

# Vitamin A Depletion and Repletion in Thoroughbred Horses

by

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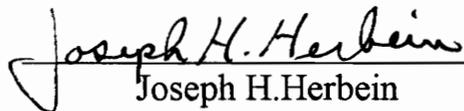
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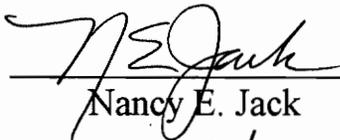
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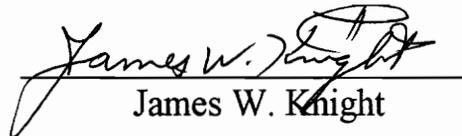
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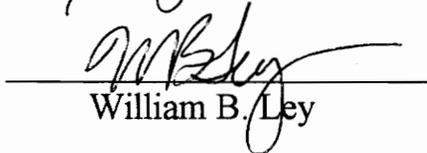
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# **VITAMIN A DEPLETION AND REPLETION IN THOROUGHBRED HORSES**

by

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## **(ABSTRACT)**

The purpose of this research was to study vitamin A status in grazing horses throughout the year and to evaluate the effectiveness of vitamin A and  $\beta$ -carotene as supplements. Vitamin A status was assessed by serum retinol concentrations (SR) and the relative dose response (RDR) which was adapted for use in the horse. The horses (45 Thoroughbred mares) were divided into three diet groups: pasture and hay only (PH); pasture, hay and vitamin A-free concentrate (PHC); and hay and vitamin A-free concentrate (HC). The mares, as well as their foals, were assessed for vitamin A status during the summer, fall and winter. After eight months, each diet group was subdivided and supplemented with either: retinyl palmitate at two times the recommended level (A), the equivalent in water dispersible  $\beta$ -carotene (B), or a placebo (C). Supplementation continued for 20 months during which the vitamin A status was assessed every 60 days in the mares, and at birth in the neonates. During both the depletion and the repletion phase the mares were kept on a regular breeding schedule and the reproductive rates were determined as well as the general health of the mares and their offspring.

The RDR proved more sensitive at detecting changes in vitamin A status than SR, and a combination of both was used. A measurable decline in vitamin A stores was seen in the HC group within 2 months, and in PH and PHC groups during the winter. The HC

group remained lower in vitamin A status throughout the study. A seasonal fluctuation of vitamin A status was observed regardless of supplementation. Supplementation with retinyl palmitate improved vitamin A status in all three diet groups, however, supplementation with  $\beta$ -carotene did not.

Both neonates and young growing horses were lower in vitamin A status than the adult. A respiratory infection observed in the weanlings affected vitamin A status as well. Supplementation of the dam had no effect on neonatal vitamin A status. Deleterious effects on reproductive rates and health were also observed with vitamin A depletion. Supplementation of  $\beta$ -carotene had a negative effect on reproductive rates in this study.

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## INTRODUCTION

Vitamin A is responsible for many critical functions in the body and may be the most important nutrient in public health worldwide (Underwood, 1990). For thousands of years humans and animals have suffered from blindness and infertility known to be alleviated by consumption of liver by carnivores or green grass by herbivores. For many years, attention was focused on vitamin A depletion and blindness in tens of thousands of children around the world. In the last 20 years, focus has shifted to subclinical vitamin A depletion and the deaths of tens of millions of children due to lowered resistance to infections such as measles (Sommer, 1989). Subclinical vitamin A deficiency has also been found to have an effect on growth rates in children (Muhilal et al., 1988; West et al., 1988). Deficiency in the grazing animal can be insidious as well, having an effect on growth and metabolic functions as well as immune function without showing the outward signs of severe deficiency (McDowell, 1989).

Vitamin A is a fat soluble vitamin that is light sensitive, so degradation is a problem in conserved forages. Loss of carotenes (provitamin A found in plants) during the drying process of hay making may be as much as 80% within the first 24 hours (Russell, 1929) and may continue at a rate of 7% per month while in storage (McDowell, 1989). Grass or mixed hays that were harvested late, or left too long in the field before baling or stored for more than 6 months have negligible amounts of carotene (Waite and Sastry, 1949). Excepting yellow corn, practically all grains used in concentrates are low in carotene (Howell et al., 1941).

As long as the horse is receiving adequate green forage such as pasture or good quality alfalfa hay, vitamin A depletion may not be a problem (Rudra, 1946). Many horses

have little opportunity to consume green grass, especially in winter or dry seasons, and hay quality can be very inconsistent. Bioavailability of carotenes to horses is low (Guilbert et al., 1939). Blood concentration of vitamin A and carotenes fluctuate with the availability of carotenes in the forage (Fonnesbeck and Symons, 1967), and subsequently with the season (Garton et al., 1964; Mäenpää et al, 1988a). The requirement of the horse is also affected by age, stage of production, and stress (McDowell, 1989).

Research is at the point of fine tuning nutrient requirements for the prevention of subclinical deficiencies. Newer techniques of nutritional assessment (i.e. function tests) are more sensitive to nutrient status than static tissue concentrations. Many of these techniques are being adapted to production animals. Defining what is *optimal nutrition* for our animals should be possible with use of these techniques (Gibson, 1990). Much is still not known about the *optimal* requirements of the horse under the many different growth stages, training programs and reproductive circumstances.

## REVIEW OF THE LITERATURE

### **Metabolism of Vitamin A**

A better understanding of how a nutrient functions in the body is essential for testing and developing requirements for optimal nutrition. Since the discovery of vitamin A 82 years ago by E. V. McCollum, much has been elucidated about the absorption and transport of vitamin A. Fervent research still continues into the exact nature of how all the metabolites of vitamin A (retinoids) function in the body.

### ***Intake and absorption***

Vitamin A is a nearly colorless, fat-soluble, long-chain, unsaturated alcohol with 5 double bonds (Karrer et al., 1931). Because of the double bonds, vitamin A can exist in different isomeric forms. These isomers and other metabolites of vitamin A are called retinoids. Most of the preformed vitamin A in the diet are retinyl esters (e.g. retinol and palmitate or acetate). These esters are hydrolyzed in the intestinal lumen by pancreatic hydrolases or at the brush border membrane then are taken up as retinol into the mucosal cell (Hollander and Muralidhara, 1977). Bile acids and adequate dietary fat digestion products in micellar form are essential for this process (Olson, 1961). In the mucosal cell, retinol is once again esterified (mainly with palmitate) and transported in chylomicrons via the lymphatic system to the liver (Ong, 1993).

Vitamin A is found in animal products such as fish oils, liver, eggs, cheese and milk (McDowell, 1989). Herbivores are not likely to ingest food with preformed vitamin A. Therefore, they must derive their vitamin A from carotenoids found in abundance in green forages. The most biologically active source of vitamin A is  $\beta$ -carotene (Bondi and Sklan, 1984).

There are approximately 600 known carotenoids. Originally they were thought of principally as vitamin A precursors, but only about 50 actually are provitamins in mammals (Bendich, 1988). Conversion of carotenes to retinol occurs in mucosal cells by the central cleavage enzyme,  $\beta$ -carotene 15,15' dioxygenase (Goodman et al., 1967). In theory,  $\beta$ -carotene is capable of splitting to form 2 retinol molecules. However, the biological efficiency of conversion is lower than 50% in all species, and a factor of one sixth is assumed in equine nutrition. Generally accepted conversion factors in the horse are 1 mg of  $\beta$ -carotene to 555 IU of vitamin A for growth and 333 IU for pregnancy (McDowell, 1989). Conversion is dependent upon an individual's vitamin A status. Carotene conversion to vitamin A will decrease with an increase in carotene intake as well as an increase in vitamin A intake (Brubacher and Weiser, 1985). Between 25-75% of carotenoids consumed in the diet are not absorbed (either as  $\beta$ -carotene or vitamin A) and are excreted (Bondi and Sklan, 1984). Absorption of carotenoids are enhanced by the presence of bile salts, lipids, proteins, antioxidants and zinc (Bendich, 1988). Beta-carotene conversion to retinol is not complete as evidenced by  $\beta$ -carotene found in plasma, tissues, organs and milk (Parker, 1989). Similarly to vitamin A,  $\beta$ -carotene absorbed intact is incorporated into the lipid core of the chylomicron and carried to the liver.

### ***Storage***

Upon arrival to the liver, the chylomicron remnants are taken up into the parenchymal cells by receptor-mediated endocytosis (Norum et al., 1983). Retinyl esters are transferred to stellate cells for storage (Blomhoff et al., 1985). About 90% of the vitamin A in the body is stored in the liver, the remainder in the kidneys, lungs, adrenals and blood. The adult horse generally has vitamin A stores of 50 - 600  $\mu\text{g/g}$  liver (Rudra,

1946). Horses have the ability to store enough vitamin A in the liver to meet their requirement for 4 to 6 months after they have had access to sufficient amounts in their diet for about a 4-6 week period (Guilbert et al., 1940). Hepatic vitamin A stores of newborn foals (pre-suckle) are much lower than in adults (Irwin et al., 1991), and foals rely on the vitamin A supplied in the milk of their dam postnatally to build up their stores (Stowe, 1982).

### ***Transport***

Retinol is secreted from the liver in association with plasma retinol-binding protein (RBP) and circulates to peripheral tissues as the retinol-RBP-transthyretin complex (Blaner, 1989). Complexation to RBP occurs before secretion from the liver, and to transthyretin in the circulation. Plasma retinol concentration is regulated in a narrow range, mainly by control of the rate of retinol-RBP release by the liver. In contrast, liver vitamin A reserves may vary over a wide range. Various RBPs facilitate the transport of retinoids either between organs and cells or within them. Postulated roles of RBP are to stabilize the retinol from chemical or enzymatic attack and to spare it from glomerular filtration (Sklan et al., 1982).

Upon arrival at target tissues, retinol dissociates from RBP and diffuses into the plasma membrane (Creek et al., 1993). Once through the membrane, conversion of retinol to retinyl ester or to retinoic acid in the target cell is directed through its association with specific cellular RBPs. Retinoic acid regulates the transcription of specific nuclear genes (De Luca, 1991).

## ***Excretion***

Vitamin A is excreted in several forms in both the urine and feces. In general, vitamin A metabolites with intact carbon chains are excreted in the feces, whereas, the chain-shortened metabolites are excreted in the urine. The relative amounts of vitamin A metabolites in the urine and feces vary with vitamin A intake and the hepatic vitamin A reserve (Sklan, 1983).

## **Functions**

Functions of vitamin A have been under intensive study in recent years which has lead to considerable information on its modes of action. Vitamin A is known to have functions in vision, differentiation of epithelial cells, immune function, gene expression, growth, embryogenesis, and reproduction.

