al. 1973).

Mitogens stimulate blastogenesis without the requirement of specific antigens. They are specific for either B or T cell populations (Anderson et al. 1972). The major mitogens are as follows:

1. Phytohemagglutinin (PHA) - involved in T lymphocyte reactions.
2. Concanavalin A (Con A) - involved in T lymphocyte reactions.
3. Bacterial Lipopolysaccharide (LPS) - involved in B lymphocyte reactions.
4. Pokeweed mitogen (PWA) - involved in stimulation of mixed lymphocyte reactions.

Other substances can inhibit lymphocyte function (Weissman 1967). For example, glucocorticoids inhibit RNA and protein synthesis in lymphocytes (Werthamer et al. 1969) by directly inhibiting the RNA polymerase (Fox and Gabourel 1967) or by directly binding to the nuclei or nuclear histones (Wira and Munck 1969). A subsequent decrease in protein synthesis and decrease in mitotic activity occurs (Zeman et al. 1972), resulting in a depression in lymphocyte transformation.

Colostrum and Immunity

Newborn calves are agammaglobulinemic and depend on colostrum as a source of immunoglobulins (Pierce 1962).

Maternal immunoglobulins are selectively concentrated in the mammary gland and secreted into the colostrum (Pierce and Feinstein 1965). Only IgG, IgM and IgA are transferred to the colostrum. Quantitatively, IgG is the major immunoglobulin (Porter et al. 1972) while IgM and IgA are found in decreasingly smaller quantities, respectively (Penhale et al. 1973). These immunologulins supply the newborn calf with passive immunity.

Passive immunity is acquired by the calf from colostral immunoglobulins only when they are absorbed in an unaltered form. Absorption of intact immunoglobulins occurs by non-selective pinocytosis through an apical tubular system in the absorptive cells of the small intestine (Staley et al. 1972). Transport from the intestine into the circulation occurs through the lymphatics within 1 to 2 hours after colostral ingestion (Comline et al. 1951). Gut permeability to intact immunoglobulin macromolecules declines with calf age. It decreases rapidly after 12 hours postpartum (Stott et al. 1979), and is essentially absent (termed gut closure) by 24 to 36 hours of age (Penhale et al. 1973).

These immunoglobulins are important for supplying the newborn calf with protection against common infectious agents (Irwin 1974). Susceptibility to infection, especially from E. coli, is related to the colostral immunoglobulin levels in the sera of calves (Fey and Margadant 1961, McEwan et al. 1970), thus calves deprived

of colostrum or having low circulating immune globulins can be experimentally infected with coliform bacteria (Dunne et al. 1956, Glantz et al. 1959). Corley et al. (1977) illustrated that colostrum-deprived calves absorb E. coli through the ileal cells of their small intestine.

The protection by colostrum against infectious agents occurs through two mechanisms. Besides conveying systemic immunity, colostrum has a local intestinal protective function (Logan and Penhale 1971ab). Logan and Penhale (1971ab) illustrated systemic protection against coli septicemia with IgM injections, but not enteritis which indicated the latter to be a local effect. Logan et al. (1974) also demonstrated that neither IgM nor IgA alone provided the full spectrum of protection and thus all three immunoglobulins (IgG, IgM, IgA) are required for optimal disease resistance.

Factors influencing the absorption of maternal immunoglobulins have been explored. These include factors such as parturition problems, temperature variation, food and water inconsistencies, injuries, pathogenic organisms and others (Cannon 1932) which stimulate the production of corticosteriod hormones. Investigations on the influence of these hormones on immunoglobulin absorption by bovine neonates are conflicting. Stott (1980) showed no effect while Stott et al. (1976) showed a depressive effect on immunoglobulin absorption with increased steriod levels. Johnston and Oxender (1979), however, reported of immunoglobulin absorption when corticosteriods increase.

The presence of maternal antibodies normally inhibits the calf's active immune response to specific foreign antigen (Husband and Lascelles 1975, Logan and Penhale 1971ab, Renshaw et al. 1976), until four weeks of age (Logan et al. 1973). Smith and Ingram (1965) and Caroll et al. (1968), however, provided evidence that colostrum-deprived calves produce antibodies at birth (active immunity), depending on the type of antigenic stimulation. Nonetheless, the calf is generally susceptible to infection in the absence of passive immunity (Dunne et al. 1956, Fey and Margadant 1961, Glantz et al. 1959, McEwan et al. 1970). However, high average immune globulin concentrations do not always mean complete resistance (Fey and Margadant 1971, Gay et al. 1964, Smith 1962).

Nutrition and Immunity

The cells involved in immunity require nutrients for their developmental, biochemical, metabolic and functional activities (Chandra 1972). Malnutrition decreases resistance to infection and infection exaggerates malnutrition. This is illustrated through changes in antibody formation, phagocytic activity, tissue integrity, inflammatory response, intestinal flora, endocrine metabolism and nonspecific protective mechanisms (cited by Scrimshaw 1975). High morbidity and mortality rates from infections are observed in countries afflicted with malnutrition problems, another indication of nutrient

importance in the immune defense system (Selvary and Sbarra 1966). Additionally, Alexander (1980) illustrated that nutritional repletion will improve immunological functioning and decrease susceptibility to infection.

The nutritional status of individuals and animals is antagonized by pathogens. The mechanism for this antagonism involves the direct destruction of host tissue or competitive leeching of the host nutrient pools to sustain the pathogens' nutritional requirements (Wilgus 1980). Infection also stimulates the host's immune defense mechanisms (Wilgus 1980) increasing metabolism of the host, increasing nutrient needs above maintenance requirements (Wilgus 1980), and resulting in added stress to the host's nutritional status. Additionally, disease states often cause anorexia, fever and stress (Scrimshaw 1977). Thus, nutritional needs are antagonized by infection (Wilgus 1980). Fever (Scrimshaw 1977) and other stresses which increase adrenocorticoid levels (Cannon 1932) increase urinary nitrogen losses and increase the need for vitamin A, ascorbic acid (Scrimshaw et al. 1968), iron, zinc and other nutrients (Beisel et al. 1967). Parasitic infections, e.g. hookworms, ascarids, and coccidia, affect absorption of nutrients, especially protein and vitamin A. Diarrhea also contributes to the nutrient depletion coinciding with disease processes (Scrimshaw 1977).

Nutritional depletion directly impairs immunity in the host. Insufficiencies in amino acids (Wilgus 1980),

vitamins (particularly pantothenic acid, vitamin B_6 and pteroylglutamic acid and, to a lesser extent, thiamin, biotin, riboflavin, niacin, and vitamin A) have been shown to impair the immune response (Axelrod 1971). Malfunctions in the immune response are associated with histological changes found in the spleen, thymus and liver, organs important in immunocyte development. The thymus appears to be the most prone to damage, especially during prenatal development (Olusi and McFarlane 1976). Both humoral and cell-mediated immune responses are affected (Chandra 1972).

B lymphocytes are not as influenced by malnutrition as T lymphocytes (Olusi and McFarlane 1976). Law et al. (1973) and Suskind et al. (1976) have shown that serum immunoglobulin levels are not changed significantly in malnutrition; however, Passwell et al. (1974) have shown that there is a decrease in functional immunoglobulins.

During malnutrition, the number of thymus-dependent lymphocytes is significantly reduced (Chandra 1979). In addition, delayed hypersensitivity development (Chandra 1974, Edelman, et al. 1973, Scrimshaw et al. 1968) and immunity against infections, such as measles and small pox, are also impaired (Scrimshaw et al. 1968). There is also an increased septicemia from fungal, viral and gram negative invasion (Bang et al. 1975, Chandra 1972, Ferguson et al. 1974). Susceptibility results from impairment in bactericidal, chemotactic and opsonin functions (Seth and Chandra 1972). Hence, insufficient

nutrients often lead to immunodeficiency and, frequently death (Scrimshaw 1975, Steihm 1980) from impairment of both humoral and cellular immunity.

Vitamin A and Immunity

Numerous disease processes have been associated with vitamin A deficiency. Vitamin A-depleted experimental animals commonly suffered lung infections (Cramer and Kingsburg 1924), and broncho-pneumonia. Septic abscesses, pneumonia and enteritis coincided frequently (Green and Mellanby 1928) with disruption in epithelial integrity in the larynx, trachea, and ducts of many glands in vitamin A-deficient rats (Mori 1922). Green and Mellanby (1928), as a result of these past observations, initiated experiments to relate vitamin A specifically to disease. Rats were placed on vitamin A-deficient diets and did well until infective processes overtook them. Microorganism invasion was recognized by pus or acute inflammation. Vitamin A was therefore considered as an anti-infective agent (Green and Mellanby 1928).

McLaren et al. (1965) observed that serum vitamin A and carotene levels of children dying with protein-energy malnutrition were significantly lower than of those children surviving. Rats on vitamin A deficient- diets have shown a marked leukopenia and a decreased number of circulating lymphocytes (Nauss et al. 1979), impairment in antibody response (Krishnann et al. 1974, Ludovich and Axelrod 1951), decreased phagocytosis (Perla and

Marmorston 1941), and atrophy of the lymphoid organs, spleen and thymus (Krishnann et al. 1974). An increase in infection also occurred (Cohen et al. 1979). In vitamin A-deficient subjects there were higher incidences of pneumonia, rheumatoid arthritis, acute tonsillitis, and rheumatoid fever (Jacobs et al. 1954, Shank et al. 1944). Further investigations revealed an increase in bacterial, viral, protozoal and parasitic (especially hookworm, ascarid, and coccidia) disease (Scrimshaw et al. 1968).

High doses of vitamin A significantly increase the immune response (Cohen et al. 1979), antibody formation (Cohen and Cohen 1973), and nonspecific resistance to infection in mice. Repletion of vitamin A-deficient mice restored lymphocytic proliferative response to approximately control values. Lymphocytes from the vitamin A-depleted mice were viable, yet their responsiveness was one third normal (Nauss et al. 1979). Others have researched the influence of vitamin A on cancer treatment and cancer prevention (Basu 1979, Gusland 1980, Mettlin et al. 1979, Micksche et al. 1977), with induction of immunity (Dressler 1968, Spitznagel and Allison 1979) occuring through the action of vitamin A on lysosomes to initiate lymphocyte proliferation (Spitznagel and Allison 1970). Vitamin A supplementation has also proven protective against infection under stressful conditions (Alexander 1980, Cohen et al. 1979, Weissmann 1967). Others have directly studied the role of vitamin A on both humoral and cell-mediated immunity (Cohen and

Cohen 1973, Jurin and Tannock 1972, Mickshe et al. 1977).

Humoral Immune Response

Immunoglobulins, are glycoproteins, which depend on vitamin A for synthesis (De Luca et al. 1970). As a result, impairment in membrane binding and proliferative response of both B and T lymphocytes occurs in hypovitaminosis A animals (Santer et al. 1973). With rats on deficient diets, however, IgG levels in the intestinal fluid, and serum IgA and IgG levels were unaffected while intestinal levels of secretory IgA were significantly lower. This effect hinders the local immune response and may result from the involvement of vitamin A in epithelial cell integrity (Sirisinha et al. 1980). Serum antibody levels observed in vitamin A-deficient swine are lower than control animals (Harmon et al. 1963).

Enhancement of antibody production by vitamin A supplementation has been demonstrated in mice (Cohen and Cohen 1973, Jurin and Tannock 1972). Tengerdy and Brown (1977) showed that vitamin A-supplemented chickens had an increased protection against E. coli and antibody levels 2-5 times higher than chickens not supplemented with vitamin A (Leutskay and Fair 1977). Potentiation of the local and systemic antibody response to an antigen was also attributed to administration of vitamin A (Falchuk et al. 1977).

Cell-Mediated Immune Response

Depletion of T lymphocytes (Bang et al. 1972, 1973) and atrophy of the thymus have been observed in vitamin A deficient animals (Jurin and Tannock 1972). Krishnann et al. (1974) has also noted a decrease in immune responsiveness to diphtheria and tetanus toxoids in vitamin A deficient animals. An increase in epithelial cancer susceptibility was noted in vitamin A deficiency (Park 1978) coincident with rearrangement in mucous glycoproteins (Roberts 1978) and uncontrolled interferon production (Blalock and Gifford 1977). As a result of epithelial malfunction associated with vitamin A deficiency, there was reduced production of normal carcinogenic deactivating enzymes (Park 1978).

However, cell-mediated immune responses, e.g. skin graft rejection (Jurin and Tannock 1972) and tumor rejection (Jurin and Tannock et al. 1972) are enhanced by vitamin A supplementation. Low doses of retinoic acid (Dennert and Lotan 1978) and some vitamin A derivatives (Lotan and Dennert 1979) have inhibited the development of chemically induced epithelial tumors and transplant tumors. Vitamin A induces its specific action on T killer cell precursors for T-cell mediated cytotoxicity (Dennert and Lotan 1978). Vitamin A therapy alone has been used successfully in the control of lung cancer. More prominent delayed cutaneous hypersensitivity reactions were observed in these patients compared with prior treatment (Micksche et al. 1977). An increase in vitamin

A intake has also been associated with a decreased incidence of lung cancer (Mettlin et al. 1979).

MATERIALS AND METHODS

Experiment I

Sixteen Holstein calves from the Michigan State University (MSU) dairy herd were used in this experiment. The calves, described in table 1, were assigned randomly at birth, and prior to colostrum consumption, to one of four feeding regimes, representing deficient, low and high levels of vitamin A supplementation and a naturally-fed group. They were kept on these regimes for 6½ weeks. All but the naturally-fed calves were fed colostrum from a pooled source acquired from the MSU dairy herd and rendered vitamin-A-low by removal of the butterfat with a milk separator. The pooled skimmed colostrum was stored frozen in 3.8 liter quantities. At feeding time the thawed colostrum was reconstituted with a vitamin A-low animal fat source[a]. Supplemental vitamin A, as water dispersable retinyl palmitate[b], was incorporated into the first colostral feeding at 0, 10,000, and 80,000 IU/calf assigned to the deficient, low and high vitamin A supplementation regimes, respectively. Each calf was bottle-fed a total of 3.8 liters of the pooled colostrum daily divided into two feedings, approximately 12 hours apart. This provided a uniform quantity of passive

[a]Land'O'Lakes, Webster City, Indiana
[b]Sigma Chemical Co., St. Louis, Mo.

Table 1. Description of calves on experiment I.