### ***Vision***

One function of vitamin A reasonably well understood at the molecular level is that in visual pigments, the photoreceptor molecules of the retina. In the retina, the protein opsin combines with 11-*cis*-retinaldehyde to form rhodopsin (visual purple), the direct recipient of light energy during vision in reduced light (Wald, 1968). Many of the steps leading from "activation" of rhodopsin by light to the visual signal in the brain via the optic nerve have been elucidated (see Sporrán et al., 1994). Following the split of the all-*trans*-retinaldehyde, in a few seconds, there is a spontaneous recombination of opsin with 11-*cis*-retinaldehyde. While most of the vitamin is recycled, a small portion of it must be replaced daily, thus the requirement for a continual supply of vitamin A either from storage depot or from the diet.

Another aspect of importance of vitamin A in vision is health of the epithelium and the proper functioning of the cornea and the goblet cells in the conjunctiva.

### ***Epithelium***

Vitamin A is also important in maintaining the health of all of the epithelia in the body: skin, respiratory, gastrointestinal, and urogenital tracts. This action of vitamin A may be the result of involvement in three different cellular systems. Vitamin A is involved in the terminal differentiation of keratinocytes of epidermis. Without vitamin A, cells synthesize keratins of excessive size (Fuchs and Green, 1981). Another action of vitamin A may be its role in the formation of glycoproteins (De Luca and Wolf, 1972); which are important in proper functioning of mucous secreting cells. The third is the differentiation of tissues by mediation of changes in intercellular recognition, intercellular interactions, adhesion and aggregation processes (De Luca, 1977).

Breakdown and keratinization of the mucous membranes lower the resistance of animals to the entrance of infective microorganisms into the tissues (Bondi and Sklan, 1984).

### ***Immune function***

Vitamin A has been found to help the body to resist infections. It is thought, at present, that retinoids and carotenoids affect the immune system differently (Ross and Hämmerling, 1994). Retinoids act on the differentiation of immune cells, increasing mitogenesis of lymphocytes and phagocytosis of monocytes and macrophages. Carotenoids affect immunosurveillance of activated NK cells and T-helper cells by modifying the release of at least some cytokine-like products by activated lymphocytes and monocytes.

Retinoids are chemoprotective against tumorigenesis (Sporn et al., 1976). They have inhibitory effects on tumor development and the growth of neoplastically transformed cells. The chemoprotective nature of carotenoids will be discussed later.

### ***Growth***

Growth is retarded by vitamin A deficiency (Bieri et al., 1968). This retardation may reflect impaired cell proliferation and differentiation. Also, bone remodeling in the growing animal is modulated by vitamin A. Vitamin A is important in the proper functioning of osteoclasts, which are responsible for resorption of bone. Without vitamin A, excessive deposition of periosteal bone occurs apparently from the unchecked function of osteoblasts. In deficiency, bones become shorter and thickened (Fell and Mellanby, 1950). This effect is associated with a reduction in the degradation of glycosaminoglycans and the synthesis of proteoglycans (Dingle et al., 1972). In addition, effects of overall bone changes may result in mechanical pressure on certain nerves, such as the optic or auditory nerves, which can result in blindness and/or deafness.

Retinoic acid has been found to affect growth hormone regulation. Retinoic acid can synergize with either thyroid hormone or glucocorticoids to enhance the transcriptional activity of the growth hormone gene, and subsequently of growth hormone secretion from cells (Bedo et al., 1989). It is possible that some of the systemic effects of retinoids on growth, as well as the poor growth usually associated with vitamin A deficiency, are related to retinoid effects on growth hormone secretion.

### ***Embryogenesis***

Retinoic acid controls differentiation in embryonal carcinoma cells (De Luca, 1991). It is directly related to embryonal development by controlling spatial positioning of

various cell types to eventually define morphological and functional differences among different segments along the axis of the body. Retinoid deficiency blocks the development of the heart and vascular system and the embryo eventually disintegrates. Retinoic acid has been implicated in the morphogenesis of the vertebrate limb (Thaller and Eichle, 1987).

### ***Reproduction***

Vitamin A is necessary for the support of spermatogenesis, oogenesis, placental development, and embryonic and fetal growth (Zile and Cullum, 1983). A deficiency can alter the epithelial lining of the reproductive tract (keratinization) and may result in irregular estrus and delayed breeding (Chew, 1993). In males, vitamin A deficiency leads to degeneration of the germinal epithelium and cessation of spermatogenesis (Huang and Hembree, 1979). There is strong evidence for a relationship between vitamin A and the production of steroid hormones in males (Rich and de Kretser, 1977) and females (McDowell et al., 1995). Retinol has been shown to stimulate progesterone secretion by porcine luteal cells which would effect early embryonic survival (Talavera and Chew, 1988). Vitamin A might impair follicular development, since epithelial tissues are highly dependent on a sufficient supply of vitamin A for normal cellular differentiation. The concentration of vitamin A in bovine follicular fluid was found to be a function of follicular quality, with the highest concentrations occurring in the non-atretic follicles and the lowest in the greatly atretic ones (Schweigert and Zucker, 1988).

Vitamin A passes through to the fetus and the fetus returns appreciable vitamin A to the mother in sheep (Donoghue et al., 1982). Yet calves and foals are known to be born with very low liver reserves (Branstetter et al., 1973; Irwin et al., 1991). With a vitamin A deficiency, lower conception rates may result in sows; those that do conceive,

abortion or the birth of weak, malformed, blind, or dead young may follow (Chew, 1993). Retained placenta may be characteristic of vitamin A deficiency in some species (McDowell, 1989).

Vitamin A is transported readily by the mammary gland to the milk in response to dietary intake in sheep (Donoghue, 1987). Maternal vitamin A status directly determines the supply of vitamin A to the neonate (Stowe, 1982).

### ***Interactions with other nutrients***

The absorption and function of vitamin A may be influenced by interactions with other nutrients.

***Protein.*** Protein deficiency interferes both directly and indirectly with vitamin A metabolism. The direct effect is that it impairs synthesis of proteins involved in cleavage of carotene to retinol (Stoeker and Arnich, 1973) and in transport of vitamin A, the RBPs (Golden, 1982). The quality of the protein at low intakes is also of importance in supplying amino acid precursors in correct proportions for specific protein synthesis (Muhilal and Glover, 1974).

The indirect effect of protein deficiency is impaired synthesis and release of proteolytic and lipolytic enzymes of the gut and pancreas. With a reduced amount of such enzymes, the hydrolysis of carotenoid and retinyl esters is slower, and the formation of micelles and their diffusion to the mucosal cell membrane are impaired (Hollander and Muralidhara, 1977).

***Fat.*** Fat in the diet markedly stimulates the absorption of carotenoids and vitamin A, not only by enhancing gallbladder contraction but also by providing a lipid vehicle for vitamin A transport and absorption. Extremely low fat diets possibly interfere with carotenoid and vitamin A absorption (Bondi and Sklan, 1984).

*Iron.* Vitamin A deficiency is accompanied by anemia characterized by a reduction in red cell hemoglobin (Hodges et al, 1980) and serum iron also appears to be depressed (Donoghue et al., 1981). Within the lumen of the gut, iron may act as a pro-oxidant, either in its ionic form or in combination with heme. Prevalences of anemia and of vitamin A deficiency are correlated (Mejia et al., 1979).

*Zinc.* Zinc and vitamin A interact on at least two levels. Zinc-deficient animals have low circulating levels of retinol-RBP and increased hepatic vitamin A stores (Solomans and Russell, 1980). This may be due to a reduction in the synthesis or release of RBP from the liver (Smith and Smith, 1974) or to reduction in the activity of retinyl palmitate hydrolase (Sklan and Donoghue, 1982a). Another level of interaction is the presence of zinc in both retinol oxidase and reductase. Activities of both enzymes decrease on zinc depletion (Sundaresan et al., 1977). These interactions may explain the night blindness and abnormal dark adaptation found in some cases of zinc deficiency (Solomans and Russell, 1980).

*Copper.* Serum copper has been found in inverse relation to vitamin A levels (Moore et al., 1972). At low dietary levels of copper or zinc the conversion of retinol from carotene is depressed (Halevy and Sklan, 1986). On the other hand, high levels of copper in feed has a detrimental effect on the stability of the vitamin A (McDowell, 1989).

*Vitamin E.* Vitamin E serves as a lipid soluble antioxidant both in the intestinal lumen and in tissues, exerting a protective effect on the conjugated double-bond system of vitamin A. High dietary tocopherol apparently spared vitamin A, increasing liver stores and reducing turnover in the chick (Sklan, 1983). Massive tocopherol supplementation also alleviated signs of hypervitaminosis A in rats (Jenkins and Mitchell, 1975). Conversely, surplus vitamin A enhanced the appearance of nutritional encephalomalacia induced by vitamin E deficiency in chicks (Dror et al., 1980). High vitamin A affected

tocopherol turnover and enzymes connected with protection of cells against oxidation such as superoxide dismutase and glutathione peroxidase in chicks (Sklan and Donoghue, 1982b). In addition, vitamin A appeared to reduce the stability and availability of tocopherol in the gastrointestinal tract (Sklan, 1983).

## **Availability**

### ***Sources of vitamin A for the horse***

Provitamin A carotenoids, mainly  $\beta$ -carotene in green feeds, are the principal source of vitamin A for grazing livestock. All green parts of growing plants are rich in carotene and, therefore, have a high potential vitamin A value. The degree of green coloring of roughage is a good index of its carotene content (McDowell, 1989). Good pasture plants, whether grass or legume, appear to provide a liberal supply of carotenes. At maturity, however, carotene concentration is higher in the leaves than in stems, hence it is higher in legumes than in grasses (Maynard et al., 1979). With all forage, vitamin A value decreases after the bloom stage. Plants at maturity can have 50% or less of the maximum carotenoid value of immature plants.

Much of carotene content is destroyed by oxidation in the process of field curing. More than 80% of alfalfa carotene may be lost during the first 24 hours of the curing process (Russell, 1929). The loss occurs chiefly during the hours of daylight, owing in part to photochemical activation of the destructive process. In alfalfa leaves, sunlight-sensitized destruction is 8% of the total pigment present, while enzymatic destruction amounts to 28% (Bauernfeind, 1972). Enzymatic destruction requires oxygen, increases at higher temperatures, and ceases after complete dehydration.

Hays that are cut in the bloom or earlier stages and cured without exposure to rain or too much sun retain a considerable proportion of their carotene content, while those cut

in the seed stage and exposed to rain and sun for extended periods lose most of it. Green hay curing in the swath may lose one half its vitamin A activity in a day of exposure to sunlight and almost all if left exposed to rain as well as sunlight. Thus, hay usually has only a small proportion of the carotene content of fresh grass. Under similar conditions of curing, alfalfa and other legume hays are much richer than grass hays in carotenes because of their leafy nature, but a poor grade of alfalfa hay may have less than a good grade of grass hay (Maynard et al., 1979).

The carotene content of dried or sun-cured forages decreases in storage with the rate of destruction depending on factors such as temperature, exposure to air and sunlight, and length of storage. Under average conditions, carotene content of hay can be expected to decrease about 7% per month. In artificial curing of hay with a "hay drier," there is only a slight loss of carotene because of the rapidity of the process and protection against exposure to oxygen, with the final product having 2-10 times the values of field-cured hay. Severe heating of hay in the mow or stack reduces its provitamin content.

Several factors can influence the loss of vitamin A from feedstuffs during storage. Trace minerals in feed and supplements, particularly copper, are detrimental to vitamin A stability. Dash and Mitchell (1976) reported the vitamin A content of 1293 commercial feeds over a 3-year period. The loss of vitamin A was over 50% in one year's time. Vitamin A loss in commercial feeds was evident even if the commercial feeds contained stabilized vitamin A supplements. The stability of vitamin A in feeds and premixes has been improved tremendously in recent years by use of antioxidants, by chemical stabilization as an ester and by physical protection with emulsifying agents, gelatin, and sugar in spray-dried, beaded or prilled products (Shields et al., 1982). Nevertheless, vitamin A supplements should not be stored for prolonged periods prior to feeding.

Vitamin A and carotene destruction also occurs from processing of feeds with steam and pressure. Pelleting effects on vitamin A in feed are caused by die thickness and hole size, which produce frictional heat and a shearing effect that can break supplemental vitamin A beadlets and expose the vitamin. In addition, steam application exposes feed to heat and moisture. Running fines back through the pellet mill exposes vitamin A to the same factors a second time. Between 30 and 40% of vitamin A present at mixing may be destroyed during pelleting (Shields et al., 1982).

The circumstances most conducive to vitamin A deficiencies in horses are 1) extended periods of drought, resulting in pastures becoming dry and bleached with no green color; 2) diets composed primarily of concentrates and poorly conserved forages with no green pasture; 3) young animals fed milk from mothers on a low intake of vitamin A or carotene (McDowell, 1989). Mild deficiencies of vitamin A, especially in winter (or during the dry season) and early spring, are probably fairly common. These mild deficiencies may be asymptomatic but nevertheless influence the total well-being and performance of the animal.

## Vitamin A Requirement

### *Current recommendations*

The first requirements of vitamin A in horses were based on clinical signs of deficiency. Horses were fed typical diets very low in carotene until they began to exhibit symptoms of night blindness, a period that varied from 284 to 627 days (Guilbert et al., 1940). If vitamin A therapy was not instituted soon after the appearance of night blindness, the severity of signs increased gradually over a period of 3 to 4 months leading to death. Other symptoms were excessive lacrymation, keratinization of the cornea, respiratory symptoms, reproductive difficulties, capricious appetite, lameness, joint lesions, progressive weakness and death (Howell et al., 1941). Using cod liver oil as a vitamin A source, the minimal requirement to support normal growth, freedom from clinical signs and little or no tissue storage was 6  $\mu\text{g}/\text{kg}$  of body weight (Guilbert et al., 1940). For significant tissue storage, optimal dark adaptation, and reproduction, a requirement of 18  $\mu\text{g}/\text{kg}$  of body weight was proposed. Dietary requirements for carotene as a source of vitamin A were estimated similarly using dehydrated alfalfa meal. Minimum requirements of carotene were approximately 5 times that of vitamin A, between 20 and 30  $\mu\text{g}/\text{kg}$  of body weight to support normal growth, freedom from clinical signs and little or no tissue storage. For significant tissue storage, optimal dark adaptation, and reproduction, a requirement of carotene was 125  $\mu\text{g}/\text{kg}$  of body weight was proposed.

The vitamin A daily requirements of the horse are affected by age, stage of production, and stress. The daily recommendations (NRC, 1989) range from 9  $\mu\text{g}$  (30 IU)/kg body weight for maintenance, to 18  $\mu\text{g}$  (60 IU) for pregnant/lactating mares, with 13.5  $\mu\text{g}$  (45 IU) for all others. The daily requirement for carotene given by the NRC are 36

to 72 µg of provitamin A activity or, specifically, 72 to 144 µg of β-carotene/kg body weight.

The requirements for the horse recommended by the National Research Council are among the lowest in western countries (NRC, 1989; Meyer, 1986; Martin-Rosset, 1990). A summary of recommendations in Germany, France and Great Britain are compared to the current U.S. recommendations (Table 1).

### ***Current questions***

The current recommendations (NRC, 1989) are based on the amounts of vitamin A required to prevent gross signs of deficiency. A low vitamin A status may be insidious and effect health and performance before gross lesions or clinical signs are manifested (Campos et al., 1987). Also, none of the horses in Guilbert's studies were used for work and all lived in small drylots their entire life (Guilbert et al., 1940). They also were kept in a mild climate (Davis, California) where extreme temperatures fluctuations, especially cold, are rare. Thus, their vitamin A requirements may not have been as high as horses in less controlled situations. Extrapolating from highly controlled experimental conditions to the wide variation in the field should be judicious. Results of other studies under more extreme conditions should therefore be taken into consideration in evaluating *minimal* requirements. On the other hand, deciding upon the *optimum* for vitamin A supplementation in the horse may be easier. One obvious standard would be the amount of vitamin A or β-carotene that would maintain vitamin A status equivalent to that of horses receiving good green pasture (Garton et al., 1964; Ahlswede and Konermann, 1980; Mäenpää et al., 1988a).

**Table 1. Comparison of recommendations for the daily nutrient requirement of vitamin A in the U.S., Germany, France and Great Britain (respectively)**

Reference	Adequate concentrations ( $\mu\text{g}/\text{kg}$ body weight)				
	Maintenance	Pregnancy	Lactation	Growth	Work
NRC, 1989	9	18	18	13.5	13.5
Meyer, 1986	22.5	45	30	45-60	22.5
Martin-Rosset, 1990	15	25	21	24	25
Frape, 1986	7	21	18	20	11

Other observations have indicated that the NRC minimum requirement may be suboptimal. Mäenpää et al. (1988b) supplemented pregnant mares and foals with a commercial vitamin mix that delivered approximately the recommended intake (NRC, 1989) during the whole year. The horses were kept on pasture from June to early October and in stalls the rest of the year. Vitamin A supplementation during the winter months at this level was not adequate to maintain serum levels of retinol equivalent to those during the months on pasture.

In another study, growing ponies were fed one of four levels of vitamin A: mildly deficient (0 µg retinol/kg body weight), control (12 µg retinol/kg body weight), mildly intoxicated (1200 µg retinol/kg body weight), or severely intoxicated (12,000 µg retinol/kg body weight) (Donoghue et al., 1981). Ponies on deficient and mildly toxic vitamin A intakes appeared clinically normal but had depressed serum iron, albumin and cholesterol (anemia). These and four other variables (rate of gain, heart girth increase, red blood cell count and packed cell volume) exhibited quadratic relationships with vitamin A, and were plotted to show that the recommended intake (NRC, 1978) was below maximal values of the seven criteria. Based on these data, the *optimal* intake of vitamin A for growing foals was 1.5-5 times (60 - 200 IU/kg bwt) the NRC recommendation.

The estimated conversion rate of β-carotene to vitamin A is 1 mg of β-carotene to 400 IU vitamin A activity (NRC, 1989). It may vary greatly, however, for different forages. Carotene in alfalfa hay appeared to be more available as a source of vitamin A activity than carotene in grass hays, and alfalfa hay was consistently more effective than grass or clover hays at maintaining the plasma vitamin A concentration (Fonnesbeck and Symons, 1967). The overall ability of hay alone to maintain adequate concentrations of plasma vitamin A or β-carotene was questioned in this experiment. The average carotene

intake in this experiment was 14 times the requirement (NRC, 1961). Nevertheless, the average carotene intake was not sufficient to maintain the plasma carotene or vitamin A concentration of the blood during the 24 week experiment. The average plasma vitamin A concentration declined to less than 10  $\mu\text{g}/\text{dl}$ , which was considered deficient, in the second part of the experiment despite horses receiving as much as 172 mg of carotene per day from grass hay.

Values determined by chemical analysis of  $\beta$ -carotene do not necessarily establish biopotency (Ullrey, 1972). Certain widely-used chemical assays tend to lack specificity, while biopotency is dependent on precise and specific structural and stereochemistry. In addition, the presence or absence of other substances in the diet may influence the efficiency with which these vitamins are absorbed and metabolized. Significant differences in vitamin utilization between species and between individual animals, coupled with differing quantitative requirements dependent upon the criterion chosen, also contribute to the discrepancy between biopotency and chemically determined concentration. For example, 1 mg of corn carotene should have 500 IU of vitamin A activity for the pig according to the NRC (1988), when in fact it was found to have only 261 IU (Wellenreiter et al., 1969). Further elucidation on the actual bioavailability of carotenes in conserved forages versus fresh forages is needed in horses.

### *Seasonal effects*

The carotene content of pastures and the vitamin A status of grazing animals varies with the season (Garton et al., 1964; Mäenpää et al., 1988a). Fluctuations in vitamin A status may be due partly to a decrease in the total intake of carotenes, or to an increase in utilization of vitamin A associated with environmental stresses. No seasonal change in

plasma retinol was found in stabled horses maintained on hay from May to November in England (Butler and Blackmore, 1982). In another study, however, horses on pasture had  $\beta$ -carotene concentrations in plasma 8 to 13 times higher than horses kept in stables (Ahlsvede and Konermann, 1980). Blood was analysed at approximately 6-week intervals for carotene and vitamin A concentration from 24 broodmares maintained mainly on pasture on 6 farms in New Jersey (Garton et al., 1964). The plasma concentrations of carotene and vitamin A were found to be influenced by the season: there was a 93% increase in plasma  $\beta$ -carotene from February to June, and a 41% increase in plasma vitamin A over the same time period. Similar changes in blood concentrations were seen by the following October. Overall ranges for carotene and vitamin A concentrations were 0-366 and 8.3-36.2  $\mu\text{g}/\text{dl}$  plasma, respectively. Pasture samples were analyzed for their carotene content in the summer and fall; the average amount of carotene found for all pastures was 42 mg/kg on a dry basis.

There was a seasonal variation in serum levels of vitamin A in adult horses undergoing active training and racing (Mäenpää et al., 1987). Another study by Mäenpää et al. (1988a) revealed a pronounced seasonal variation in serum levels of retinol in pregnant mares and foals receiving commonly used diets in Finland. The winter levels of serum retinol were half the summer values on pasture. The low winter values were taken to reflect increased utilization of these vitamins in the two groups of horses compared with adult non pregnant horses and also low levels of these vitamins in the stored feeds provided during the winter months. Feeding acid-stored hay in winter did not prevent the decrease in serum retinol. Of the retinol values determined in this study, about 10% were less than 10  $\mu\text{g}/\text{dl}$ , and about 50% were less than 15  $\mu\text{g}/\text{dl}$ . In the weanlings, the corresponding incidence were about 15% and 90%.