Group	Calf No.	Sex	Retinyl Palmitate Supplementation		Birth Wt. (kg)	Final Wt. (kg)	Days Expt.	Brucella vac.	
			Colostral[a]	Daily[b]				Age (days)	Date (1979)
1[c]	1	F	0	0	33.1	34.4	7	-	11/26
1[c]	2	M	0	0	*	-	3	-	11/30
1[c]	3	M	0	0	39.0	-	4	-	12/9
1[c]	4	F	0	0	*	-	3	-	12/12
2	5	F	10,000	800	*	47.6	38	31	11/28
2	6	F	10,000	800	39.9	45.4	33	27	11/28
2	7	F	10,000	800	*	54.9	43	35	12/2
2	8	M	10,000	800	29.0	37.6	46	38	12/15
3	9	F	80,000	5,000	41.7	40.4	46	39	12/8
3[c]	10	F	80,000	5,000	*	-	7	-	12/12
3	11	F	80,000	5,000	28.1	31.3	40	32	12/15
3	12	M	80,000	5,000	36.7	42.2	40	33	12/27
4[c]	13	F	42,000[d]	3,200[e]	32.7	-	2	-	12/19
4	14	F	42,000	3,200	41.7	54.4	44	39	12/20
4	15	F	42,000	3,200	45.4	53.5	42	35	12/22
4	16	M	42,000	3,200	35.4	47.6	36	28	12/26

* Weights were unavailable

[a]Colostrum without supplementation provided an additional 18,000 IU/calf of vitamin A for groups 1,2,3.

[b]Milk replacer without supplementation provided 1,300 IU/calf/day of vitamin A for groups 1,2,3 1979.

[c]Calves that did not live for entire experimental period.

[d]Total vitamin A (IU) supplied naturally to each calf/day in group 4 in the colostrum fed on day one.

[e]Total vitamin A (IU) supplied naturally to each calf/day in group 4 in the whole milk fed daily.

immunity (immunoglobulins) per calf[c]. The naturally-fed calves remained with their dams for 24 hours and obtained colostrum directly from them ad libitum.

All calves were given BoSe[d], subcutaneously (SQ) at 3cc/45 kg body weight and placed, at 24 hours of age, in individual calf hutches in the natural outdoor environment. In addition, calves on the natural diet were given SQ injections of vitamins A, D and E before going out to the hutches. Environmental extremes occured during this experiment conducted between 26 November and 6 February 1980. Damp and southernly winds predominated and the temperatures ranged between -13 and 11°C (The daily weather conditions are presented in appendix 1).

The calves were then bottle-fed a commercial milk replacer[a], specially prepared without supplemental vitamin A, 2x/day at a maximum rate of 10g/kg calf/day. This milk replacer was supplemented once/day with water-dispersable retinyl palmitate, providing 0, 800, and 5,000 IU vitamin A/day/calf, respectively, for the deficient, low and high vitamin A groups. The naturally-fed calves received pooled fresh milk from MSU

[c]Joint study was done with Dr. J.V. Marteniuk (MSU), whose emphasis was immunoglobulin absorption and production in these experimental calves.
[d]Burns Bio Tec. (1 mg Se and 50 IU vitamin E/ml)
[e]500,000 IU vitamin A
75,000 IU D_2
5 IU Vitamin E/ml

dairy cows. The calves were on this experiment for a maximum of 46 days. Feeding intervals and daily quantities of milk replacer were occassionally adjusted for calves developing diarrhea. Antibiotic therapy was instituted in calves with body temperatures above 37.8°C.

Blood samples were drawn from the external jugular vein pre-and postcolostrally and prior to the morning feeding at 1, 2, 3 and 4 weeks of age. Each calf was then given 1 cc (½ normal dosage) of strain 19 Brucella abortus vaccine[f], and resampled at 3 and 7 days post vaccination.

Modification in treatment occurred for individual calves depending on their health status and weather conditions. To prevent added stress in such circumstances, sample times were missed. As a result, for some calves alteration in Brucella vaccination schedule occurred. However, postvaccination sampling was consistent at 3 and 7 days following vaccination.

On each sampling day, two 10 ml sterile Vacutainer[g] tubes of blood were collected, one with 7 units/ml of heparin[h] and the other without. An extra precolostral sample was obtained in the heparinized tube containing 1 ml of culture media (98 ml RPMI 1640 media[i] + 1 ml

[f]Jen Sal, Jensen Salsbery Laboratories, Kansas City, Mo.
[g]Becton and Dickinson; Rutherford, N.J.
[h]UpJohn Co.; Kalamazoo, MI.

penicillin (10,000 Units)-streptomycin (10,000 ug)[i] + 1 ml heat-inactivated fetal calf serum[i]). Heparinized blood samples for white blood cell counts (WBC), pack cell volumes (PCV), and lymphocyte cultures were stored at room temperature (25°C) and processed within 2-6 hours after being obtained, depending upon interval between calving and notification of same. The precolostral samples and weights of calves were obtained by the herdsman within 1-2 hours after birth. Calf weights were obtained once weekly, thereafter.

Serum was collected from the nonheparinized blood and stored frozen until assays for vitamin A and cortisol.

Experiment II

Since too many variable conditions existed in experiment I, experiment II was designed to avoid environmental, sampling and other inconsistencies of the first experiment.

Nine healthy Holstein bull calves described in table 2, were obtained at birth from a private herd[j], and assigned at random precolostrally, 3 per group, to the vitamin A-deficient, low and high supplementation programs as in experiment I. Each group of calves was housed in separate, temperature-controlled (20-25°C) rooms.

[i]Grand Island Biological Co.; Grand Island, N.Y.
[j]Green Meadows Farm; Ovid, Mi.

Table 2. Description of calves on experiment II.

Group	Calf No.	Birth Date (1980)	Sex	Retinyl Palmitate Supplementation		Calf Weights (Kg)		Days on Expt.	Brucella Vaccination Age (days)
				Colostral[a]	Daily[b]	Birth	Final		
1	1	2/8	M	0	0	45.4	61.2	35	28
1	2	2/12	M	0	0	43.1	57.2	35	28
1	3	2/20	M	0	0	49.9	49.0	35	28
2	4	2/24	M	10,000 IU	800 IU	45.4	57.2	35	28
2	5	2/25	M	10,000 IU	800 IU	44.0	60.8	35	28
2	6	2/26	M	10,000 IU	800 IU	37.2	53.5	35	28
3	7	2/21	M	80,000 IU	5,000 IU	44.9	57.6	35	28
3	8	2/8	M	80,000 IU	5,000 IU	47.6	63.5	35	28
3	9	2/14	M	80,000 IU	5,000 IU	43.1	63.0	35	28

[a]Colostrum without supplementation provided an estimated 18,000 IU vitamin A/calf, for groups 1, 2, 3.
[b]Milk replacer without supplementation provided an additional 1,300 IU vitamin A/calf/day for groups 1, 2, 3.

Individual calves were tied with a neck strap and rope to prevent sucking each other.

Feeding, sampling and vaccination regimes were similar to experiment I, except calves were pail-fed after 1 week of age and the experiment ended when calves were 35 days of age. All calves remained on schedule for treatments and sampling times.

Experiment III

Experiment III was designed to study the direct in vitro effects by retinyl palmitate on lymphocyte blastogenesis.

In this experiment, heparinized blood samples from coxygeal veins were collected with Vacutainer[g] tubes four different times from 16 randomly-chosen lactating MSU Holstein cows, described in table 3. Peripheral lymphocytes were collected from these blood samples and cultured with graded quantities of water-dispersable retinyl palmitate. Vitamin A analyses were conducted in one plasma sample for each of the 16 cows.

Table 3. Description of cows on experiment III[a].

Cow No.	Age (years)	Plasma[b] Vitamin A Concentrations (IU/dl) Retinyl palm.	retinol	Total	Leukosis Test
1	7.5	16	104	120	+
2	6.0	10	85	95	+
3	8.5	16	69	85	+
4	5.5	20	75	95	+
5	5.5	17	59	76	+
6	5.0	15	93	108	+
7	6.0	17	94	111	+
8	5.7	11	60	71	+
9	4.5	21	87	108	-
10	4.0	14	63	77	+
11	5.5	21	69	89	+
12[c]	8.0				
13	8.0	20	65	85	+
14	4.0	10	93	103	+
15	5.3	24	106	130	-
16	3.5	19	76	95	+

[a]These cows were on no standard feeding regime in the Michigan State University herd

[b]Plasma collected for all cows 10/9/80, stored frozen and assayed 11/3/80.

[c]This cow was culled from MSU dairy herd prior to plasma collection for vitamin A analysis.

ANALYTICAL PROCEDURES

White Blood Cell Counts (WBC) - Packed Cell Volumes (PCV)

Total WBC counts were determined with a hemocytometer (Bright-Line, 0.1 mm deep)[k]. Packed cell volumes were obtained by centrifugation of microcapillary tubes[l].

Lymphocyte Cultures

Reagents were stored at 5°C and brought to room temperature prior to use. Sterile techniques and materials were used in all phases, up to but excluding harvesting.

Isolation

Lymphocytes were isolated from heparinized blood samples by a modification of the Ficoll-Hypaque procedure described by Muscoplat et al. (1974). Eight ml of blood were diluted to 50 ml volume and gently mixed with Hanks balanced salt solution (HBSS)[i]. Twenty ml of this dilution were then layered over 10 ml of Histopaque[b] in a 50 ml polycarbonated centrifuge tube, and centrifuged at

[k]American Optical Co.; Buffalo N.Y.
[l]International Microcapillary centrifuge Model MB.

600 rpm for 25-45 minutes. The lymphocyte layer (buffy interphase) was carefully removed, placed in another 50 ml/tube with enough HBSS to cover cells, mixed gently and centrifuged for 10 minutes at 150 xg. This wash step was repeated with HBSS and culture media for a total of 3 washes. The supernate was discarded each time. After the final wash, 2 ml of culture media were added and cells were counted by suspending 0.05 ml of the mixture into 0.45 ml of a 0.40% Trypan blue mixture prepared with HBSS. Counts were made by filling a hemocytometer and counting the unstained cells in the 1 mm squares (cells/2 ml = cell count x $10^6/mm^2$). The final cell mixture was diluted with culture media to 1 x 10^6 cells/ml, and cultured in covered microtiter plates[m] with 96 flat-bottom, 0.35 ml wells.

In Experiment I, lymphocytes were cultured in triplicate in wells containing 0.00, 0.25, 0.50 and 1.00 ug of Con A. The commercial Con A was diluted to a concentration of 1000 ug/ml culture media and filter-sterilized through a 0.20 um Millipore filter. A working stock of 200 ug Con A/ml culture media was then prepared from this. The remainding dilutions were prepared from the working stock. All dilutions were stored frozen. Each culture well contained 1 x 10^5 cells in sufficient

[m]Linbro; Hamden, Conn.

culture media to make 0.20 ml total volume. Cultures were incubated at 35°C and in 4% CO_2 for approximately 29 hours, at which time 0.5 uCi of methyl ^{3}H-thymidine[n], from a working stock of 2/uCi/ml prepared in culture media, was added to each well. Cells were reincubated and harvested with a multiharvester[o] at 48 hours on glass fiber filter strips[p].

In Experiment II, cultures were set up as in experiment I except 0.00, 0.10, 0.25 and 0.50 ug Con A/ culture well were used, the ^{3}H-thymidine was added at approximately 55 hours of culture and harvesting was done at 72 hours.

In Experiment III, cultures were set up as in experiment II, except each sample was cultured with 0, 10, 100, and 1000 ng of water-dispersable retinyl palmitate for each set of four mitogen concentrations used.

Counting

After the filter paper dried, each filter disc was placed in individual, glass scintillation vials (15 x 45mm)[q]. Two ml of scintillation fluid (42 ml Spectrofluor[r]/liter toluene) were added to vials which were counted, 1 minute/sample, in a beta-scintillation

[n]New England Nuclear (Sp. Act. 6.7Ci/mMol in sterile aqueous soln.; Boston, Mass
[o]Cell Harvester Model 12V Brandel; Rockville, Md.
[p]MA Bioproducts; Walkersville, Md.
[q]Rochester Scientific; Rochester, N.Y.
[r]Amersham Searle; Arlington Ht., Ill.

counter[s] having an open ^{3}H range. Counts from triplicate wells were averaged and recorded as counts per minute (CPM) representing ^{3}H thymidine uptake by the cellular deoxyribonucleic acid (DNA) and indicating degree of lymphocyte transformation.

Serum Vitamin A

Serum samples were thawed over night under refrigeration and processed under subdued lighting by a modified procedure described by Dennison and Kirk (1977). One ml of serum was mixed with 1 ml of absolute glass-distilled ethanol in 10 ml disposable test tubes, stoppered, and vortexed 10 seconds. Two ml of filtered, glass-distilled, UV grade hexane[t] were then added and the mixture was then vortexed 1 minute and centrifuged for 10 minutes at 600 x gravity. The hexane supernate layer was removed with a Pasteur pipet, and passed through a 0.45 um Millipore filter into a 10 ml glass tube. One hundred ul of this extract were injected onto a Microporosil[u] column (3.9 mm ID x 30 cm) in an isocratic HPLC system[u]. The solvent, a 60:40 ratio of degassed UV grade hexane and chloroform[t], was pumped at 2.5 ml/minute @ 60kg/cm^{2} across the column and through a 35 ul flow cell in a

[s]Beckman LS 9000; Irvine, Ca.
[t]Burdick and Jackson, Muskegon, Mi.

spectrofluorometer[v] with excitation and emission wavelengths set at 330 and 470 nm, respectively. The detection signals of the fluorometer were recorded by an integrating data module[u].

Serum Cortisol

Serum cortisol determinations were assayed in the endocrine section of Michigan State University Animal Health Diagnostic Laboratory, using Gamma Coat ^{125}I Cortisol Radioimmunoassay kits[w], and ^{125}I detection with a gamma counter[x] set at 1 minute count time/sample. This assay was modified to make it valid for bovine cortisol determinations by increasing the ANS (8-Anilino-1-Naphthalene Sulfonic Acid) concentration (adding 0.20 mg ANS/20.00 ul bovine serum sample).

[u]Waters Assoc.; Milford, Ma.
[v]Aminco Bowman spectrofluorometer.
[w]Travenol Laboratories; Inc., Cambridge, Mass.
[x]Micromedic 4/600.

RESULTS

A. Experiment I

General Calf Health

Nine of the original sixteen calves completed this trial. All calves in the vitamin A deficient group (group 1, Table 1) died acutely from scours within 7 days of age. One calf from each of the other groups, low vitamin A (group 2), high vitamin A (group 3) and naturally-fed group (group 4) died in a similar manner (Table 1). Calves supplemented with high concentrations of vitamin A (group 3) seemed to have greater digestive and respiratory difficulties than the other surviving groups of calves.

The mean PCV values are shown in Table 4. The PCV values of all calves 24 hours postcolostrum were significantly ($P < 0.05$) less than at birth. Thereafter, there were no significant differences in mean PCV among any surviving groups of calves; however, the PCV tended to decline in groups 2 and 3 during the course of the experiment and tended to be higher in the naturally-fed group throughout the experiment than groups 2 and 3.