In a follow up study, dietary vitamin A supplementation of pregnant mares at 1.3 times the recommended level (NRC, 1973) was inadequate to prevent the decreases in serum levels of retinol in the winter (Mäenpää et al., 1988b). The serum levels of retinol observed in June were much higher than those with or without vitamin supplementation in winter. No consistent difference in retinol between feeding groups (with 40,000 IU/d or without) were observed in the mares during this experiment. In the weanlings, however, dietary vitamin A (40,000 IU/d) increased serum retinol levels by 23.1% in late winter or early spring. The dietary vitamin supplementation of foals and weanlings was more effective than that of mares during the winter months. As with the mares, the high summer levels were not reached in late winter and early spring. However, the increases occurring in June (after 2 weeks on pasture) were much greater than those obtained by dietary vitamin supplementation in winter. These authors speculated that the relatively poor pregnancy rates in Finland in horses with inseminations prior to June are due at least in part, to low tissue levels of retinol.

Some of the seasonal fluctuations in blood vitamin A and carotenes in horses may be explained partly by analogy with carotene digestibilities in cows (Wing, 1969). Overall mean digestibility of naturally occurring carotenes in forage was 77.7%. Carotene digestibility in cattle was higher than average during warmer months and lower than average during the winter. Also drought and subsequent decreased pasture quality had a negative effect on digestibility. The same differences in carotene digestibilities were seen in summer pasture versus winter. Higher absorption of carotenes occurred from grazed forage than from conserved forage, although alfalfa hay was consistently high in carotene digestibility. There was no consistency in carotene digestibilities among the other grasses and legumes. Higher serum carotene levels in cows on pasture were observed than in

similar animals receiving a nonpasture ration containing more carotene than did the average of the pasture forage (Whitnah et al., 1939).

Little research has been done in the horse to examine if supplementation with enough vitamin A during the winter months would be able to counteract the seasonal drop (Mäenpää et al., 1988b). The supplementation of large quantities of vitamin A may increase the risk of vitamin A intoxication (Donoghue et al., 1981).

### ***Hypervitaminosis***

Availability of numerous equine supplements to the consumer with varying vagueness as to actual contents and the old adage "if a little is good, much more is better" may inadvertently lead to vitamin A intoxication in horses. Fortunately, moderate excess of vitamin A can be consumed without harm; it merely increases the liver reserves of retinyl esters. The rate of excretion of vitamin A can be readily exceeded with intakes of 50 - 500 times the requirements (Bondi and Sklan, 1984). When liver storage becomes saturated, increasing amounts of retinyl esters appear in the circulating lipoproteins (Mallia et al., 1975). Non-specific delivery of either retinyl esters or retinol, following hydrolysis by lipoprotein lipase causes membranolytic effects and cell damage (Sklan and Donoghue, 1982). An upper safe level of 320 µg (1067 IU) of retinol/kg of body weight/day or 16,000 IU/kg diet has been proposed for horses (NRC, 1989).

The upper limit of the recommendation of vitamin A intakes (20,000 IU/kg diet) was fed to growing-finishing pigs for 90 days and no evidence was found that this level of dietary vitamin A interfered with performance or with blood serum or tissue  $\alpha$ -tocopherol concentrations (Anderson et al., 1995). This indicates that the NRC has built in a buffer zone for its upper safe levels of vitamin A. Perhaps chronic supplementation to the pigs would have eventually had an adverse effects on production. Gross symptoms of

hypervitaminosis did not manifest themselves until 15 weeks of supplementation at 1000 times the recommendation (NRC, 1978) in growing horses (Donoghue et al., 1981).

The proposed upper safe limit of vitamin A for cattle is 66,000 IU/kg diet (1320 IU/kg bwt/d). A lowering of cerebrospinal fluid pressure was seen in 6 chronic hypervitaminotic (3700 IU/kg bwt/d) Holstein calves due in part to a reduced rate of formation of cerebrospinal fluid and possibly to a decreased resistance to bulk absorption (Hurt et al., 1967). The level at which the varying symptoms of hypervitaminosis occurred for various intakes of vitamin A ranging from 225 to 2500 IU/kg bwt/d was determined in Holstein male calves (Hazzard et al., 1964). Cerebrospinal fluid pressure began to drop at intakes of 225 IU/kg bwt/d (67,500 IU/kg diet). Heart rates increased with intakes of 285 IU/kg bwt/d, but live weight gain did not decrease until intakes of 520 IU/kg bwt/d. Vitamin A concentrations in plasma increased across all intakes and in liver up to an intake of 880 IU/kg bwt/d, thereafter decreasing.

Plasma retinol was observed to increase then decrease in a quadratic manner with increasing supplementation of vitamin A to growing ponies (Donoghue et al., 1981). The ponies were fed diets containing retinyl acetate in differing levels; control (35 IU/kg bwt/d), mildly intoxicated (3500 IU/kg bwt/d) and severely intoxicated (35,000 IU/kg bwt/d). Signs of severe intoxication appeared after 15 weeks of supplementation including rough hair coats, poor muscle tone and depression. By week 20, ponies were losing large areas of hair and epidermis, were severely depressed and spent much time in lateral recumbency. They also suffered from periodic bouts of ataxia or extreme hyperextension of carpal, tarsal and metapalangeal joints. Ponies on mildly toxic vitamin A intakes (3.75 times the upper safe limit recommended by the NRC, 1989) appeared clinically normal throughout the 40-week experiment, only slightly less well fleshed and

their hair coats were slightly dull compared to controls. Growth rates were lower than in the normal ponies.

### ***Beta-Carotene***

In contrast, there is no evidence of deleterious effects from large doses of  $\beta$ -carotene, which has not been found to cause hypervitaminosis A (Hathcock et al., 1990). Therefore, the use of  $\beta$ -carotene should be considered as an alternative to vitamin A supplementation. The non provitamin A properties of  $\beta$ -carotene have been subject to much research in recent years.

***Antioxidant.*** Carotenoids have photo-protection, radical quenching and antioxidant functions apart from being a precursor of retinol and retinoic acid (Bendich, 1993). Free radicals are highly reactive and may react with almost any biochemical component of a cell. They can promote lipid peroxidation in the membrane, causing increased cell permeability. They can attack nucleic acids and may have a lethal or mutagenic effect.

Free radicals are formed daily through normal internal biological processes: formation of prostaglandin, normal intracellular oxygen metabolism, and reactions in the presence of transition metals (Chew, 1993a). Free radicals can also be formed by other stresses such as dietary imbalances, inflammation, and infection (Brevard, 1989). External factors of much concern in our modern day environment which cause free radical formation are radiation, pollution, cigarette smoke and herbicides (Miller et al., 1993). The body has its own endogenous mechanisms to prevent or limit injury by free radicals, such as: mitochondrial cytochrome system which consumes most of the oxygen available within the cell, preventing 95-99% from forming toxic metabolites; enzymes which detoxify metabolites such as superoxide dismutase (SOD), catalase and glutathione

peroxidase; bile pigments such as bilirubin and biliverden which have been described as effective singlet oxygen quenchers (Krinsky, 1992). Overproduction of free radicals can be more than endogenous antioxidants can handle, so nutritional antioxidants may be required. These are lipid soluble ( $\beta$ -carotene, vitamin E, vitamin A and ubiquinone) and water soluble (ascorbate and citrate). The long conjugated, double bonds of  $\beta$ -carotene readily combine with free radicals, and this activity is more effective at lower oxygen tensions than higher, 2% versus 20% (Burton, 1989). Beta-carotene molecule may react directly with peroxy radical possibly quenching at least 2 or more at one time. Singlet oxygen is not considered a free radical but is also reactive, highly energized and unstable. Singlet oxygen may participate in reactions to generate free radicals such as the superoxide radical and has been shown to be capable of inducing DNA damage and to be mutagenic (Di Mascio et al., 1991). Carotenoids readily accept the energy of singlet oxygen ("quench") and then dissipate energy to the solvent system.

***Immune response.*** Beta-carotene supplementation has an effect on the immune response, possibly through its antioxidant action. The immune system is responsible for protection against infection by pathogens: bacteria, viruses and protozoan parasites (Miller et al., 1993). It also responds to cells that have undergone changes such as cancer cells. The invaders are destroyed by immune cells and their secretions. Conditions that depress immune function increase risks of infection and development of certain cancers.

During the immune response, free radicals and reactive oxygen molecules are used as weapons against bacterial invaders (Bendich, 1989). However, during the killing of pathogenic organisms, free radicals may be overproduced and result in injury to white blood cells as well as neighboring cells and tissues (Brevard, 1989). Beta-carotene could be beneficial in boosting the immune system during the overproduction of toxins from free radicals or reactive oxygen metabolites.

When human neutrophils were incubated with bacteria and  $\beta$ -carotene, the bacteria were killed efficiently and the neutrophils were not damaged or destroyed by their own oxidative products (Bendich, 1989). Beta-carotene has been found to induce lymphocyte proliferation in Holstein cows during the peripartum period (Heirman et al., 1990), in non-lactating, primiparous cows (Daniel et al., 1986), and in mature and newborn pigs (Hoskinson et al., 1989 and 1992).

**Cancer.** In vitro studies on cancer in cells and cell cultures have demonstrated  $\beta$ -carotenes ability to affect cancer cells. Chinese hamster ovary cells treated with a tumor promoter had a significant reduction in the number of sister chromatid exchanges after the addition of  $\beta$ -carotene to these cells (Weitberg et al., 1985). Genotoxic compounds can induce chromosomal aberrations, translocation or the development of micronuclei in Chinese hamster ovary cells, but these actions were inhibited by the addition of  $\beta$ -carotene (Stich and Dunn, 1986). In vivo studies with  $\beta$ -carotene supplementation also find similar effects. Beta-carotene was found to enhance tumor immunity in mice, tumors developing in  $\beta$ -carotene fed mice were significantly smaller than controls (Tomita et al, 1987). Administration of  $\beta$ -carotene (intraperitoneal injection) resulted in a longer latent period and slower growth of skin tumors induce by exposure of mice to UV-A and UV-B light (Epstein, 1977). Oral  $\beta$ -carotene protected mice by decreasing the number of tumors formed following administration of benzo[a]pyrene and UV-A light (Santamaria et al., 1981). Colon cancer induced mice fed high levels of  $\beta$ -carotene reported a significant decrease in total number of colon cancers (Temple and Basu, 1987). Increasing concentrations of dietary  $\beta$ -carotene were effective in decreasing the number of salivary gland tumors induced by injecting DMBA (Alam and Alam, 1987). Beta-carotene inhibited DMBA induced tumors in hamster cheek pouches by topical application of  $\beta$ -carotene in mineral oil (Suda et al., 1987). Supplementation of  $\beta$ -carotene was found to

cause partial or complete regression of oral leukoplakia (pre-cancerous lesions of the mouth) in betel nut (form of tobacco) chewers (Stich et al., 1991). Beta-carotene does not always eliminate the cancer cells completely but it has been useful in slowing down the proliferation of certain cancers.

Antioxidant nutrient status may be a determinant of susceptibility to inflammation related tissue damage and carcinogenesis. Decreased plasma and leukocyte levels of vitamins C, E and  $\beta$ -carotene are found in conditions associated with increased numbers and oxidative metabolism of neutrophils such as microbial infections, trauma and cigarette smoking. This probably reflects an increase in the use of these antioxidant vitamins during neutralization of phagocyte derived reactive oxygen metabolites. Low serum or plasma levels of  $\beta$ -carotene are consistently associated with the subsequent development of lung cancer (Anderson, 1991). Epidemiological studies in humans have shown that a low intake of vegetables, fruits and carotenoids is consistently associated with an increased risk of lung cancer - vegetable and fruit intake may reduce the risk of certain other cancers but epidemiological evidence is at present less persuasive than for lung cancer (Ziegler, 1991).

**Reproduction.** Beta-carotene has been suggested to have a non provitamin A effect on reproduction (Bonsembiante et al., 1980). Beta-carotene is absorbed from the intestine and found in the plasma, tissues and organs of the body. Five consecutive trials with beef heifers and cows showed that  $\beta$ -carotene supplementation induced an increase of  $\beta$ -carotene content of plasma, corpora lutea, liver and colostrum and of the vitamin A content of the liver (Bonsembiante et al., 1980).

Beta-carotene may be integral for the establishment of conception and maintenance of pregnancy. A low level of  $\beta$ -carotene in the diet of Italian Brown and Simmental heifers produced a marked reduction in the percentages of first-service conception, 58.8% for the cows supplemented with beta-carotene versus 38.8% for the controls

(Bonsembiante et al., 1980). It was also associated with an increase in the number of poorly recorded and irregular estruses, and an increase in the number of inseminations necessary for conception. In another study with Friesian cows with lower plasma  $\beta$ -carotene values showed cyclic irregularities or appeared to have depressed steroid hormone production (Jackson et al., 1981).

Supplementation of 400 mg  $\beta$ -carotene to 36 pony mares was found to increase conception rates, enhance estrus symptoms and reduce early embryonal mortality (Van der Holst et al., 1984). In other horse studies,  $\beta$ -carotene supplementation improved pregnancy rates (Ferraro and Cote, 1984; Arbeiter and Lorin, 1986; Schubert and Hennig, 1986), increased the intensity of heat shown by mares, decreased early embryonic loss (Ferraro and Cote, 1984) and improved induction of estrus (Ahlsweide and Konermann, 1980).

Young and mature female rabbits supplemented with  $\beta$ -carotene along with vitamin A demonstrated better reproductive performance as evidenced by increased conception rates, higher percentages of pups born alive and of pups surviving the lactation period (Kormann and Schlachter, 1984; Kormann et al., 1989). In both the study with mares and the studies with rabbits vitamin E was also supplemented, as an interdependence of vitamin A and vitamin E has been reported for several animal species (Sklan, 1987).

Crossbred gilts injected weekly with  $\beta$ -carotene starting on the day of breeding and continuing through weaning at 3 weeks postpartum had lower embryonic mortality, larger litter size, and heavier litter weight at birth and at weaning than did unsupplemented gilts (Brief. and Chew, 1985). This study was in general agreement with a later study in which multiparous sows were injected once at weaning with 1, 50, 100, or 200 mg of  $\beta$ -carotene

(Coffey et al., 1989). A linear increase in litter size at birth was associated with increasing dosage of  $\beta$ -carotene. Also, the number of pigs born dead was lower in treated sows.

A lower incidence of retained placentas was reported in dairy cows supplemented with  $\beta$ -carotene compared with unsupplemented cows or cows fed vitamin A (Chew et al., 1977). Retained placenta often leads to metritis and decreased reproductive performance.

A specific reproductive role has been hypothesized for  $\beta$ -carotene independent from its precursor function as a provitamin A. High rate of progesterone synthesis and pregnancy cannot be maintained in vitamin A deficient rats (Tjoelker et al., 1988). Retinol, retinoic acid and  $\beta$ -carotene have been shown to stimulate progesterone secretion by porcine luteal cells in vitro with greatest stimulation caused by  $\beta$ -carotene (Juneja et al., 1969). This suggests that  $\beta$ -carotene may play an important role in regulating luteal cell function, especially in the cow in which high concentrations of  $\beta$ -carotene in the corpora lutea (Wang et al., 1988). In vitro,  $\beta$ -carotene stimulated progesterone production by bovine luteal cells (Talavera and Chew, 1987). Again,  $\beta$ -carotene stimulated progesterone production by collagenase-dispersed pig luteal cells by 10-fold after a 24-hour incubation period (Talavera and Chew, 1988). Further evidence for the specific role of  $\beta$ -carotene in regulating reproduction was seen when  $\beta$ -carotene was found to form an integral component of the microsomal membrane of bovine luteal cells (O'Fallon and Chew, 1984).

Because uterine secretions are important in the survival and development of the embryo and because these uterine secretions are progesterone-induced (Bazer, 1975), it is possible that lower embryonic mortality observed in any of the before mentioned studies may be due to changes in the uterine secretions and ovarian progesterone production. Gilts injected every other day starting on the day of breeding with 16.4 mg of  $\beta$ -carotene had quantities of uterine-specific proteins on day 15 of pregnancy higher than those of untreated pigs (Chew et al., 1982).

Beta-carotene has been describe to have protective effects against numerous infectious organisms, including mastitis pathogens. Beta-carotene supplementation has been shown to interact with lactational status in dairy cows to influence responsiveness of mammary-derived lymphocytes and neutrophils in vitro (Tjoekler et al., 1988a, 1988b). Fewer treatments for clinical mastitis were required in a group of dairy cows supplemented with  $\beta$ -carotene (Wang et al., 1988).

Other studies have not found any added effects to  $\beta$ -carotene supplementation on reproduction. No effect of 300 mg supplemental  $\beta$ -carotene on the interval from the time of parturition to uterine involution, ovulation, first observed estrus, or conception was found in lactating Holstein cows (Wang et al., 1988). In addition, feeding  $\beta$ -carotene did not influence incidence of cystic follicles, ovarian cyclicity based on blood progesterone patterns, peak progesterone concentrations, or first service conception rate. In a study at Virginia Tech (Bindas et al., 1984), supplementation of 600 mg  $\beta$ -carotene to Holstein cows had no effect on luteinizing hormone, progesterone, insulin, glucose, or glucagon in blood plasma, or somatic cells in milk. There was no difference in reproductive measures (days to first heat, days to first breeding, days open and services per conception) either. All of the studies mentioned here as having no effect on reproduction supplemented with a water dispersible form of  $\beta$ -carotene (Rovimix, Hoffman LaRoche, Nutley, NJ). Absorption was not the problem, because all of the studies reported an increase in serum  $\beta$ -carotene after commencement of supplementation and higher serum  $\beta$ -carotene concentrations in supplemented than in control animals.

In contrast, Watson et al. (1996) reported no marked differences in plasma  $\beta$ -carotene or vitamin A between supplemented and control mares. They also found no benefit of 810 mg  $\beta$ -carotene (Rovimix) to Thoroughbred mares on reproductive characteristics such as length of estrus, interovulatory interval, follicle diameter on day

before ovulation, and plasma progesterone concentration during diestrus. Other horse studies have failed to show an effect on fertility following  $\beta$ -carotene supplementation, although plasma  $\beta$ -carotene was increased (Eitzer and Rapp, 1985; Enbergs and Klemt, 1987).

A negative effect from supplementation of  $\beta$ -carotene on reproduction has also been observed. The intervals from prostaglandin F<sub>2</sub> $\alpha$  administration to onset of estrus, leuteinizing hormone peak and ovulation were shorter in control heifers as compared to the respective intervals in heifers receiving 300 mg supplemental  $\beta$ -carotene (Wang et al., 1982). Another experiment used high yielding multiparous cows supplemented with vitamin A or  $\beta$ -carotene in Israel (Folman et al., 1987). Compared to the vitamin A supplemented group, the  $\beta$ -carotene supplemented group had conception rates 48% lower, days open 17% more for young cows and 33% more for older cows, number of inseminations per conception 28% higher in the young cows and 47% higher in the old cows, and fat-corrected milk yields 9% lower in the older cows. All cows were fed a basal diet with adequate levels of vitamin A and supplemented with either vitamin A palmitate or  $\beta$ -carotene (Rovimix). Unfortunately, the researchers do not give an indication of the vitamin A status of the animals prior to the study and there was no unsupplemented control group with which to compare reproductive measures.

## Methods of Assessment of Vitamin A Status

Several methods are used to assess vitamin A status; each has advantages and disadvantages. These methods include growth, blood values of vitamin A and  $\beta$ -carotene, the relative dose response, rapid dark adaptation test, and conjunctival impression cytology. Only growth, blood values and the relative dose response will be discussed in this review.

### ***Growth***

In the absence of dietary vitamin A, young animals will cease to grow and eventually die. The classic biological assay method of vitamin status is based on measurement of growth responses of weanling rats to graded doses of vitamin A (Underwood, 1994). The same principle has been applied to many of studies done in Third World countries in conjunction with the RDR (Flores et al., 1984; Campos et al, 1987; Tanumihardjo et al., 1990c).

### ***Blood values***

***$\beta$ -Carotene.*** Measurement of  $\beta$ -carotene in serum or plasma is not necessarily indicative of vitamin A status, although it is generally accepted that the presence of carotenoids in plasma is indicative of replete vitamin A status (Hoppe et al., 1996). Unlike vitamin A, there is a direct relationship between dietary intake of  $\beta$ -carotene and plasma concentrations in the cow and humans (Bindas et al., 1984; Parker, 1989). Plasma concentrations directly respond to carotene intake (Thurnham, 1994) up to a certain point where concentrations may reach a steady state regardless of increasing levels of intake (Coffey and Britt, 1993; Hoppe et al., 1996). Plasma carotene may be indicative of short

term carotene intake while organ or adipose carotene would be indicative of long term intake (Parker, 1989). Large species differences occur in an animal's ability to absorb  $\beta$ -carotene intact (Chew, 1993), even breed differences among a species (Bonsembiante et al., 1980). There are, also, considerable inter-individual differences in  $\beta$ -carotene in tissues (Brady et al., 1996). The major determinants of serum carotenoid levels are poorly determined, but may include dietary intake, destruction in the gastrointestinal tract, efficiency of absorption and metabolism and rate of tissue uptake (Parker, 1989). In addition, there may be effects of genotype, age and the physiologic condition, i.e. pregnancy, parturition and lactation (Bonsembiante et al., 1980). All these factors make interpretation of  $\beta$ -carotene concentrations in the blood difficult. However, there is no evidence of deleterious effects from large doses of  $\beta$ -carotene nor has it been found to cause hypervitaminosis A.