WBC data are also presented in Table 4. The mean values ranged from 4,300 - 13,000 among all groups of calves. The initial (precolostral) mean WBC for the calves for the deficient vitamin A group was significantly higher ($P < .02$) than the initial means of any of the other

Table 4. Comparison of packed cell volumes and white blood cell counts of naturally fed calves and calves fed milk replacer with different levels of retinyl palmitate from birth to 46 days of age (Expt. I).

Vitamin A Feeding regimen	Days of age										
	0	1	7	14	21	28	31	35	38	42	46
	% packed cell volumes										
Natural	30.1	27.0	---	29.3	30.9	30.6	31.0	33.5	29.9	32.2	33.0
Deficient	31.6	26.1	---	---	---	---	---	---	---	---	---
Low	31.7	23.2	27.2	28.7	29.1	31.5	30.3	30.6	23.9	26.9	30.0
High	29.4	26.5	31.8	27.2	25.5	28.5	25.8	25.9	26.8	25.0	28.0
	white blood cell counts x $10^3/mm^3$										
Natural	4.5	5.7	---	10.0	7.3	7.7	9.2	8.8	13.0	6.6	8.8
Deficient	11.7	8.2	---	---	---	---	---	---	---	---	---
Low	7.9	4.9	5.3	7.5	5.8	6.8	8.2	6.7	8.9	5.2	5.2
High	5.7	4.3	9.3	6.4	8.2	7.8	9.0	9.8	8.7	8.4	6.5

groups. During the course of the experiment, there was no consistent trends or significant differences in mean WBC counts among any of the groups.

Serum vitamin A

Retinyl palmitate

The mean serum retinyl palmitate (rp) concentrations are presented in Figure 1. There was an immediate increase in rp following consumption of colostrum in all groups to a maximum of approximately 26 IU/dl serum. Thereafter, the serum concentrations of rp did not reflect differences in rates of vitamin A supplementation and varied widely. There was no consistent response of serum rp to Brucella vaccination of the calves.

Retinol

The mean serum retinol (r) concentrations are presented in Figure 2. The mean serum retinol levels of group 2 and 3 increased gradually during the course of the experiment, during which group 3 tended to be higher than group 2. The mean serum retinol levels of group 4 were significantly ($P < .01$) greater that the other groups within 24 hours postpartum and remained significantly

Figure 1. Comparison of serum retinyl palmitate concentration of calves fed different levels of retinyl palmitate in milk replacer and of calves naturally fed from birth to 48 days of age. (Experiment I)

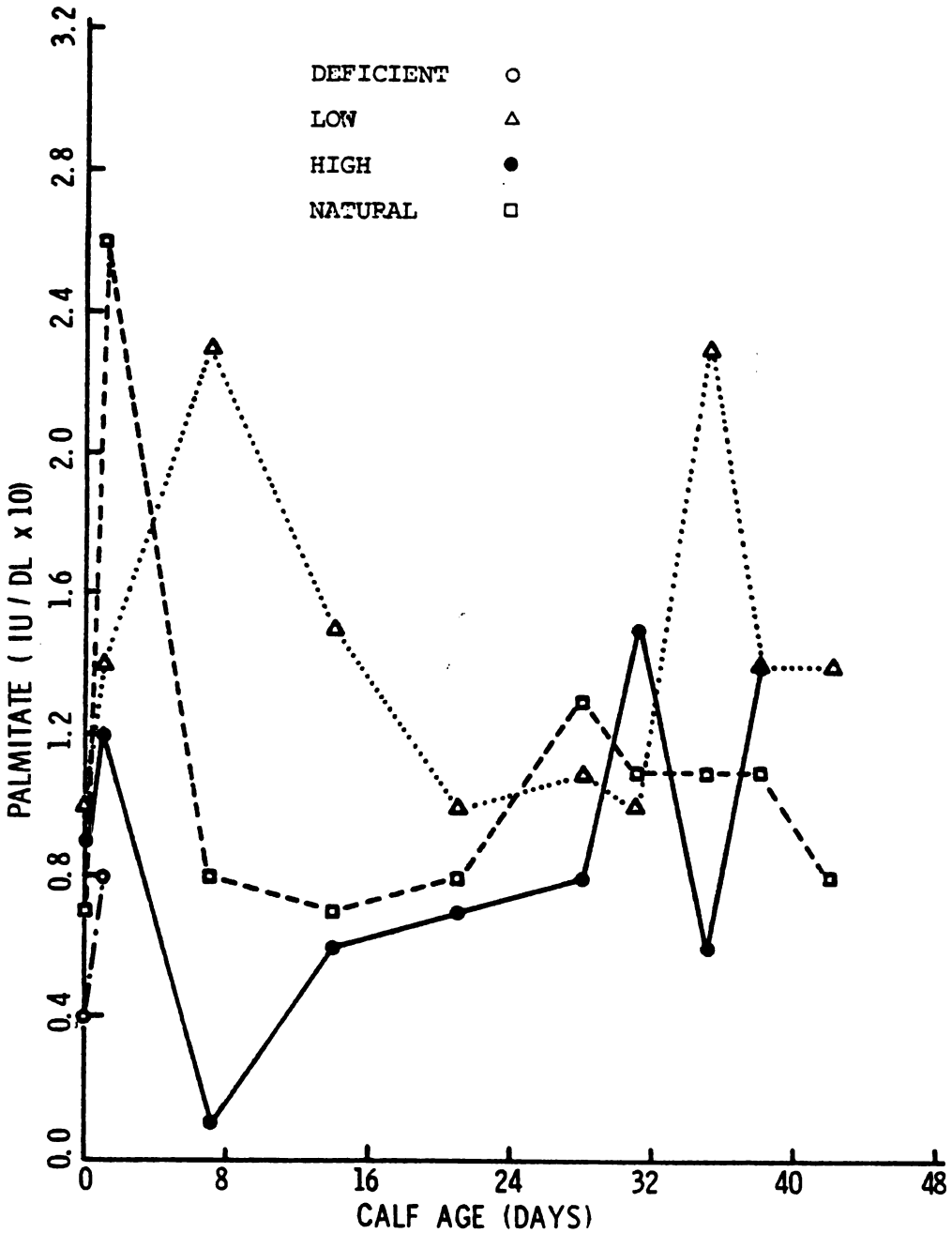

DEFICIENT
LOW
HIGH
NATURAL
PALMITATE (IU / DL x 10)
0.0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
0
8
16
24
32
40
48
CALF AGE (DAYS)

Figure 1

Figure 2. Comparison of serum retinol concentrations of calves fed milk replacer containing different levels of retinyl palmitate and of calves fed naturally from birth to 48 days of age. (Experiment I)

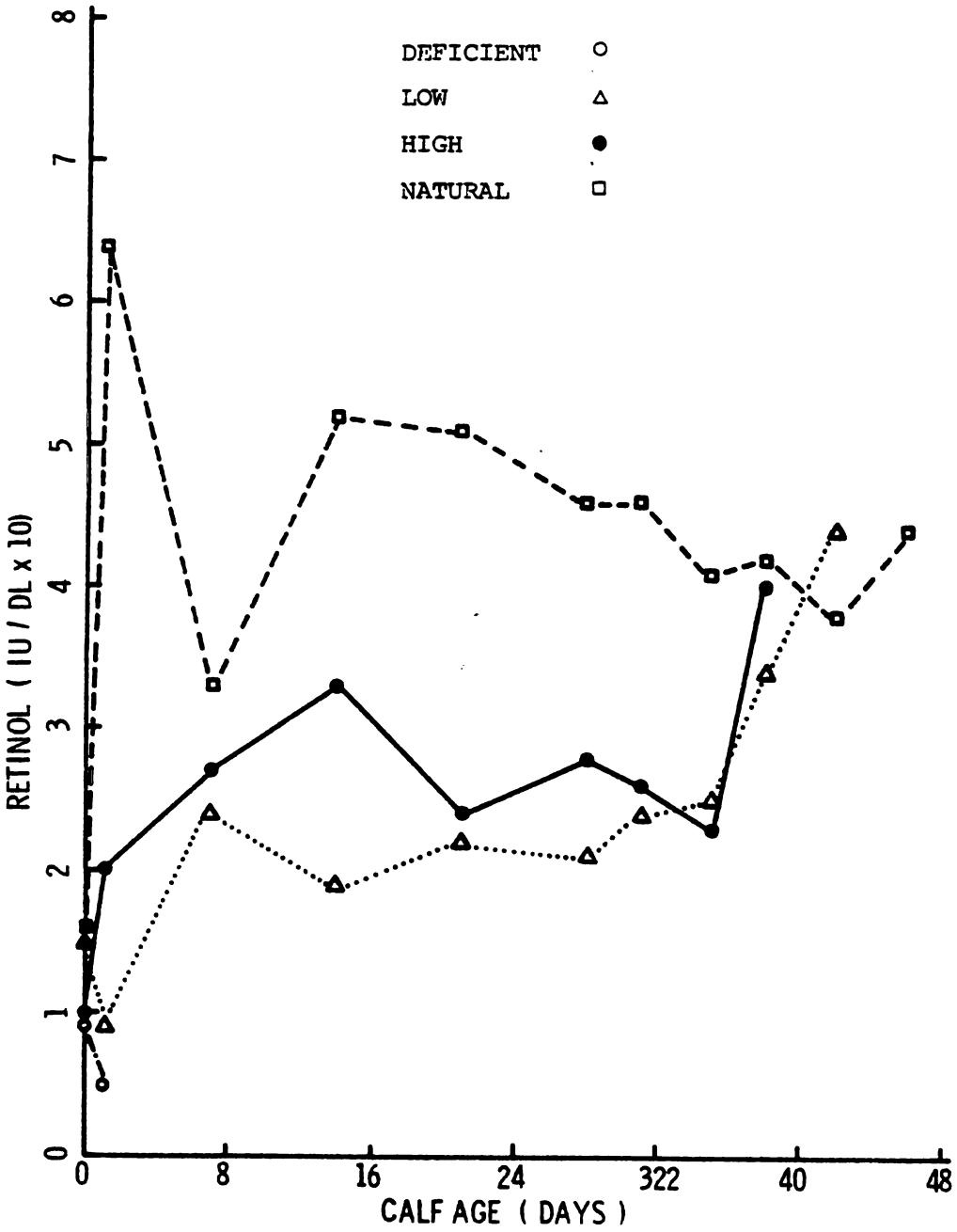

DEFICIENT
LOW
HIGH
NATURAL
RETINOL (IU / DL x 10)
0
1
2
3
4
5
6
7
8
0
8
16
24
322
40
48
CALF AGE (DAYS)

Figure 2

higher between days 7 and 36 of the experiment. There was a tendency for a postvaccinal increase in serum retinol for groups 2 and 3.

Total Vitamin A (retinyl palmitate and retinol)

The mean total serum vitamin A concentrations are illustrated in Figure 3. These reflect changes in serum retinol concentrations during the experiment more so than changes in rp. As with rp, they did not consistantly reflect the dietary vitamin A supplementation.

Cell Mediated Immunity

In vitro lymphocyte transformation data are presented in Table 5 and Figs. 4 and 5. These data were from culture wells containing 0.10 ug Con A, which generally resulted in optimum blastogenic response. Uptake of H^3-thymidine by control cultures of lymphocytes ranged from 215-7950 cpm for all calves during the experiment and there were no significant differences between groups.

Uptake of H^3-Thymidine by mitogen-stimulated calf lymphocytes expressed as CPM, Δ CPM and SI increased significantly ($P < 0.0041$) in all surviving groups (2,3, and 4) within 24 hours of calf age. Lymphocytes from low vitamin A-supplemented calves showed no further increase in blastogenic activity for the remainder of the trial period. However, lymphocytes from calves on high

Figure 3. Comparison of total serum vitamin A concentrations of calves fed milk replacer containing different levels of retinyl palamitate and of calves fed naturally from birth to 48 days of age. (Experiment I)

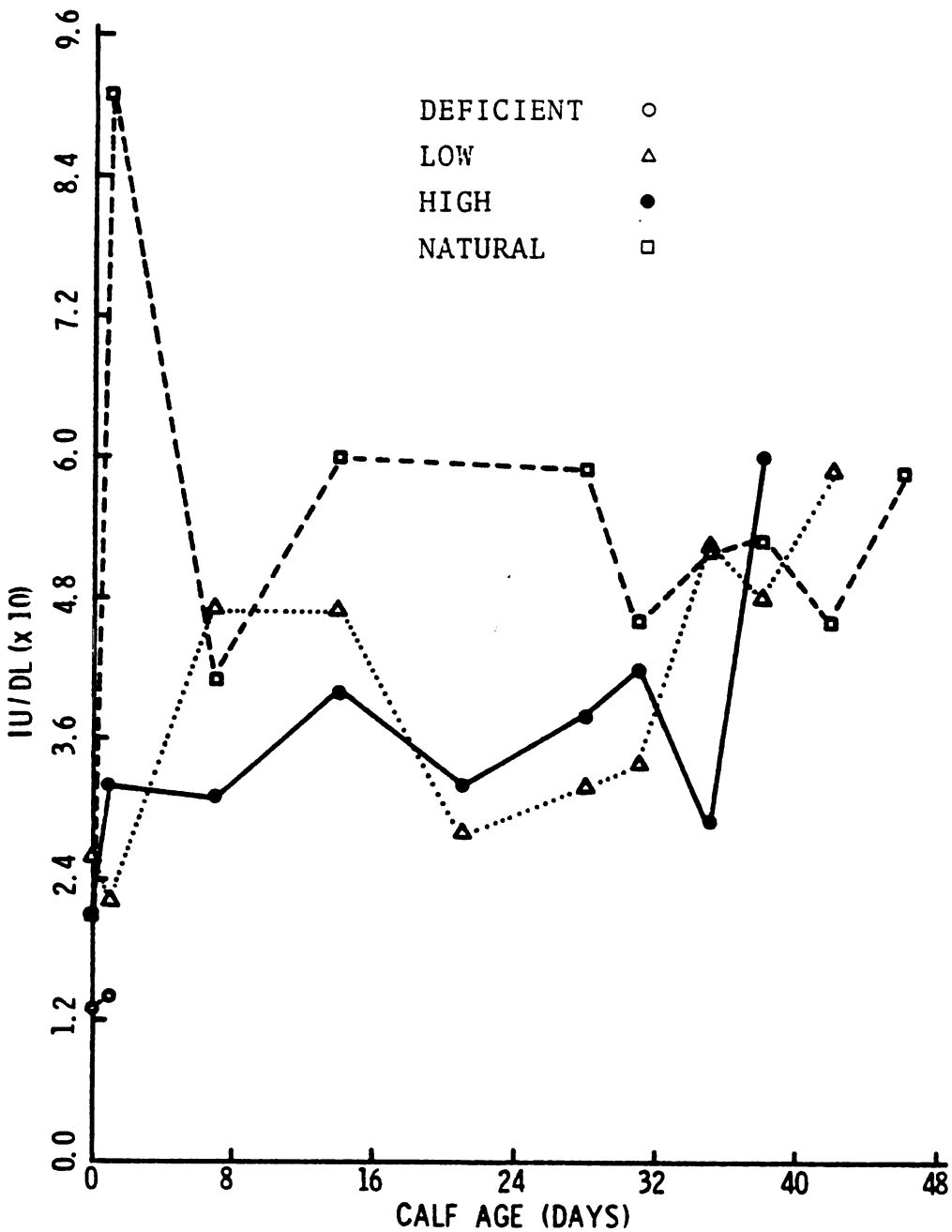

DEFICIENT
LOW
HIGH
NATURAL
IU/DL (x 10)
0.0
1.2
2.4
3.6
4.8
6.0
7.2
8.4
9.6
0
8
16
24
32
40
48
CALF AGE (DAYS)

Figure 3

Table 5. Comparison of in vitro blastogenic responses of lymphocytes of naturally-fed calves and calves fed milk replacer with different levels of retinyl palmitate (Experiment I).