***Vitamin A.*** Serum retinol levels of vitamin A are the most commonly used biochemical measure of vitamin A status, even though it is well known that these are closely regulated by physiological controls (Underwood, 1990b). Vitamin A in the serum circulates largely in the form of a 1:1 complex of retinol and retinol-binding protein (RBP); the remainder is in the form of retinyl ester and small amounts of retinoic acid and other metabolites (Olson, 1984).

In normal healthy humans, retinyl esters constitute less than 5% of the total vitamin A content of fasting serum samples (Gibson, 1990). In a study on 2 and 3 year old Thoroughbreds in race training, retinyl palmitate, retinyl acetate, retinal and retinoic acid were not detected in any of the samples analyzed (Butler and Blackmore, 1982). The retinol:retinyl palmitate ratio ranged from 2.1:1 at parturition to 3.6:1 by 3 weeks postpartum in a study on brood mares from different farms in Michigan (Stowe, 1982).

In humans, results for both serum retinol and total serum vitamin A assays are in general comparable for levels below 35 µg/dl (1.22 µmol/L) (Driskell et al., 1982). At higher concentrations, the discrepancy between the two assays is greater; approximately 4 µg/dl (0.14 µmol/L) higher for total serum vitamin A compared to that for serum retinol.

When the capacity of the liver to store vitamin A is exceeded, elevated concentrations of serum retinyl esters are observed. The percent of retinyl esters of total vitamin A was 56% in moderately intoxicated and 75.8 % in severely intoxicated ponies (Donoghue et al., 1981). The percent of retinyl palmitate was 56.6%, retinyl acetate 4.0% and retinol 43.1% of total vitamin A in mature horses receiving 8 times the recommended daily intake of vitamin A (Jarrett et al., 1987).

Serum retinol levels reflect vitamin A status only when liver vitamin A stores are severely depleted in humans or rats, below 20 µg/g liver, or excessively high, above 300 µg/g (Olson, 1984; Zachman and Chen, 1991; Green and Green, 1994). When liver vitamin A concentrations are between these limits, serum retinol concentrations are homeostatically controlled; levels remain relatively constant and do not reflect total body reserves of vitamin A.

Supplementation of vitamin A at levels approximating the requirement have been found to have little or no effect on serum retinol levels. Abrams (1979) followed plasma retinol levels for 2 years in 8 racing Thoroughbreds, 4 of which were supplemented weekly with the equivalent of 50,000 IU/d of retinol. No differences between supplemented and unsupplemented horses were found in the first year and a difference in only 2 of the 4 supplemented horses in the second year. Similarly, Finhorses were supplemented 40,000 IU/d of vitamin A without a response in serum retinol as compared to controls (Mäenpää et al., 1988b). No differences were found in total serum vitamin A

or serum retinol in horses fed varying levels (0, 10,000 or 80,000 IU/d) of vitamin A palmitate for 30 days (Jarrett et al., 1987).

***Factors affecting blood values.*** Independent of the size of vitamin A stores in the liver, serum vitamin A or retinol concentrations may be affected by a variety of extraneous factors (Gibson, 1990).

- 1) Liver disease decreases plasma retinol levels, probably as a result of a combination of decreased synthesis and secretion of RBP (Mobarhan et al., 1984).
- 2) Stresses of various kinds increase the requirement for vitamin A. Both an increased body temperature and exposure to low temperature may increase the requirement of vitamin A (Sundaresan et al., 1967). The increased requirement has been found to lower blood levels.
- 3) Protein-energy malnutrition decreases RBP production because of a limited supply of protein substrate. Consequently hepatic release of vitamin A is impaired resulting in decreased serum retinol levels (Underwood, 1980; Russell et al., 1983).
- 4) Zinc deficiency decreases plasma retinol levels via its role in the synthesis or release of RBP from the liver (Solomons and Russell, 1980) or reduction in the activity of retinyl palmitate hydrolase (Sklan and Donoghue, 1982).
- 5) Chronic renal disease increases plasma retinol levels with the reduced catabolism of vitamin A and its carriers (Webb et al., 1968; Webb et al., 1970; Ritter et al., 1975; Gerlach and Zile, 1990).
- 6) Infections and parasitic infections lower plasma retinol levels by interfering with vitamin A absorption, utilization and excretion (Campos et al., 1987; West et al., 1989).
- 7) Low fat diets impair absorption of vitamin A, lowering plasma retinol concentrations (Bondi and Sklan, 1984).

8) Estrogens increase plasma retinol and RBP apparently by stimulating protein synthesis in the liver (Supopark and Olson, 1975).

9) Age, sex and race influence serum retinol levels in humans (Pilch, 1985). Preterm infants were found to have plasma retinol concentrations between 2.5 to 20.5 ug/dl (0.087 - 0.72 umol/l) while normal adults range from 20 to 60 ug/dl. In children, serum retinol levels have been found to be directly related to age (Underwood, 1974).

Serum retinol in adult horses over seasonal fluctuations ranged from 12 to 30 µg/dl (Mäenpää et al., 1988a). Serum retinol in yearling horses was lower than adult horses and ranged from 11 ug/dl to 18 ug/dl over the seasons (Mäenpää et al., 1988a). Two and 3 year old Thoroughbreds were found to have plasma retinol levels with a mean of 16.5 ug/dl and ranged from 6 to 30 ug/dl (Butler and Blackmore, 1982). The same researchers found no significant differences in plasma retinol levels between 2 and 3 year olds or between fillies and colts.

Individual variation and homeostatic control of release of vitamin A from the liver make blood values not reliable as a definitive measure of vitamin A status. Therefore, the use of other tests have been explored.

### ***Relative Dose Response***

The nutritional status of an individual or a population exists in a continuum ranging from deficiency through an optimal zone to toxicity (Underwood, 1990a). At the extremes of the continuum for vitamin A status, clinical signs or symptoms are usually present, as well as changes in blood concentrations. At the intermediate stages of marginal, adequate, optimal and excessive nutrient status, however, it is more difficult to ascertain where the individual or population stands on the continuum. The blood

concentration of vitamin A may not be directly proportional to the liver stores. In this situation, a dose-response test may prove useful.

The relative dose response (RDR) is a function test for the estimation of liver stores of vitamin A. The test is based on the observation that in vitamin A deficiency, retinol-binding protein (RBP) accumulates in the liver as apo-RBP (i.e. RBP that is not bound to retinol) to levels which are 4- to 10-fold higher than those observed in corresponding control livers (Blaner et al., 1985). Retinol deficiency specifically inhibits the secretion of RBP from the liver by preventing the movement of newly synthesized RBP from the endoplasmic reticulum to the Golgi apparatus (Ronne et al., 1983). Following a test dose of vitamin A, all of the retinol is taken up rapidly by the liver and binds to this relative excess of apo-RBP. Then holo-RBP (i.e. RBP bound to retinol) is subsequently released from the liver (Loerch et al., 1979). Consequently, in vitamin A depleted individuals, a rapid sustained increase in serum retinol (holo-RBP) occurs after the ingestion of a dose of vitamin A. This increase peaks at 5 hours in humans and rats, and return to baseline by 12 hours. In some domestic species, this peak may occur later than 5 hours and the return to baseline may take as long as 24 hours (Kronfeld et al., 1990).

For the test, a baseline blood sample is taken immediately before the administration of an oral dose of vitamin A (as retinyl acetate or retinol palmitate), followed by a second blood sample, five hours later. Serum is analyzed for retinol ( $SR_0$  and  $SR_5$ ). The relative dose response (RDR, %) is calculated by this equation:

$$RDR = 100 (SR_5 - SR_0) / SR_5$$

Ranges for the RDR values in rats have been described. A RDR of > 50% was associated with low plasma levels (10 - 30  $\mu\text{g}/\text{dl}$ ) and low liver stores (< 1  $\mu\text{g}/\text{g}$ ) of

vitamin A, and a RDR of < 40% was associated with plasma levels above 30 µg/dl and liver stores from 0.32 - 10 µg/g (Loerch et al., 1979). Vitamin A replete humans have RDR values ranging from 0% to 14% (Mobarhan et al., 1984). Depending upon the precision of the methodology used to determine plasma levels of retinol, two alternative cut-off criteria have been proposed to define an abnormal RDR: 20% and 14% (Flores et al., 1984). By their calculations, methodologies that result in intra-assay coefficients of variation less than 5% can be used with the 14% critical value and still provide diagnostic discrimination within a 95% confidence interval.

Research in developing the RDR had subjects fasting for 12 hours before taking the first blood sample. In human studies, a fasting blood value is not always feasible. Certainly, in a field situation with studies on large numbers of grazing animals like horses, who consume several small meals in a day, starving for 12 hours is not practical. Jarrett (1987) found no differences in serum retinol in fasted horses than 4 hours after a meal in fed horses. Mejia (1983) showed that adults ingesting a meal rich in vitamin A did not have significant postprandial changes in serum retinol, RBP and carotenoid concentrations up to a period of 4 hours. The same experiment was repeated with 5 to 8 year olds, with the same results (Mejia et al., 1984). The rationalization for the difference between the dose given for the RDR test and the vitamin A in the meal was that the vitamin A was an intrinsic part of the meal, and that the rate of gastric emptying may not be fast enough to provide at one point in time an amount of vitamin A for intestinal absorption sufficient to cause detectable changes in the serum concentration of the vitamin.

The validity of the RDR test as an index of body stores of vitamin A has been studied by comparing vitamin A concentrations in liver biopsy samples with corresponding RDR test results (Loerch et al., 1979; Underwood, 1980; Zachman and Chen, 1991). Another comparison was done in otherwise healthy surgical patients (Amédée-Manesme et

al., 1984). The 2 subjects with the lowest liver vitamin A concentrations had the highest RDR values (i.e. 14  $\mu\text{g}/\text{dL}$  and RDR 28%; 30  $\mu\text{g}/\text{g}$  and RDR 15%). Following supplementation with vitamin A, RDR values fell to < 5%. Subjects with liver vitamin A concentrations ranging from 58 to 434  $\mu\text{g}/\text{g}$  had corresponding RDR values ranging from 0% to 12%.

In a study of Brazilian children from low-income families, all children with serum retinol concentrations < 20  $\mu\text{g}/\text{dL}$  had elevated RDR values (Flores et al., 1984). Moreover, 86% of the children with serum retinol concentration 21 to 29  $\mu\text{g}/\text{dL}$  had elevated RDR values. Following supplementation with vitamin A, all the elevated RDR values reverted to normal, < 14%. These results indicate that RDR is a more sensitive index of marginal vitamin A status than using serum vitamin A levels < 20  $\mu\text{g}/\text{dL}$ . An infective episode of the chicken pox caused a deterioration in the hepatic stores of vitamin A in a portion of the same group of Brazilian children (Campos et al., 1987). At 180 days after a massive dose of vitamin A, 74% of the children who had been infected tested positive by the RDR in contrast to only 10% who had not been infected.

In a study on Guatemalan adults, who were expected to show a moderate prevalence of hypovitaminosis A, only 9.8% of the subjects had RDR tests above 14% (Solomons et al., 1990). The lack of sensitivity of the RDR in this case may have been due to the dose of vitamin A given, which was only 480  $\mu\text{g}$  retinyl esters (similar to the dose used in children). To get the full effect of the RDR test there must be sufficient substrate (retinol) to elicit a complete response from the stored RBP (Loerch et al., 1979).

Anorectic women were found to have significantly higher RDRs ( $31 \pm .05\%$ ) than normal ( $10 \pm .02\%$ ) or reformed anorectic ( $14 \pm .03\%$ ) women (Vaisman et al., 1992). The dose given was much higher than those used in other studies (15,000  $\mu\text{g}$  versus 450

µg retinol equivalents) but it did not affect the magnitude of the RDR, which is dependent on stored RBP.

Few studies using the RDR have been reported for domestic animals. In a study on neonatal and 13 day old lambs, neonates from ewes fed low (120 µg/kg/d) levels of vitamin A were extremely deficient with an RDR of 80% (Kronfeld et al., 1990). Neonates from ewes fed high (12,000 µg/kg/d) levels of vitamin A were normal (12.9%). Dose responses were normal in both groups after 13 days of suckling (7.5% and 2.9%, respectively). In this study, blood samples were taken for 8 hours after the initial dose of vitamin A palmitate (5000 IU/kg). It is interesting to note that even after 8 hours the plasma levels had not begun to drop to predose levels. The postdose retinol value used in calculation of the RDR was obtained at 8 hours.

To determine the time of the peak after a dose of vitamin A in the horse, one was fed an unspecified amount of vitamin A palmitate and blood samples were collected hourly for 10 hours (Jarrett and Schurg, 1987). The peak was found to be 4 hours for total plasma vitamin A. Therefore, their equation was  $\%RDR_{(horse)} = 100(A_4 - A_0/A_4)$ . Also, total plasma vitamin A (including esters) was used in the equation for the RDR (Jarrett and Schurg, 1987), as opposed to serum or plasma retinol which was used in humans and rats by other researchers (Loerch et al., 1979; Underwood, 1980; Mobarhan et al., 1984; Flores et al., 1984).

Fifteen mature horses with adequate vitamin A status were fed a diet either deficient, control or excess in vitamin A (0, 10,000, or 80,000 IU/d, respectively) for 30 days (Jarrett and Schurg, 1987). After 1 month on the experimental diets, total plasma vitamin A levels did not change but the RDR was significantly higher in the deficient group (42%) than in the control (5%) and the excess (-11%).

An oral dose of vitamin A is not always possible or practical, so the use of injected vitamin A may be desirable. An RDR using an intravenous dose of 1000 µg retinyl palmitate has been tested in 12 children with liver disease, and it proved to be a reliable and sensitive index of vitamin A status (Amédée-Manesme et al., 1987). The intravenous vitamin A was given over a 30-minute infusion, which may be a disadvantage in field. The use of an intramuscular injection of retinyl palmitate has been tested in rats by comparing the RDR results with actual liver vitamin A values (Zachman and Chen, 1991). A clear relationship to liver retinyl palmitate concentration was demonstrated. Differing sizes of intramuscular dose ranging from 1 to 9 µmol/kg body weight were tested, with no variation in the resulting RDR. The doses ranged from 3 to 25 times the oral dose given in the original work on the RDR (Loerch et al., 1979). The intramuscular method avoids potential variables in the dietary absorption process.

A typical RDR involves 2 blood samples. Only one sample is needed for a modified relative dose-response test (MRDR) using dehydroretinol a naturally occurring form of vitamin A that is rare in most diets (Tanumihardjo and Olson, 1988). Analytical techniques are available for differentiation of these two forms of vitamin A. Only one blood sample is taken approximately 5-8 hours following oral dosing. The ratio of dehydroretinol to retinol is the measured response.

The MRDR was developed in rats (Tanumihardjo et al., 1990a), then applied to preschool children in the US (Tanumihardjo et al., 1990b) and in Indonesia (Tanumihardjo et al., 1990c). A positive (abnormal) response was found in < 10% of healthy American preschool children and in 63% of Indonesian children. After children were dosed with 50,000 IU of vitamin A, all children showed a normal response 7 days later. These results support the value of the MRDR as an indicator of vitamin A status.

The main benefit of using the MRDR instead of the RDR is that only one blood sample needs to be drawn. The time required for the procedure is no shorter for the MRDR than the RDR; there is still a lag time of 5 to 8 hrs between dosing and collecting the blood sample. A major drawback of the MRDR is that dehydrotetrol is not yet commercially available.

Many factors which have adverse effects on vitamin A absorption may interfere with the RDR test. Severe protein-energy malnutrition and liver disease have been found to reduce the sensitivity and the specificity of the RDR test. When an oral dose of vitamin A was given to patients with varying degrees of liver dysfunction and protein-energy malnutrition, no correlation was observed between vitamin A content of liver biopsies and the RDR test result (Russell et al. 1983; Mobarhan et al., 1984).

The RDR test for vitamin A can provide information on vitamin A status that may not be reflected by single measures of blood concentrations. Where clear cut-off points for relative nutritional states are not well defined, dose-response tests can provide a basis for assessing nutritional status in marginal zones.

## OBJECTIVES

The general objectives of this research were to measure quantitative changes in the vitamin A status of grazing horses on pasture during the seasons of the year, and to evaluate whether vitamin A or  $\beta$ -carotene would be effective for the improvement of vitamin A status in these animals.

The specific objectives were:

- 1) to adapt the relative dose response test to horses and evaluate its effectiveness for the assessment of vitamin A status;
- 2) to measure quantitative changes in the vitamin A status of horses (mares and foals) on pasture as compared to horses with no access to pasture (fed hay) during all the seasons of the year;
- 3) to evaluate the effectiveness of vitamin A and  $\beta$ -carotene for improving the vitamin A status in these animals;
- 4) to evaluate the ability to influence the vitamin A status of the neonate through vitamin supplementation of the dam; and
- 5) to evaluate the influence of vitamin A and  $\beta$ -carotene supplementation on reproductive efficiency and general health.

**Seasonal Vitamin A Depletion in Grazing Horses is Assessed Better by the Relative  
Dose Response Tests than by Serum Retinol Concentration<sup>1,2</sup>**

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**Vitamin A Assessment In Horses**

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**ABSTRACT** Vitamin A influences growth and reproduction in horses. A retinol dose response (RDR) test for retinol has been shown to be better than serum retinol concentration for assessing vitamin A status in other species, so we have compared these two methods in the horse. Forty-five Thoroughbred broodmares were assigned randomly to 3 groups fed pasture and hay (PH), pasture, hay and vitamin A-free concentrate (PHC), or hay and concentrate (HC) in early summer (May, 1991). Mares in pasture groups produced 23 foals (March through June) that had access to their dam's diets and were also studied. In the mares, significant vitamin A depletion developed in two months in the non-pasture group (HC) and in eight months in the two pasture groups (PH and PHC) according to the RDR test, and in all three groups at eight months as shown by a decrease in serum retinol concentration. In the weanlings (PH and PHC only), no differences between groups were found for serum retinol, but the RDR was significantly higher in the PH group, which had suffered a respiratory infection, than in the PHC group. These findings indicated that vitamin A depletion was detected more readily by the RDR test than by serum retinol concentration, that consumption of pasture delayed depletion in the late fall, and that infection was associated with lower vitamin A status.

**INDEXING KEY WORDS:** vitamin A relative dose response horses forage infection

Forages, such as pastures and hays, often require supplementation to be utilized efficiently, especially as the nutritional value of pastures declines during fall and winter (McDowell 1989, NRC 1989). A common supplement is vitamin A which influences many functions, notably reproduction and growth in horses (Donoghue et al. 1981, Donoghue et al. 1991). Its dietary precursor, beta-carotene, progressively deteriorates in pastures after they stop growing during the fall and winter as well as in hays and other conserved feeds (Bondi and Sklan 1984, Schryver and Hintz 1983), so additional sources of vitamin A are often required for grazing or stabled herbivores (Mäenpää et al. 1988a, 1988b).

Mean minimum requirements of vitamin A for maintenance, growth and the last month of pregnancy in horses have been estimated at 17, 25 and 33  $\mu\text{g}/\text{kg}$  body weight, respectively (NRC 1989), mainly on the basis of body weight, blood and tissue concentrations of vitamin A, and absence of deficiency signs. An optimal range of 38 to 60  $\mu\text{g}/\text{kg}$  for growth was suggested on the basis of growth variables, erythrocyte indices, serum iron and cholesterol, and liver vitamin A concentrations (Donoghue et al. 1981). Daily supplementation with up to 44  $\mu\text{g}/\text{kg}$  of vitamin A palmitate failed to fully control a decrease in the serum retinol concentration of broodmares when stabled and fed hay and oats (Mäenpää et al. 1988b).

The blood concentration of retinol is regulated mainly by the rate of its secretion from the liver in the form of retinol bound to retinol binding protein. Hepatic stores usually suffice to maintain a constant level of serum retinol during variations in dietary supply (Blomhoff et al. 1991). Thus serum retinol concentration is not usually regarded as a sensitive indicator of vitamin A status. Nevertheless, seasonal fluctuations in serum retinol concentration have been observed in horses (NRC 1989). The relative dose response (RDR<sup>3</sup>) test, based on the response of serum retinol to an oral dose of retinol

palmitate, was developed in rats as a more sensitive test of vitamin A depletion (Loerch et al. 1979). Various forms of RDR tests have been used to assess vitamin A status in humans and other species (Amadee-Manesme et al. 1984, Campos et al. 1987, Kronfeld et al. 1990, Vaisman et al. 1992, Woodruff et al. 1987). An RDR test has demonstrated depleted vitamin A status in mature horses kept in stalls and fed a diet low in beta-carotene for 30 days (Jarrett and Schurg 1987). Seasonal depletions of vitamin A have been suggested for grazing horses, cattle and sheep (McDowell 1989) but have not been demonstrated by RDRs. This seasonal depletion is extremely important in all grazing species. Our primary objective was to develop an RDR test for horses grazing on pasture, and to compare the responses of this test and serum retinol concentration in pregnant mares and weanlings fed pasture, carotene-low hay and a vitamin A-free concentrate during summer, late fall and winter.

An incidental outbreak of a respiratory infection occurred in one group of weanlings in October and appeared to influence their vitamin A status. Thus the association of infection and vitamin A depletion, which has been described in other species (West et al. 1989), became a secondary interest.