Vitamin A Feeding Regimen	Days of age										
	0	1	7	14	21	28	31	35	38	42	46
	counts per minute[a] w/o mitogen x 10^3										
Natural	1.8	3.0	2.0	3.0	3.0	2.5	2.9	1.1[c]	0.9	1.2	0.2
Deficient	1.1	4.9	---[b]	---	---	---	---	---	---	---	---
Low	0.5	5.1	2.7	3.0	5.0	4.2	2.4	2.5	4.7	5.0	0.4
High	1.1	1.2	2.6	1.0	1.4	1.1	1.8	0.6	1.0	8.0	3.3
	counts per minute[a] with 0.1 ug Con A/well x 10^3										
Natural	66.4	134.0	218.0	172.0	99.0	118.0	130.0	146.0[c]	47.0	19.0	15.2
Deficient	38.0	45.1	---[b]	---	---	---	---	---	---	---	---
Low	10.2	188.5	113.2	153.1	215.0	204.2	135.4	161.5	145.7	148.8	172.4
High	27.2	185.1	197.6	125.0	93.4	90.1	74.8	18.6	60.0	104.0	216.2

[a]geometric mean representing H^3-thymidine uptake

[b]calves died

[c]Brucella vaccination

Figure 4. Representation of the effects of different rates of retinyl palmitate supplementation in calves on the vitro blastogenic activity of their lymphocytes. Activity is expressed as the difference between the rates of tritiated thymidine (H^3) uptake of cells in the presence and absence of 0.1 ug Con A per culture well. (Experiment I)

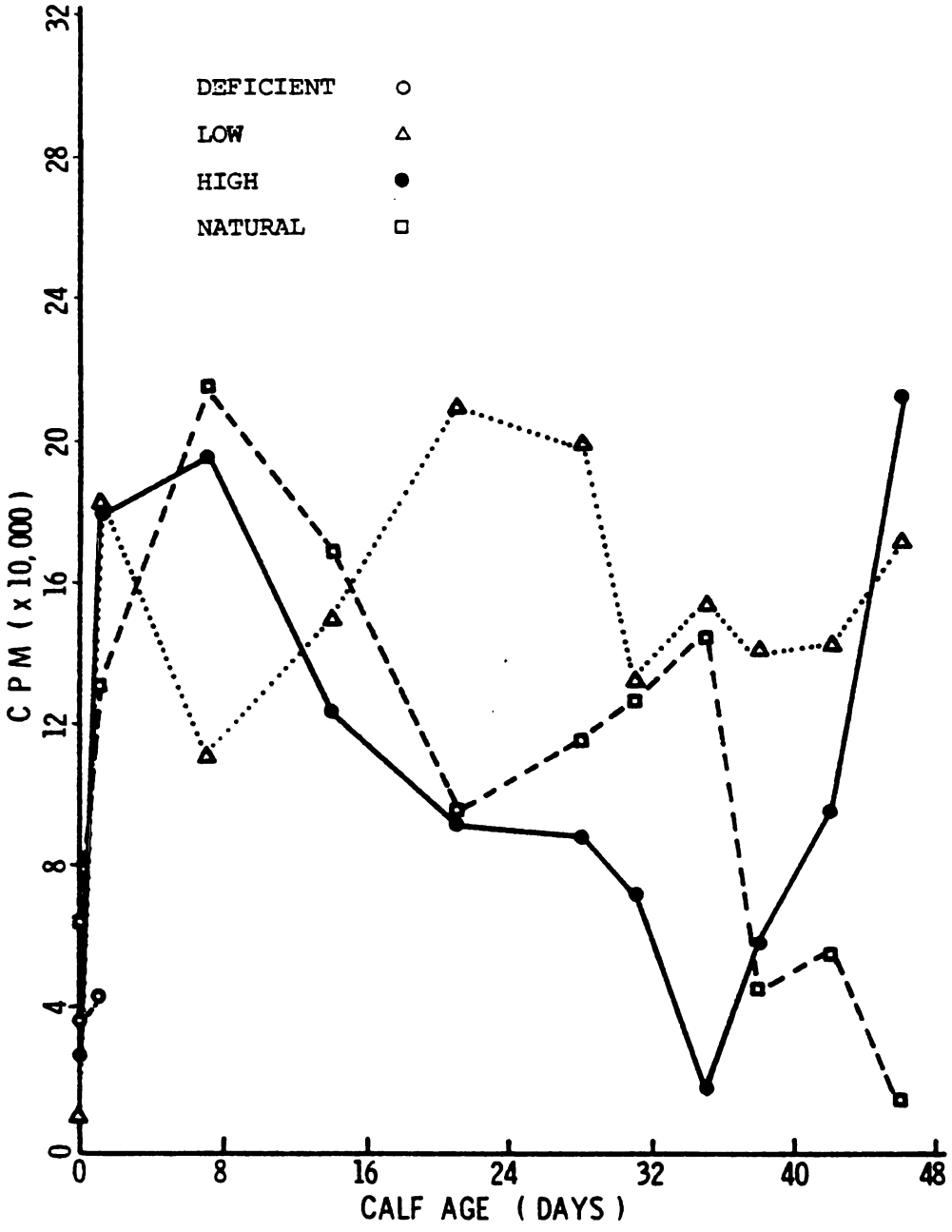

DEFICIENT
LOW
HIGH
NATURAL
CPM (x10,000)
0
4
8
12
16
20
24
28
32
0
8
16
24
32
40
48
CALF AGE (DAYS)

Figure 4

Figure 5. Representation of the effects of different rates of retinyl palmitate supplementation in calves on the in vitro blastogenic activity of their lymphocytes. Activity is expressed as stimulation index (SI) which is the ratio of the rate of tritiated thymidine (H^3) uptake of cells in the presence of 0.1 ng Con A per culture well to the rate of H^3 uptake of cells without mitogen. (Experiment I)

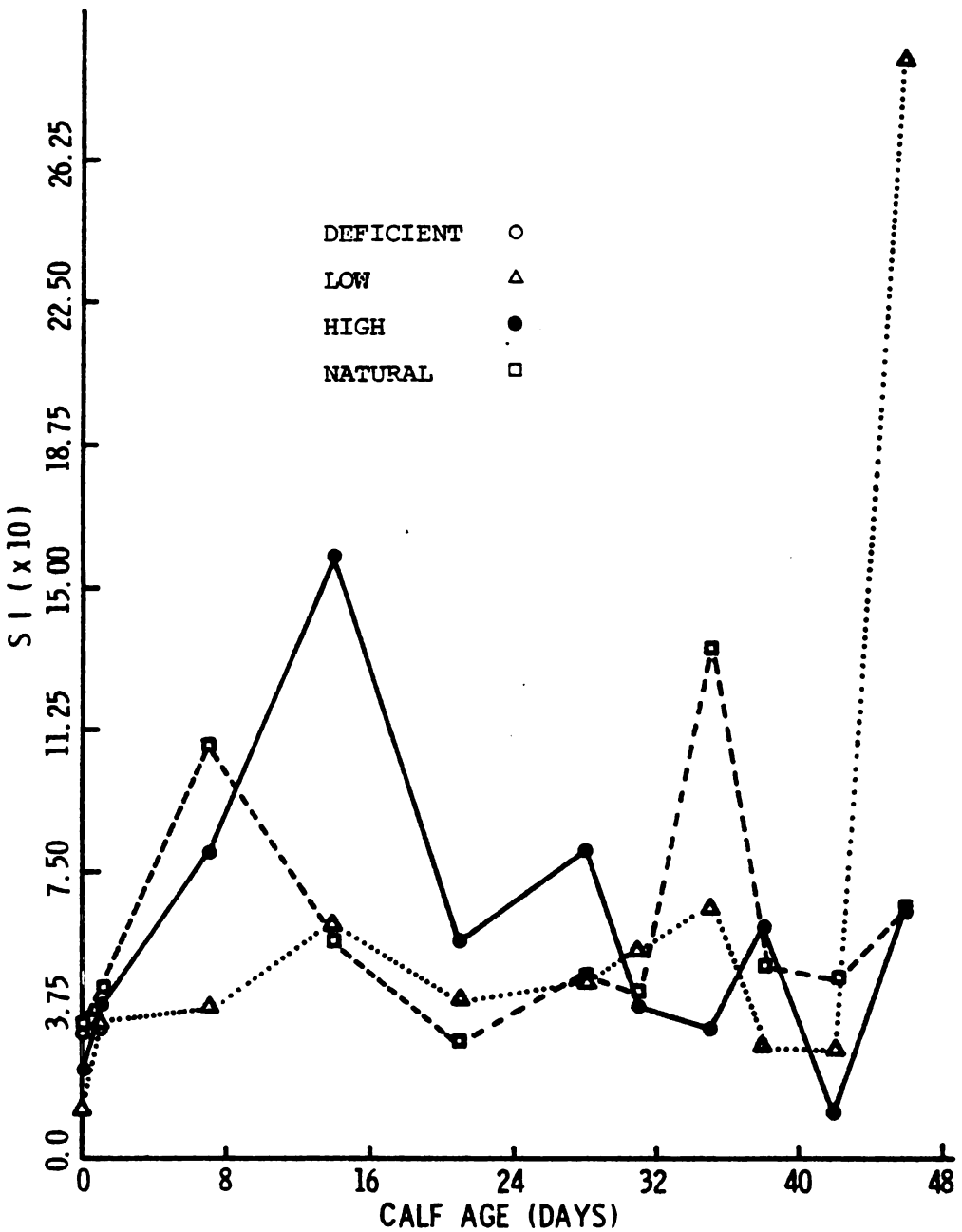

DEFICIENT
LOW
HIGH
NATURAL
S I (x 10)
0.0
3.75
7.50
11.25
15.00
18.75
22.50
26.25
0
8
16
24
32
40
48
CALF AGE (DAYS)

Figure 5

vitamin A supplementation regimes progressively decreased in blastogenic activity from one week of age until 3 and 7 days post-Brucella vaccination when an increase in activity was observed. The naturally-fed calves followed a similar trend in lymphocyte transformation as observed for the high vitamin A supplemented group, except that blastogenic response continued to decline following vaccination. Nonetheless, statistical analysis indicated that there were no significant differences between rates of lymphocyte transformation among the groups of calves surviving to experiment termination.

Cortisol

The mean serum cortisol concentrations are illustrated in Figure 6. The mean cortisol values for all calves declined significantly ($P < .01$) within 24 hours of calf age. In general there was a gradual decline thereafter in serum cortisol concentrations for all calves in all groups for the duration of the experiment with intermittent exceptions in the low and high vitamin A groups at 8 and 16 days of age, respectively. There were no significant differences in serum cortisol levels due to treatment.

Figure 6. Comparison of serum cortisol concentrations of calves fed different levels of vitamin A as retinyl palmitate from birth to 48 days of age.(Experiment I)

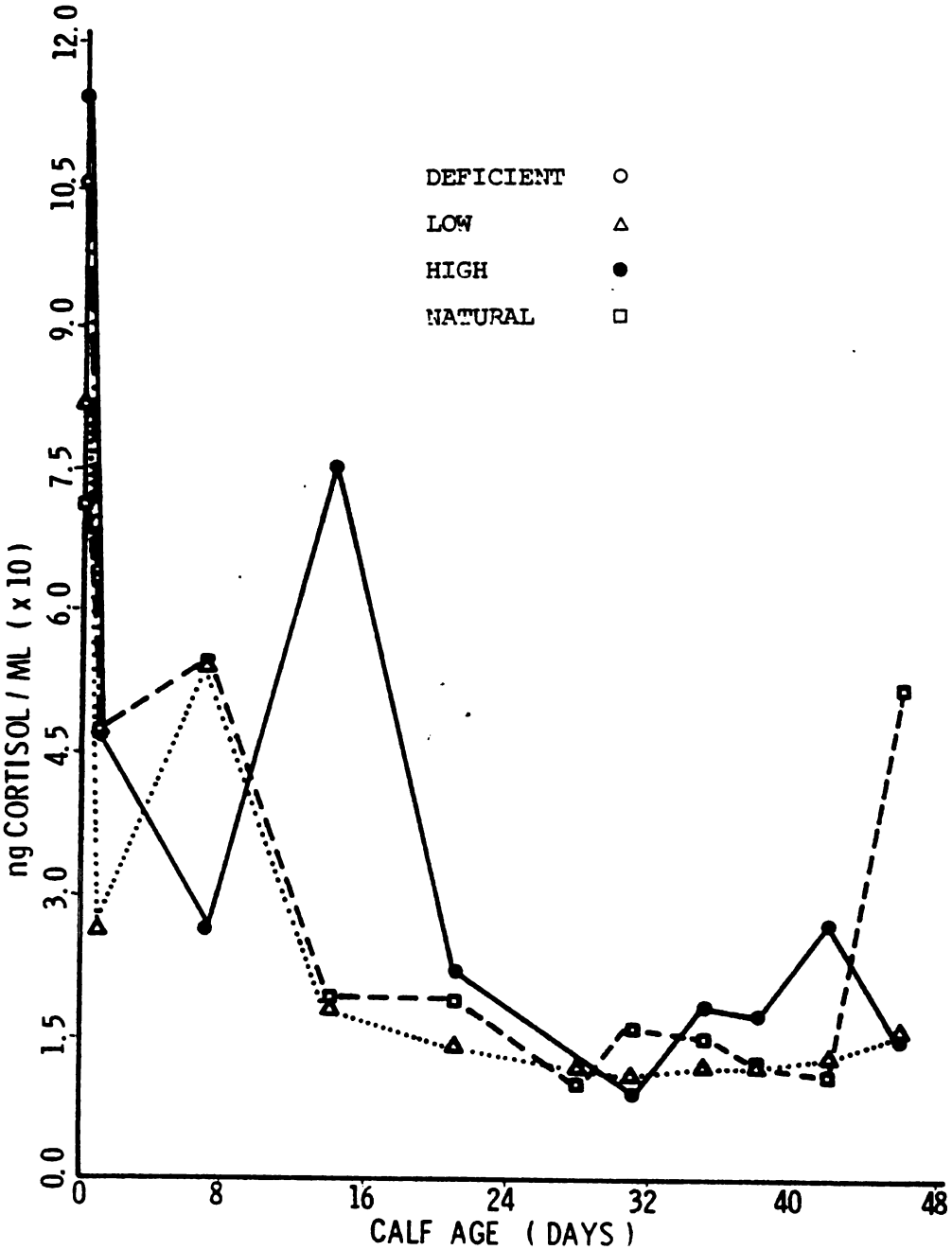

DEFICIENT
LOW
HIGH
NATURAL
ng CORTISOL / ML (x 10)
0.0
1.5
3.0
4.5
6.0
7.5
9.0
10.5
12.0
0
8
16
24
32
40
48
CALF AGE (DAYS)

Figure 6

B. Experiment II

General Calf Health

All calves survived this experiment; however, one calf from the vitamin A deficient group developed pyrexia and diarrhea at 3 days of age and remained unthrifty for the duration of the experiment despite antibiotic therapy. All calves on low vitamin A supplemented diet had mild diarrhea for the first week of age while the high vitamin A supplemented group remained healthy throughout.

The PCV data are presented in Table 6. The initial PCV values for the three calf groups ranged between 31.4-33.7%. There was a significant decrease ($P < .008$) in the PCV of vitamin A deficient calves during the experiment with the final mean value of 22.9% at 35 days of age. A similar decline in PCV to 25.9% was observed in the high vitamin A group during the experiment while PCV of calves consuming the intermediate vitamin A level remained relatively constant during the experiment.

The WBC data are presented in Table 6. The initial mean WBC values for each group were within a normal range between 8,100-11,500. WBC values of vitamin A-deficient calves remained significantly higher ($P < .05$) than the high vitamin A group, which was generally lower than the intermediate supplemented group throughout the experiment.

Table 6. Packed cell volumes and white blood cell counts for calves fed different levels of vitamin A as retinyl palmitate from birth to 38 days of age (Experiment II).

Dietary vitamin A	Days of age							
	0	1	7	14	21	28	31	35
	% packed cell volumes							
Deficient	33.3	29.0	29.0	28.3	28.4	27.2	24.7	22.9
Low	33.7	30.8	32.0	30.7	30.2	29.9	29.7	31.6
High	31.4	26.3	28.2	30.8	30.8	27.0	26.6	25.9
	white blood cell counts x $10^3/mm^3$							
Deficient	11.5	10.4	16.8	18.0	19.5	21.0	40.0	17.6
Low	10.8	5.8	13.5	12.0	9.0	8.7	13.8	11.5
High	8.1	5.0	5.8	8.8	7.3	6.7	15.2	7.5

Serum Vitamin A

Retinyl palmitate

The mean serum retinyl palmitate (rp) concentrations are presented in Figure 7. The mean rp in serum peaked in the deficient and high but not the low vitamin A supplemented groups 24 hours postcolostral intake. The mean rp for the deficient and low supplemented calves remained similar and fairly stable for the remainder of the experiment while the mean rp for the high vitamin A supplemented group was consistantly higher during the experiment than the other two groups.

Retinol

Serum retinol concentrations are presented in Figure 8. A serum retinol response was observed only in the high vitamin A supplemented group. The mean serum retinol values were significantly values higher ($P < .01$) in this group than in the other two groups over the course of the experiment. There was a slight tendency for a gradual increase in the mean serum retinol concentrations over the course of the experiment in the deficient and low supplemented vitamin A group.

Total Vitamin A

Total serum vitamin A concentrations (Figure 9) were comparable to the serum retinol concentrations illustrated in Figure 8 i.e. significantly higher ($P < .01$)

Figure 7. Comparison of serum retinyl palmitate concentrations of calves fed different levels of vitamin A as retinyl palmitate from birth to 35 days of age. (Experiment II)

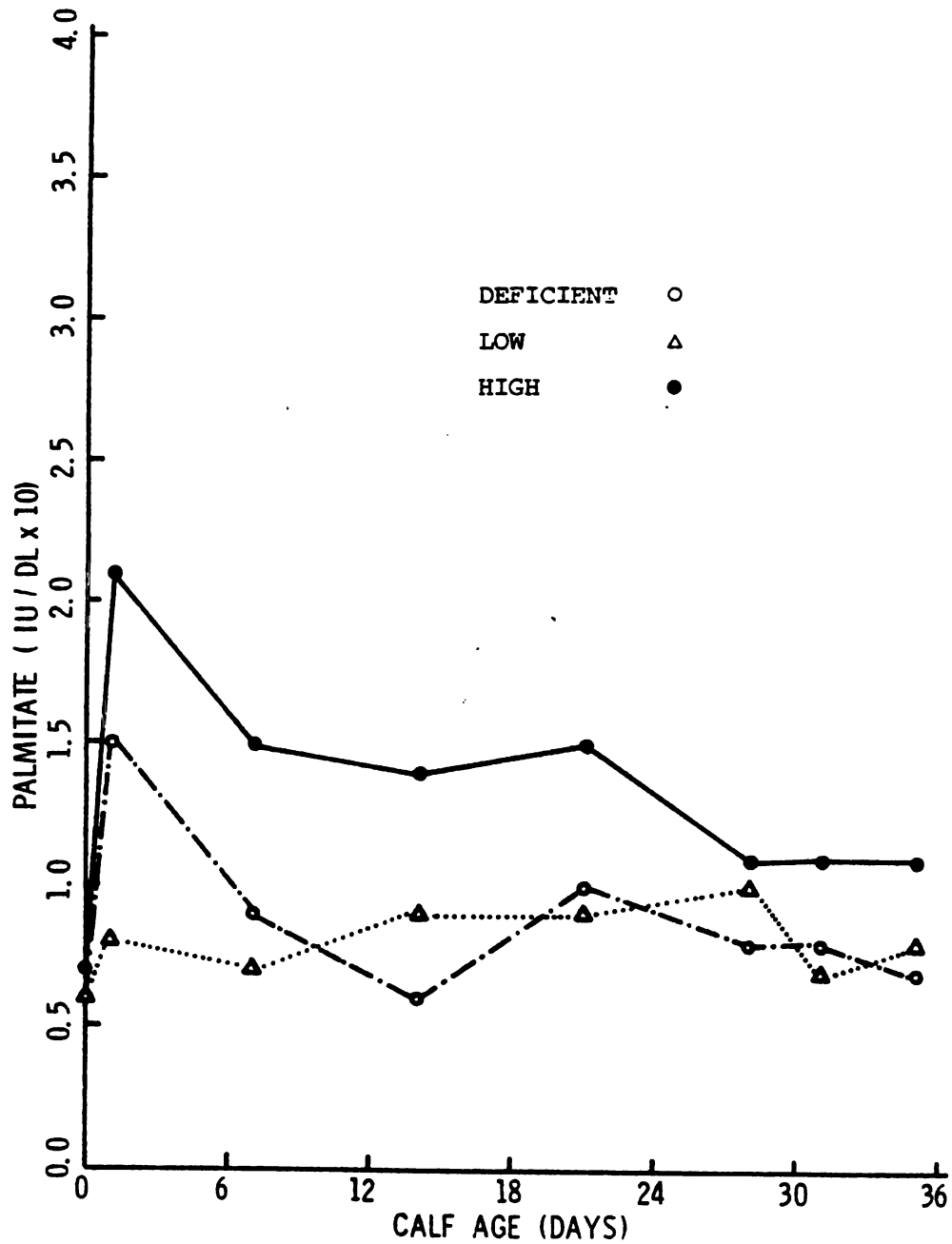

DEFICIENT
LOW
HIGH
PALMITATE (IU / DL x 10)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
6
12
18
24
30
36
CALF AGE (DAYS)

Figure 7

Figure 8. Comparison of serum retinol concentrations of calves fed different levels of vitamin A as retinyl palmitate from birth to 35 days of age. (Experiment II)

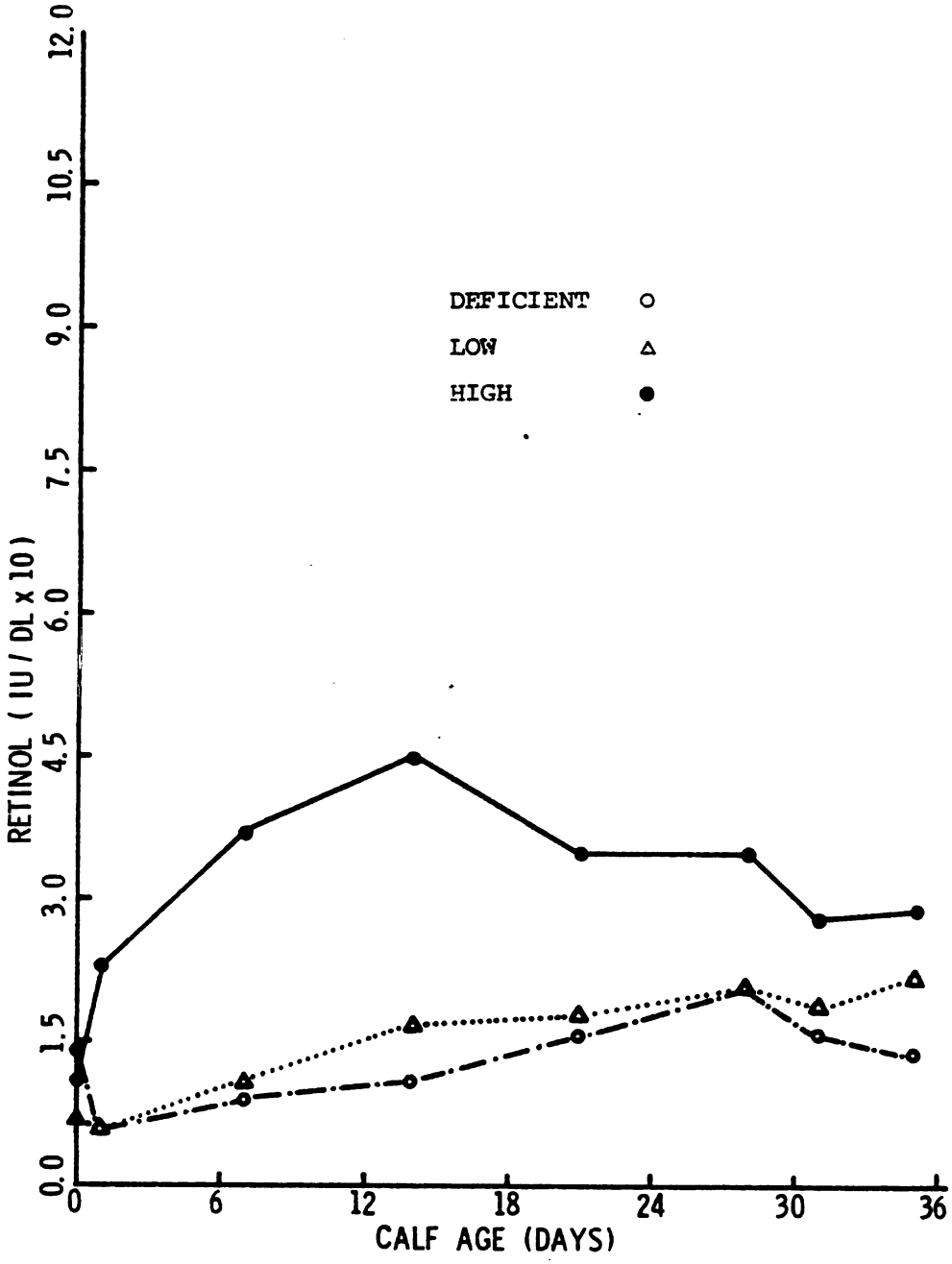

RETINOL (IU / DL x 10)
0.0
1.5
3.0
4.5
6.0
7.5
9.0
10.5
12.0
0
6
12
18
24
30
36
CALF AGE (DAYS)
DEFICIENT
LOW
HIGH

Figure 8

Figure 9. Comparison of total vitamin A concentrations of calves fed different levels of vitamin A as retinyl palmitate from birth to 35 days of age. (Experiment II)

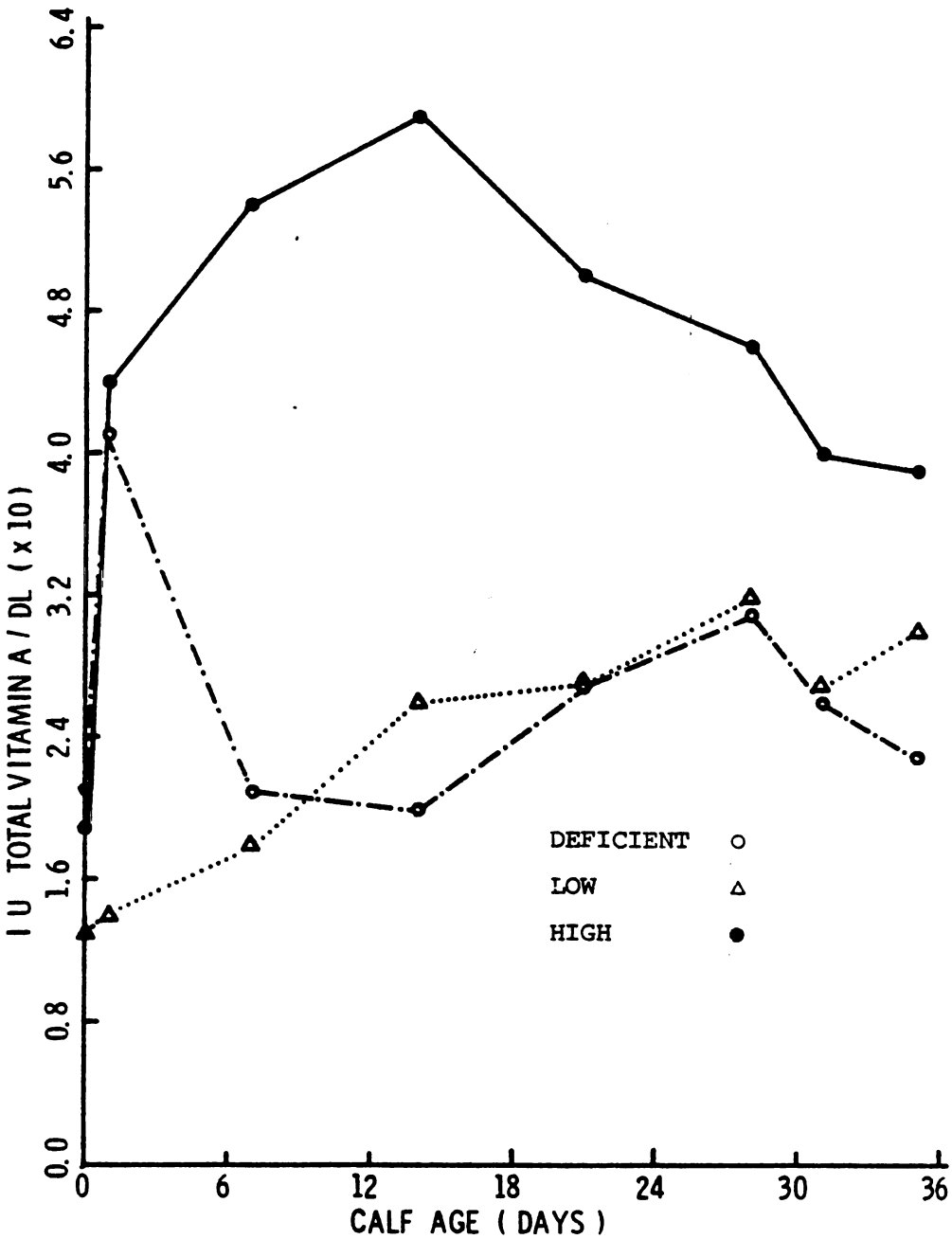

I U TOTAL VITAMIN A / DL (x 10)
0.0
0.8
1.6
2.4
3.2
4.0
4.8
5.6
6.4
0
6
12
18
24
30
36
CALF AGE (DAYS)
DEFICIENT
LOW
HIGH

Figure 9

values in the high vitamin A group in comparison to the other two groups and a gradual increase in total serum vitamin A in the deficient and low groups over the course of the experiment.

Cell-Mediated Immunity

The blastogenic responses of lymphocytes from calves in the different groups are illustrated in Table 7 and Figures 10 and 11. The control values, representing H^3-thymidine uptake by the lymphocytes in absence of mitogen, ranged between 259-4037 CPM over the course of the experiment and were not significantly ($P < .05$) different between groups. The H^3-thymidine uptake of mitogen-stimulated (0.25ug Con A/well) calf lymphocytes, expressed as CPM, Δ CPM and SI increased significantly ($P < .01$) between 0 and 1 day of age in groups 1 and 3 and between age 1 and 7 days of age in group 2. There was a significant ($P < .05$) positive correlation between degree of blastogenesis and calf age for all dietary groups. The deficient calves appeared to increase in lymphocyte transformation at a slower rate than either of the other two groups. There was a general decrease in blastogenic activity of calf lymphocytes following Brucella vaccination in all groups. There was no consistent effect of in vivo vitamin A supplementation on blastogenic response. The SI data (Figure 11) were much more variable than the Δ CPM and again showed no consistent

Table 7. Comparison of in vitro blastogenic responses of lymphocytes of calves fed different concentrations of retinyl palmitate from birth to 35 days of age (Experiment II).

Dietary vitamin A	Days of age							
	0	1	7	14	21	28	31	35
	Counts per minute[a] in absence of mitogen x 10^3							
Deficient	1.1	1.5	1.9	1.8	1.6	2.5	1.6	1.4
Low	0.6	0.7	1.0	3.1	1.1	1.5	3.3	4.0
High	0.8	1.4	0.3	1.1	1.4	2.5	3.1	1.8
	Counts per minute[a] in presence of 0.25 ug Con A x 10^3							
Deficient	36.6	81.7	89.8	111.0	128.0[b]	133.3	109.2	101.9
Low	34.8	39.6	107.8	173.2	161.6	172.1	100.4	142.2
High	20.9	85.7	43.1	92.0	138.2	163.2	106.7	103.1

[a]geometric mean representing H^3-thymidine uptake
[b]vaccinated with brucella

Figure 10. Representation of the effects of different rates of retinyl palmitate supplementation in calves on the vitro blastogenic activity of their lymphocytes. Activity is expressed as the difference between the rates of tritiated thymidine (H^3) uptake of cells in the presence and absence of 0.25 ug Con A per culture well. (Experiment II)

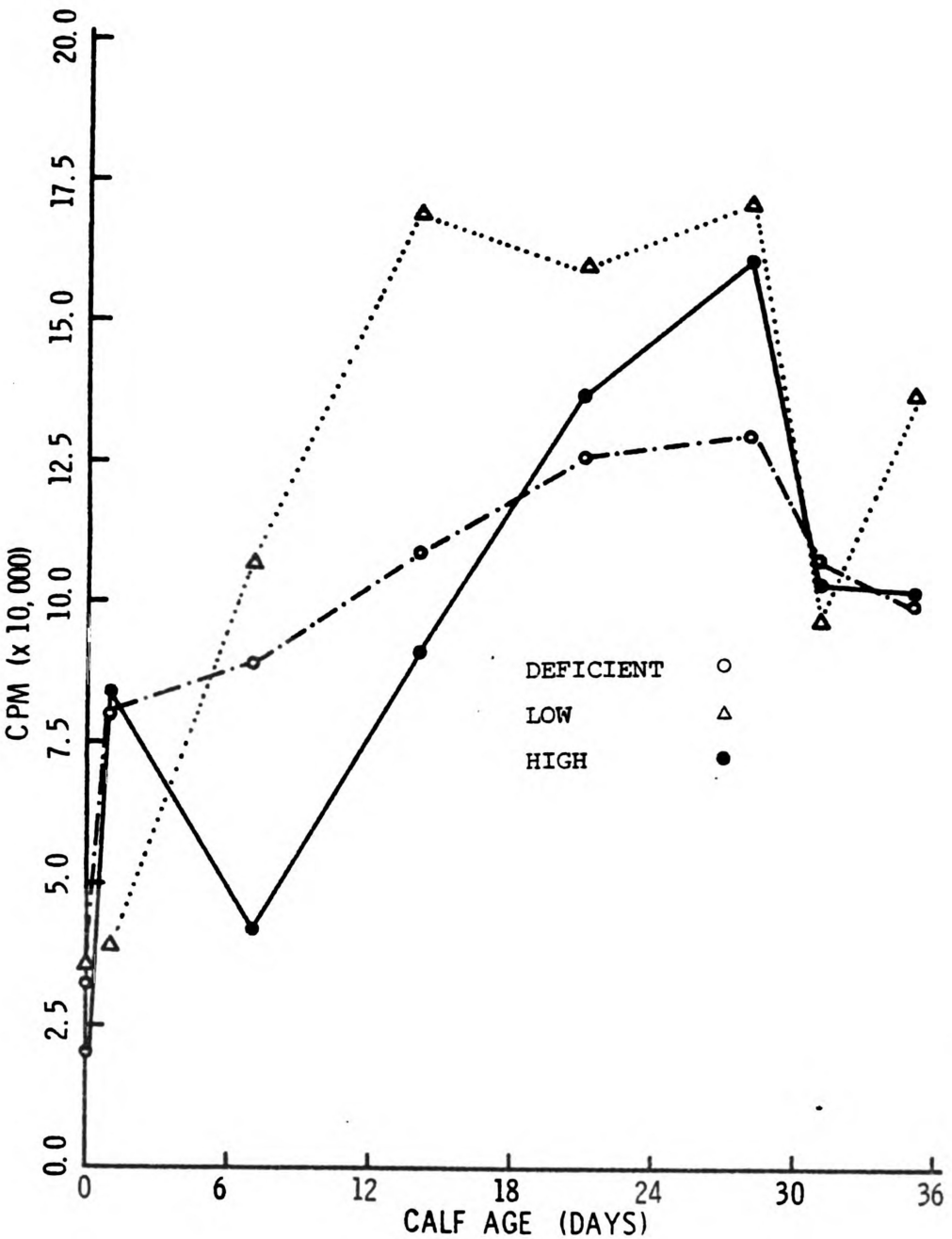

CPM (x 10,000)
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
0
6
12
18
24
30
36
CALF AGE (DAYS)
DEFICIENT
LOW
HIGH

Figure 10

Figure 11. Representation of the effects of different rates of retinyl palmitate supplementation in calves on the in vitro blastogenic activity of their lymphocytes. Activity is expressed as stimulation index (SI) which is the ratio of the rate of tritiated thymidine (H^3) uptake of cells in the presence of 0.25 ug Con A per culture well to the rate of H^3 uptake of cells without mitogen.(Experiment II)

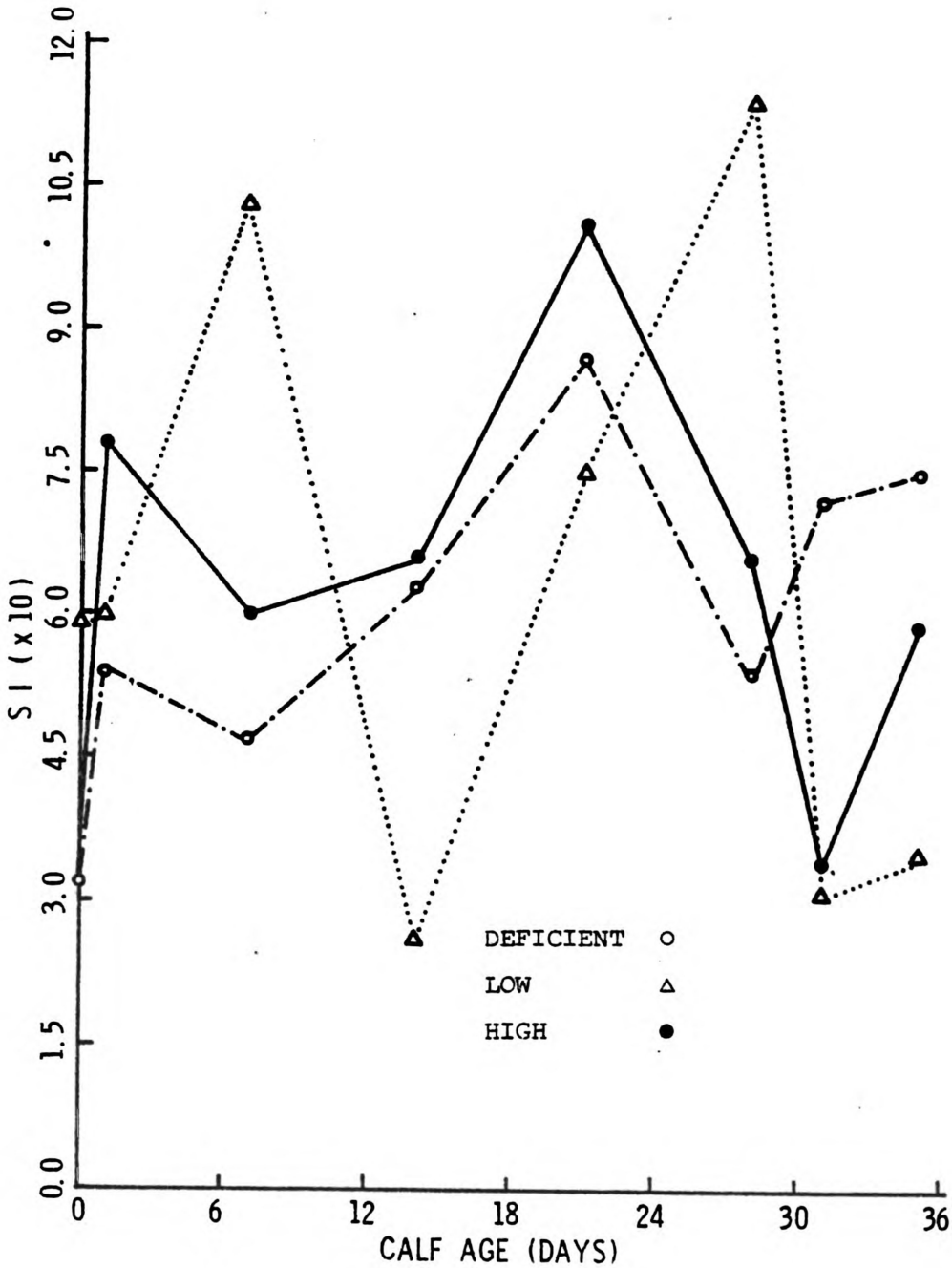

SI (x10)
0.0
1.5
3.0
4.5
6.0
7.5
9.0
10.5
12.0
0
6
12
18
24
30
36
CALF AGE (DAYS)
DEFICIENT
LOW
HIGH

Figure 11

consistent relationship between blastogenic activity and dietary vitamin A.

Cortisol

The mean serum cortisol concentrations are illustrated in Figure 12. These varied between 32.5 and 69.5 ng/ml at birth and declined significantly ($P < .01$) between 1 and 14 days of age for all groups. Thereafter, a gradual decline occurred in all groups to approximately 4-6 ng/ml at 35 days of age. There was no influence of dietary vitamin A on serum cortisol.

C. Experiment III

The lymphocyte transformation data are presented in Table 8 and represent H^3-thymidine uptake by lymphocytes in the presence and absence of .10 ug Con A/culture well. There was significant ($P < .01$) relationship between rate of spontaneous blastogenesis (Figure 13) and concentration of retinyl palmitate added to the culture wells. The H^3-thymidine uptake, expressed as CPM and Δ CPM (Table 8 Figure 14) tended to be increased by the addition of either 10 or 100 ng of retinyl palmitate per culture well and was significantly ($P < .01$) depressed in the presence of 1000 ng of retinyl palmitate per culture well. In the case of SI there was a significant ($P < .01$) relationship

Figure 12. Comparison of serum cortisol concentrations of calves fed different levels of vitamin A as retinyl palmitate from birth to 35 days of age. (Experiment II)

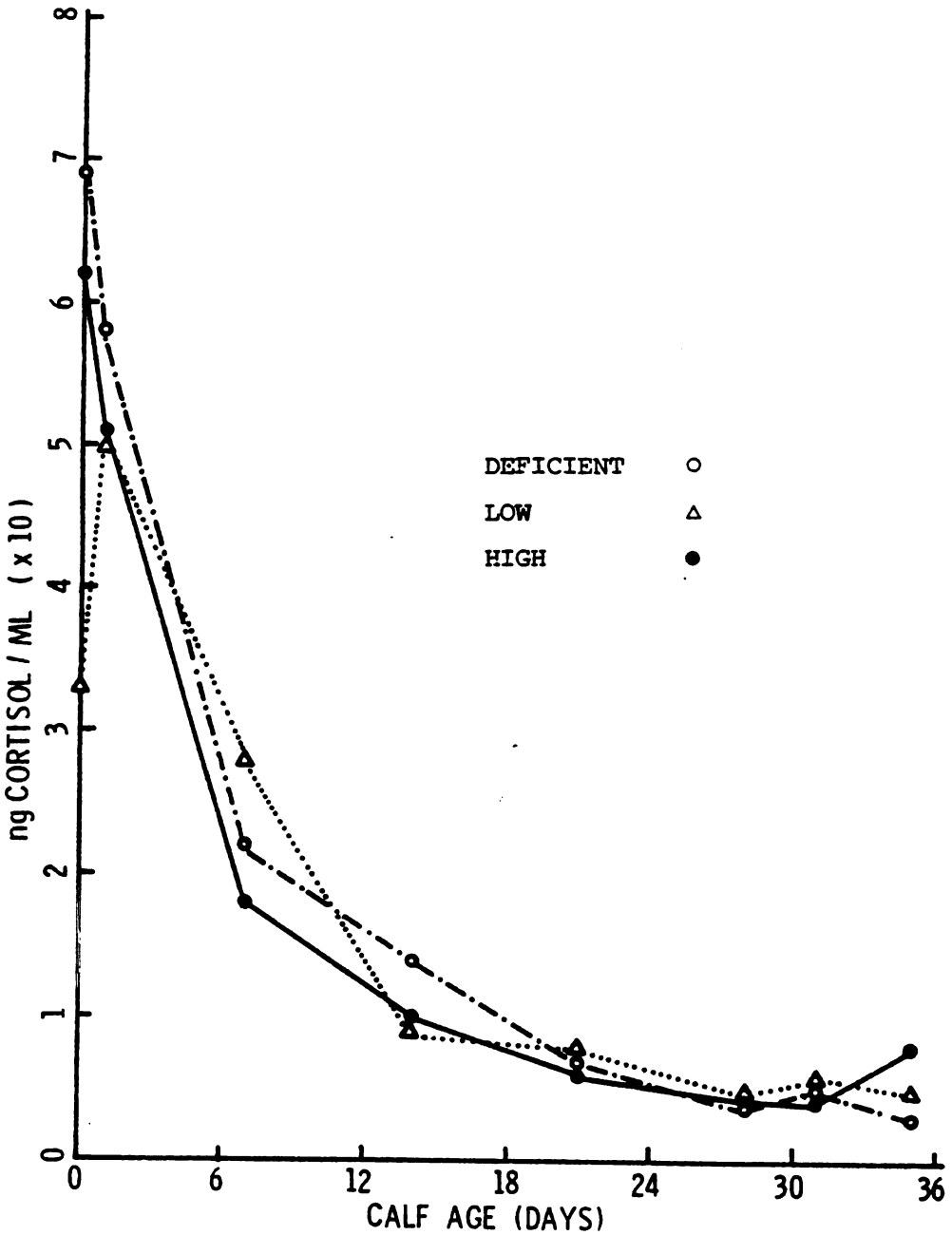

ng CORTISOL / ML (x 10)
0
1
2
3
4
5
6
7
8
0
6
12
18
24
30
36
CALF AGE (DAYS)
DEFICIENT
LOW
HIGH

Figure 12

Table 8. Representation of the effects of in vivo retinyl palmitate supplementation on lymphocyte blastogenesis in the presence and absence of Con A mitogen. (Experiment III)

Retinyl palmitate/ culture well (ng)	Method of measurement: Without mitogen (A)	With 0.1 ug Con A/well (B)	B-A	B/A
	counts/minute[a]			
0	1.1	111.2	109.3	102.0
10	.9	122.2	121.3	144.0
100	.6	120.9	120.3	211.0
1000	.3	71.3	71.0	193.0

[a] counts per minute x 10^3

Figure 13. In vitro retinyl palmitate influence on bovine lymphocyte blastogenesis in the absence of Con A. (Experiment III)

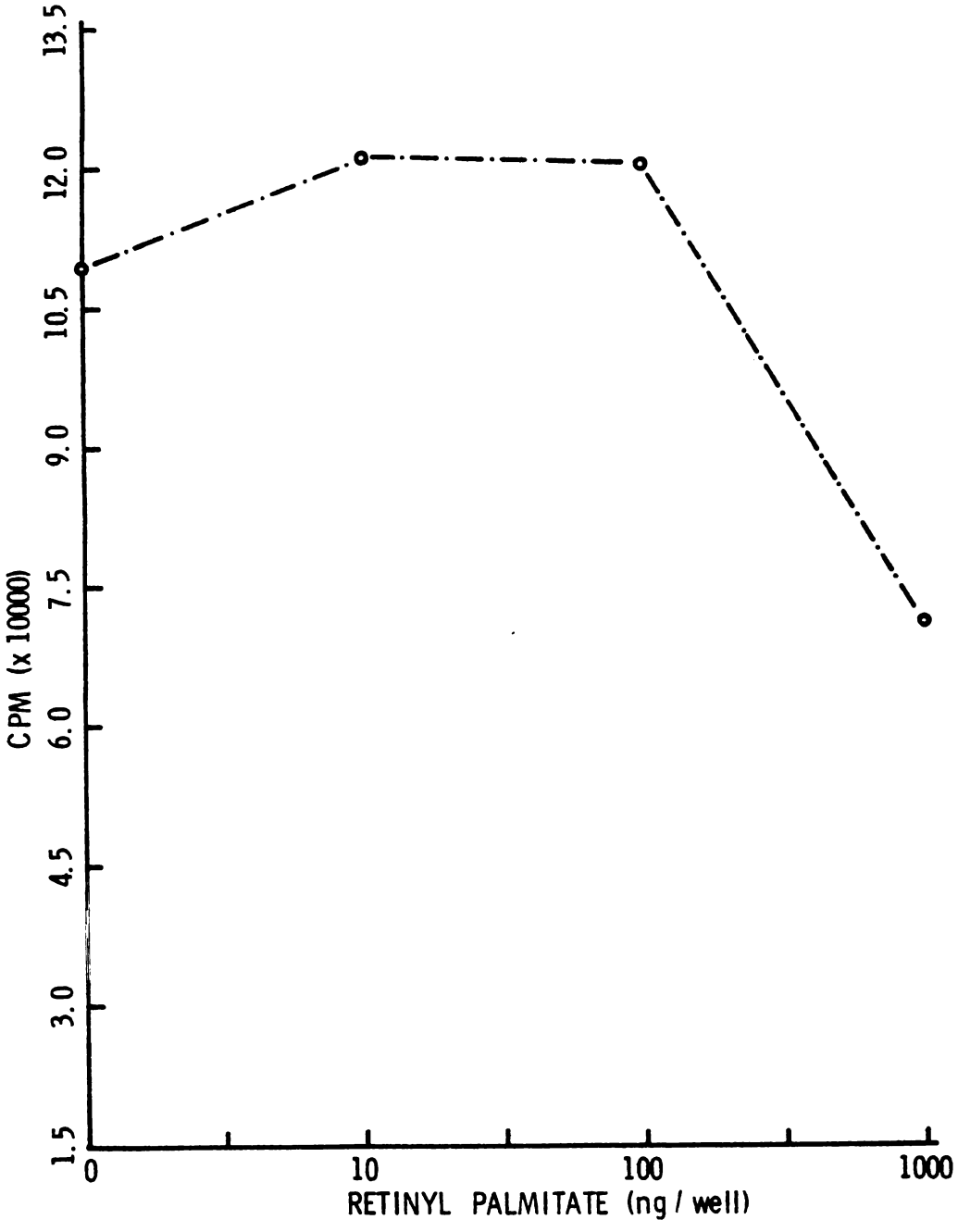

CPM (x 10000)
13.5
12.0
10.5
9.0
7.5
6.0
4.5
3.0
1.5
0
10
100
1000
RETINYL PALMITATE (ng / well)

Figure 13

Figure 14. In vitro retinyl palmitate influence on bovine lymphocyte blastogenesis (changed counts per minute) using 0.10ug Con. A. (Experiment III)

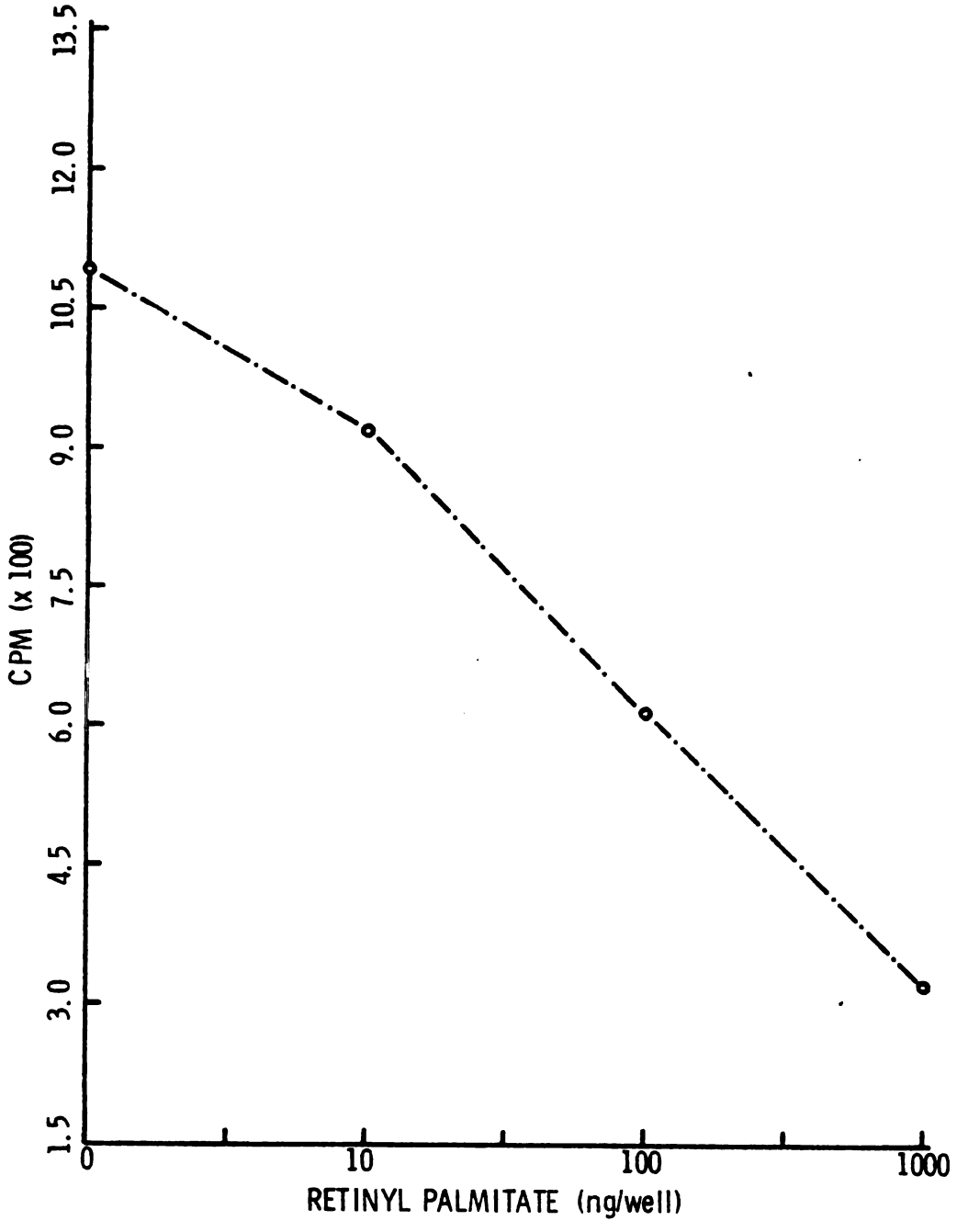

CPM (x 100)
13.5
12.0
10.5
9.0
7.5
6.0
4.5
3.0
1.5
0
10
100
1000
RETINYL PALMITATE (ng/well)

Figure 14

between levels of retinyl palmitate per culture well (0 and 100 ng) and stimulation indices (Figure 15). Beyond 100 ng retinyl palmitate/well the stimulation index tended to be depressed.

Figure 15. In vitro retinyl palmitate influence on bovine lymphocyte blastogensis (stimulation index) using 0.10 ug Con. A. (Experiment III)

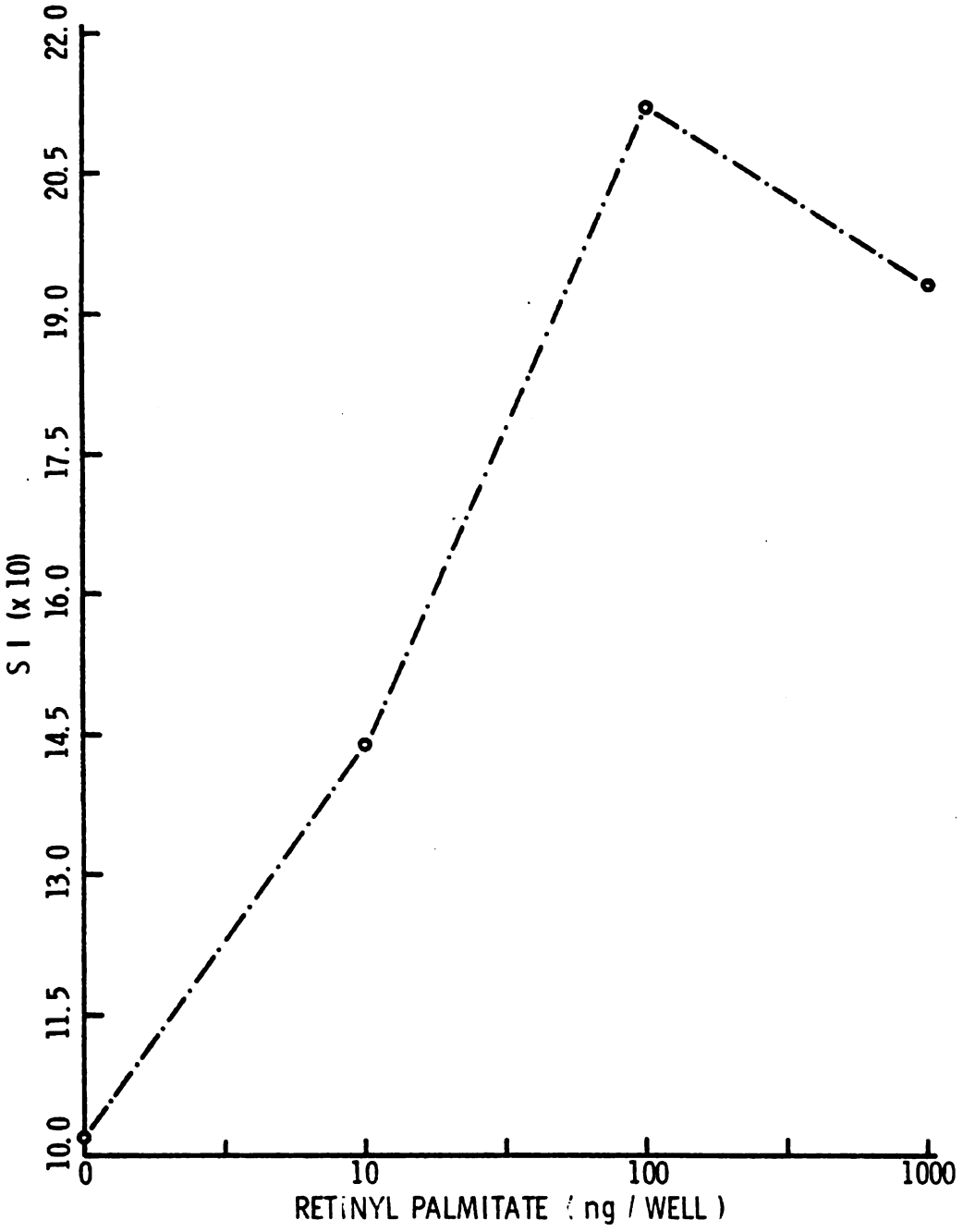

S I (x10)
10.0
11.5
13.0
14.5
16.0
17.5
19.0
20.5
22.0
0
10
100
1000
RETINYL PALMITATE (ng / WELL)

Figure 15

DISCUSSION

The experiments described were intended to characterize the importance of vitamin A in the immune response of calves (Scrimshaw et al. 1968) in a continuing effort to find ways to reduce the persistant, relatively high rate of deaths among young calves (Amstuz et al. 1965). The two experiments, I in which the calves were housed in calf hutches and exposed to inclement, late fall, Michigan weather and II, in which the calves were housed in a controlled environment provided an opportunity to observe the effect of temperature stress on calves with restricted vitamin A consumption.

The degree of vitamin A restriction, accomplished by replacing the colostrum milk fat and the milk replacer milk fat with a vitamin A-low, commercial fat source, was not as severe as intended. In fact, it is estimated that the natural vitamin A and provitamin A forms remaining in the basal calf diets represented approximately 50% of the National Research Council's estimates of the vitamin A requirement of the calves. However, all the weather-stressed calves on the most vitamin-A-restricted diets died within 7 days of age while their vitamin-A-restricted counterparts, housed in a controlled environment, survived in spite of a greater incidence of infectious disease problems than either group fed the medium or high levels of vitamin A. These observations

paralleled those of Keener et al. 1942 who reported on the increased vitamin A requirements of calves exposed to cold weather.

The packed cell volumes, used to monitor hydration status, as well as the red blood cell concentration of the calves' blood, were generally within the normal range (Schalm 1975). The final means for the PCV of the vitamin A restricted and high vitamin A-supplemented calves were somewhat lower than normal which could indicate that an optimal dietary vitamin A intake for hematopoiesis is between these two extremes.

The mean WBC values from each of the experiments were in general, within normal limits according to Schalm (1975), except for calves in experiment I on the most vitamin A-restricted diet. The WBC values for those calves were slightly higher than normal. Since white blood cells are involved in the first line of defense in an immune response, it is possible that these calves were responding to infection. Unfortunately, white blood cell differential counts, an important means of evaluating the immune defense system by estimating the proportions of cells essential for immunological activity or inhibition (Trentin 1971), were, not made.

The serum vitamin A concentrations, as measured by high performance liquid chromatography, did not reflect dietary retinyl palmitate supplementation of the respective groups. In both experiments I and II, the

serum retinyl palmitate concentrations remained relatively constant among all the calves; however, the serum retinol concentrations in both experiments did correspond to the relative level of vitamin A supplementation in the intermediate and high vitamin A supplemented groups. These results are partly because ingested retinyl palmitate is de-esterified in the Gl tract, converted to retinol and ultimately re-esterified in the liver for storage there. As required, this ester form, primarily, is de-esterified within the liver to become retinol, the most biologically active form of vitamin A (Olson 1969). Had liver biopsies from these calves been obtained, perhaps vitamin A assays on these samples would have corresponded better to the levels of vitamin A supplementation .

Nonspecific resistance to disease increases with vitamin A supplementation. This has been observed with mice (Cohen et al. 1973 and 1979), rats (Cohen et al. 1979 and Sirisinha et al. 1980) and chickens (Leutskay and Fair 1977). This resistance could be associated with indirect enhancement of antibody production and cellular immunity by vitamin A (Cohen and Cohen 1973, Jurin and Tannock 1972, Mickshe et al. 1977) through its action on lysosomes which affect cellular proliferation (Dingle and Lucy 1965). Thus, it was expected that lymphocyte transformation would be progressively enhanced as the degree of dietary vitamin A supplementation of the calves

increased. That effect was not observed in either Experiments I or II. There was, however, a tendency for calves fed the lowest and the highest levels of vitamin A to have lower lymphocyte blastogenic activity than the control calves. This suggests that the vitamin A supplementation often found in commercial calf milk replacers (i.e. 5-10 times the requirement levels as indicated by NRC), might impair the immune response of calves and be more detrimental to calf health than if only normal vitamin A concentrations were provided in the diets.

The Brucella vaccinations were used to test the immune responsiveness of the calves because an increase in blastogenic activity usually occurs following vaccination. In these experiments, however, there was an immediate depression of lymphocyte transformation observed after the Brucella vaccinations for all calves in both experiments. By 7 days postvaccination, the expected stimulated blastogenic response seemed to be occurring. This biphasic immune response to vaccination may represent an initial vaccination stress increasing serum cortisol concentrations and depressing the immune response followed by the normal cell-mediated response by 7 days postvaccination. This response may have been more pronounced had a longer postvaccination observation period been provided.

To understand stress effects, serum cortisol concentrations were measured in all the serum samples

collected from each calf. The cortisol values were than correlated positively with serum and dietary vitamin A concentrations, and with blastogenic activity. Calves under the constant weather-stress maintained higher serum cortisol concentrations than the nonweather-stressed calves and the elevated cortisol levels correlated positively with lymphocyte blastogenic activity. This observation, in conjunction with the postvaccination cortisol levels, suggests that the effect of cortisol on immunity may be bidirectional and not simply depress immunocyte activity, as demonstrated by Werthemener et al. 1969.

The in vitro aspect of this study (Expt. III) was an effort to pursue the observatons of Dingle and Lucy 1965, regarding the vitamin A requirements for blastogenesis and involved the direct addition of graded quantities of retinyl palmitate to the culture wells for the lymphocyte proliferation. The results seemed to depend on the presence or absence of mitogen in the cuture wells. For example, in the absence of mitogen, spontaneous blastogenesis was significantly depressed by all levels, (10, 100, or 1000 ng), of retinyl palmitate added to the wells. In the presence of mitogen, however, the two lower levels of retinyl palmitate were associated with a slight increase in blastogenesis while the highest level (1000ng) was definitely toxic to the culture. This suggests that mitogen affects the toxicity of vitamin A on lymphocyte cultures. Since retinol is the biologically active form,

and retinyl palitate is primarily the storage form, in retrospect, it would have been preferable to have tested the direct addition of retinol, instead of retinyl palmitate, to the cell cultures. Technical difficulties associated with sterilizing the retinol while maintaining its activity precluded its use in the cultures.

The results of the in vivo and in vitro vitamin A blastogenesis studies have some similarity, in that the calves fed the relatively high vitamin A concentrations and the bovine lymphocyte cultures with supplemental vitamin A each had depressed lymphocyte blastogenesis. The mechanism for this and or its significance remain to be determined.

These studies have revealed the difficulties implicit in working with lymphocyte cultures and measuring blastogenesis as Muscoplat et al. 1974 advised. In addition, the experiments emphasized the difficulties in creating immunogenically-comparable calf diets while varying the vitamin A concentrations. Obviously, removal of the milk fat from the colostrum by centrifugation and replacment of this fat with a commercial milk fat source intended to be void in vitamin A did not create milk as low in vitamin A as expected. The large standard errors in both the lymphocyte blastogenesis and vitamin A data, which could have been somewhat offset by larger numbers of calves used in the experiments, also diminished the significance of the trends observed in these experiments.

SUMMARY AND CONCLUSION

The continuing, nationwide, high, calf mortality rate of 8-23% necessitates research efforts to optimize the immune response of calves, and in particular, improve our understanding of the role of vitamin A in cell-mediated immunity. Two trials, using a total of 25 newborn calves, were conducted to determine the in vivo effect of vitamin A on blastogenesis of neonatal calf lymphocytes. Calves were housed in outside calf hutches in cold inclement weather in trial 1 and in temperature-controlled (20-25^{o}C) box stalls in trial 2. Heparinized blood samples were collected at birth, at one day and weekly for up to 7 weeks from calves fed colostrum and milk replacer containing deficient, normal and high levels of vitamin A, as retinyl palmitate. Lymphocytes were isolated by the Ficoll-Hypaque procedure and incubated for 48-72 hours in microtiter plates with an estimated 1 x 10^{5} lymphocytes and 0, 0.1, 0.25, 0.5 or 1.0 ug mitogen (Con A) per culture well.

In each trial the mortality rate and/or incidence of diarrhea was greatest among the vitamin A-deficient calves. In trial two, there was a tendency for the degree of lymphocyte blastogenesis to correlate positively with serum vitamin A levels of calves. In each trial the lymphocyte transformation of these calves significantly increased ($P< 0.0041$) between birth and 7 days of age.

Over the same time period there was a significant decrease ($P < .0001$) in serum cortisol concentrations. Due to large individual calf variance in lymphocyte blastogenesis, no significent treatment effects on this parameter were observed between groups.

The in vitro effect of retinyl palmitate on lymphocyte blastogenesis was investigated by the addition of 0, 10, 100 and 1000 ng retinyl palmitate per culture well prepared as above except using peripheral lymphocytes from 16 lactating Holsteins. In this study there was an inverse relationship between the degree of blastogenesis and level of retinyl palmitate in the culture wells in the absence of mitogen ($P < 0.0001$). In the presence of mitogen, however, 10 and 100 ng retinyl palmitate per culture well tended to enhance the blastogenic response while 1000 ng per culture well depressed lymphocyte blastogenesis ($P < 0.0001$).

These experiments were not as effective as hoped in demonstrating the role of vitamin A in the immune response of calves for several reasons. One of these was the fact that the removal of the milk fat, with its vitamin A activity, from the colostrum by centrifugation, (passing the colostrum through a cream separator) was incomplete and considerable vitamin A activity, estimated at 50% of the requirement level for calves, remained in the colostrum. Furthermore, the fat replacement for the colostrum and for the specially ordered, non-vitamin A-supplemented, commercial milk replacer contained

significant vitamin A activity also. Thus it was not possible to demonstrate very severe vitamin A deficiency as measured by serum vitamin A assays in the intended vitamin A-deficient calves.

In addition, under the best of circumstances, there is such wide variation in the estimates of cellular immune responses, as in the lymphocyte blastogenesis studies, that many more calves per group on experiment would be required to demonstrate significant differences between treatments. Lastly, the effort to study a direct effect of vitamin A on lymphocyte cultures by addition of retinyl palmitate to the culture wells was probably limited by the fat-soluble nature of the additive in an otherwise aqueous medium. Further efforts to perfect these techniques await resources and interest.

APPENDIX

Appendix 1. Daily Weather Conditions, Experiment I

Dairy Barn Calves (housed in hutches).

Date	Temp °F ($\overline{X}$)	Wind	Comments
11/26/79	48	38 SW	Fog . Rain
11/27/79	37	30 SW	Fog . Rain . Haze
11/28/79	28	16 W	Fog
11/29/79	26	21 W	Fog . . Haze . Snow
11/30/79	25	21 W	
12/1/79	23	18 N	Fog . Blowing Snow . Haze
12/2/79	14	15 NW	
12/3/79	23	27 SW	
12/4/79	28	19 S	Fog
12/5/79	42	32 SW	
12/6/79	35	14 W	
12/7/79	37	30 SW	Fog . Rain
12/8/79	25	23 NW	
12/9/79	32	32 SW	Snow . Showers
12/10/79	38	24 SW	Fog
12/11/79	52	24 SW	Fog . Drizzle
12/12/79	34	15 NW	Fog . Glaze . Rain
12/13/79	25	20 NW	Fog . Snow
12/14/79	25	17 NW	
12/15/79	32	26 SW	
12/16/79	24	28 N	Fog . Freezing Rain. Blowing Snow. Ice
12/17/79	10	25 W	Haze

Date	Temp °F $(\overline{X})$	Wind	Comments
12/18/79	19	13 S	
12/19/79	30	12 S	
12/20/79	25	17 SE	Fog . Haze . Drizzle
12/21/79	30	18 S	Fog . Haze . Drizzle
12/22/79	41	17 S	Fog . Rain
12/23/79	47	15 S	Fog . Drizzle
12/24/79	44	27 NE	Heavy Rain . Fog
12/25/79	34	30 NE	Heavy Fog . Rain . Pellets
12/26/79	30	14 W	Fog . Drizzle
12/27/79	30	17 W	Fog
12/28/79	36	17 W	Fog
12/29/79	36	15 W	Heavy Fog
12/30/79	29	8 W	Heavy Fog
12/31/79	27	5 W	Heavy Fog
1/1/80	27	10 W 24	Fog
1/2/80	28	13 W 05	Freezing Fog . Drizzle . Snow
1/3/80	23	12 W 04	
1/4/80	21	14 W 06	
1/5/80	22	15 W 04	
1/6/80	19	30 W 15	Blowing Snow
1/7/80	25	38 W 25	Blowing Snow
1/8/80	15	23 W 28	Snow Blowing Snow & Showers
1/9/80	8	15 W 27	Snow
1/10/80	24	25 W 16	Fog . Haze
1/11/80	34	38 W 28	Fog . Rain . Snow
1/12/80	14	29 W 26	Blowing Snow

Date	Temp °F $(\overline{X})$	Wind	Comments
1/13/80	31	24 W 18	Ice Pellets
1/14/80	31	17 W 23	Fog . Haze
1/15/80	32	17 W 14	Fog
1/16/80	39	23 W 15	Fog . Rain . Haze
1/17/80	42	18 W 23	Fog . Rain . Drizzle
1/18/80	33	17 W 28	
1/19/80	30	17 W 28	
1/20/80	26	20 W 26	
1/21/80	29	14 W 27	Snow
1/22/80	27	17 W 23	Fog . Snow
1/23/80	12	22 W 26	Snow Showers
1/24/80	12	13 W 14	Snow
1/25/80	14	13 W 34	
1/26/80	12	12 W 25	Fog . Snow
1/27/80	17	15 W 23	Snow Showers
1/28/80	14	14 W 28	Snow
1/29/80	14	20 W 28	Snow Showers
1/30/80	9	12 W 29	
1/31/80	8	10 W 01	
2/1/80	8	12 W 01	
2/2/80	13	9 W 02	
2/3/80	8	18 W 35	
2/4/80	13	12 W 31	
2/5/80	13	12 W 09	Snow
2/6/80	21	15 W 05	Fog . Snow
2/7/80	19	10 W 01	Snow

Date	Temp °F $(\overline{X})$	Wind	Comments
2/8/80	16	7 W 36	
2/9/80	24	8 W 34	Fog . Snow
2/10/80	17	20 W 21	Fog . Snow

Brief summary compiled from Michigan Weather Bureau

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