## **METHODS**

*Horses and diets.* This protocol was approved by the University's animal care committee. In January 1991, 38 Thoroughbred mares, aged 4-14 years, were placed on bluegrass-clover pasture and randomly assigned to 2 groups. One group (PH) was supplemented seasonally with hay sufficient to provide all of the food energy requirement through the winter. The other group (PHC) was supplemented similarly with hay and with a grain concentrate, 4 kg/day, throughout the year. In May, 4 mares without foals from

each group and 7 additional mares without foals (also kept on pasture) were used to form a third group kept on a drylot and fed only hay and concentrate (HC). All mares were bred, March through July, and of the 15 mares in each group, 13, 11 and 11 became pregnant in the PH, PHC and HC groups, respectively.

Foals were born from 12 PH mares and 11 PHC mares from March through June, 1991. These 23 foals were studied as weanlings. They had access to the feed of their dams before weaning in September and were kept on the same feed after weaning. All horses were weighed monthly.

The pastures were mainly bluegrass with some clover. They presumably provided abundant beta-carotene in May and the summer, probably 40-50 times the equivalent of the vitamin A requirement (NRC 1989), but progressively less through the fall and winter. The mixed grass hay was 2 years old, hence provided little or no beta-carotene. The concentrate was based on a commercial formula<sup>3</sup> (PurePride 200, Purina Mills, St. Louis, MO), which was modified to be low in beta-carotene and free of vitamin A. It was fed at a rate of 4 kg/d to mares and 3 kg/d to weanlings. Horses had free access to a trace-mineralized salt and to water.

Samples of pastures were collected in May, July and November, and hay and concentrate samples were obtained as new shipments arrived. All were submitted to the Virginia Tech DHIA Forage Testing Laboratory, which uses standard procedures (AOAC 1984) for proximate and mineral analysis (Table 1).

Additional trace element analysis, using inductively coupled plasma spectrophotometry was conducted by the Virginia Tech Soil Testing Laboratory (Table 1). The forages were marginal or low in phosphorus (1.4 - 3.8 g/kg dry matter) and low in selenium (<0.08 mg/kg). Deficiencies of these elements were identified by analysis of serum and urine in November and supplementation with phosphorus and selenium was

initiated in December (Greiwe-Crandell et al. 1992). Because selenium depletion has been associated with depression of serum IgG in foals (Knight and Tyznik 1990), this immunoglobulin was measured in serum from the weanlings, using a single radial immunodiffusion kit (Equine IgG SRID Kit, VMRD Inc., Pullman WA).

*Serum retinol assay.* Jugular blood samples were collected into 10 mL tubes (Vacutainer, Becton Dickinson, Rutherford NJ) and placed immediately in a closed, dark container. They were centrifuged within 30 min., and the serum stored at -20°C until thawed for assay. All samples were prepared in duplicate. Protein in serum was precipitated with ethanol and retinol was extracted with hexane after addition of retinol acetate as an internal standard (Catignani and Bieri 1982, Miller and Yang 1985), then sample was injected into a HPLC (Waters uBondapak Reverse Phase C18 Column [3.9x300 mm] with Waters 484 Tunable Absorbance Detector set at 325 nm and Waters 600E Controller, Millipore, Milford, MA). Solvent was 98% methanol and 2% water, with a flow rate of 1 mL/min for 7 min with retention time for retinol and retinyl acetate eluding at 4-5 and 5-6 min respectively.

*The serum retinol curve.* In a preliminary experiment, temporal changes in serum retinol following an oral dose of vitamin A palmitate<sup>4</sup> were determined in 4 mares. The mares were stalled overnight before insertion of a 16-gauge catheter (Abbocath-H, Abbott Laboratories, North Chicago, IL) into a jugular vein. After an hour, a blood sample was taken and an oral dose of vitamin A palmitate, 123.5 mg in 5 mL corn oil, was given by syringe directed deeply into the mouth. This dose corresponds to 7 times the daily requirement for a 535 kg pregnant mare (NRC 1989). A series of blood samples was taken at 1, 3, 6, 12, 18 and 24 hours after the dose.

*The RDR tests.* Two-term polynomial equations were fitted to the serum retinol/time curves (**Figure 1**). Peaks were found at 12 to 14 h. Recognizing that the

curves were rather flat and needing to fit our work into farm management, subsequent RDR tests used an interval of 15 h between two blood samples taken at 1600 to 1700 h then 0700 to 0800 h the following morning. The vitamin A dose, 123.5 mg vitamin A palmitate in 5 mL corn oil, was given immediately after the first blood sample was taken. During the RDR test horses were not moved from their respective fields and had constant access to pasture or hay.

The initial RDR test was done in April, 1991, on the mares before they were divided into the 3 groups and the HC group taken off pasture. Three sequential RDR tests were conducted on the mares at 2 mo (July, summer), 6 mo (November, fall), and 8 mo (January, winter) after the mares were introduced to the experimental diets in May. Two RDR tests were conducted on the weanlings (November and January).

The RDR was calculated in two ways. One followed the original RDR test (Loerch et al. 1979):

$$RDR_{15} = 100(T_{15} - T_0)/T_{15} \%$$

where  $T_0$  and  $T_{15}$  are the first and second serum retinol concentrations, respectively. The second calculation used the usual form of an increment, with the initial serum retinol concentration as the denominator (Vaisman et al. 1992):

$$RDR_0 = 100(T_{15} - T_0)/T_0 \%$$

The RDR and  $T_0$  data were summarized as least-squares means and standard errors, and examined by analysis of variance with repeated measures using the GLM procedures of SAS (SAS/STAT Version 6, SAS Institute, Cary, NC). The model initially

included horse, diet, time, pregnancy and interactions. Pregnancy and the interactions were not significant, so were dropped from the model. Probabilities for differences between least-squares means were evaluated by *t*-tests found in the analysis of variance, and these *P* values for  $T_0$ ,  $RDR_0$  and  $RDR_{15}$  were compared by Fishers exact probability test and by the nonparametric sign test (Snedecor and Cochran 1976). Significance was inferred when  $P \leq 0.05$ , a trend when  $0.05 < P \leq 0.10$ , unless otherwise stated.

## RESULTS

*Mares.* All remained in good health and flesh throughout the study. Their body weights were not significantly different among groups, and were  $542.19 \pm 42.21$ ,  $540.58 \pm 41.01$ ,  $566.73 \pm 36.10$  and  $574.74 \pm 44.01$  kg in April, July, November and January, respectively.

In July, mean serum retinol concentration was about 20% lower in the concentrate fed groups (PHC and HC) than in the pasture only (PH) group (**Figure 2, upper**). The RDR tests were 2-3 times higher in the HC group on the drylot than in the PHC and PH groups on pasture (**Figure 2, middle**); the difference was significant for both PH and PHC groups with the  $RDR_{15}$  test but for the PH group only with the  $RDR_0$  test (**Figure 2, lower**).

In November, serum retinol was lower and both RDR tests were significantly higher in the HC group than in those on pasture (Figure 2). In January, serum retinol also decreased significantly in the pasture groups (PH and PHC), compared to November. However, the RDR tests in January were significantly higher in the HC group than in the PH group, with the PHC group intermediate and not significantly different from either

other group. Thus serum retinol showed progressive depletion with time, but only the RDR tests discriminated between diet groups in January.

*Weanlings.* The body weights were the same for both groups and increased from  $277.36 \pm 37.89$  kg in November to  $307.07 \pm 34.12$  kg in January. An outbreak of respiratory disease affected the PH group in October and spread to the PHC group, though less seriously, in December. Both the clinical signs (coughing, weeping eyes and a serous or purulent nasal discharge) and the progressive transmission through the group were characteristic of infection of the upper airways and bronchi with herpesvirus-4 (Ostlund et al., 1990), which is common in this region. The nasal discharge lasted anywhere from 10 to 60 days in PH horses, and the outbreak took 3 months to run its course through the group. In contrast, the outbreak lasted only 10 to 30 days in the PHC group and the whole group was clear in a month's time. Three weanlings in the PH group developed fever and coughing severe enough to warrant treatment with antibiotics<sup>5</sup>. Serum IgG levels were  $44 \pm 4$  and  $52 \pm 3$   $\mu\text{mol/L}$  in the PHC and PH weanling groups, respectively ( $P = 0.089$ ) in November, and  $52 \pm 3$   $\mu\text{mol/L}$  in both groups in January.

Analysis of serum retinol concentration revealed no differences between diet groups in November or January, and no differences from November to January for either weanling group (**Figure 3, upper**). Both RDR tests were about 50% higher in the PH group than the PHC group in November, and about 33% higher in January, with no indication of depletion through the winter (**Figures 3, middle and lower**).

*Comparison of indices.* The 3 indices of vitamin A status were compared on the basis of the  $P$  values in the  $t$ -test table generated for the analysis of variance. Values of  $P < 0.05$  were found in 3, 7 and 4 of 11 estimates of  $P$  for serum retinol,  $\text{RDR}_{15}$  and  $\text{RDR}_0$ , respectively. Comparable  $P$  values were lower with the  $\text{RDR}_{15}$  test than the  $\text{RDR}_0$  test for 9 of 11 estimates ( $P = 0.065$ ).

## DISCUSSION

This study developed an RDR test for assessment of vitamin A status in a grazing animal, the horse, and showed that this test was more sensitive than serum retinol concentration in detecting differences associated with diet and season in mares and with infection in weanlings. The study revealed that mares fed only conserved feeds, such as hay low in beta-carotene and a vitamin A-free concentrate, became depleted of vitamin A within two months in the summer, and that pasture fed mares become depleted by mid-winter. Any tendency for pasture fed weanlings to become depleted was obscured by an outbreak of respiratory disease early in winter. These findings support the common practice of supplementing vitamin A to horses fed on pasture during winter.

An RDR test was described previously in stall-fed horses of mixed breed (Jarrett and Schurg 1987). A dose of vitamin A acetate, 450  $\mu\text{g}$ , was dissolved in oil and top-dressed on the morning meal, being soaked up largely by cottonseed hulls (S.H. Jarrett, Midway College KY, personal communication). A sharp peak increase of 0.11  $\mu\text{mol/L}$  of total vitamin A measured colorimetrically at 4 hours in a 10 hour observational period. In contrast, we found smoother peaks with maximal increments were 0.07 to 0.35  $\mu\text{mol/L}$  of serum retinol (not including retinyl ester) measured by HPLC at 12 - 14 h after an oral dose of 123.5 mg of vitamin A palmitate (a dose selected to be given twice a week to pregnant mares in a subsequent experiment). The difference in the shapes of the curves may be explained partly by the assay, one for total vitamin A, the other for retinol only. The cubic curve with a 4-hour peak for plasma total vitamin A (Jarrett and Schurg, 1987) probably represented a combination of retinyl ester being absorbed following a meal plus the subsequent release of retinol from the liver, whereas the quadratic curve for serum retinol only (Figure 1) specifically represented the hepatic response. Subsequent research

using the HPLC to measure serum retinol only indicated a peak at 12 hours (S.H. Jarrett, Midway College KY, personal communication), which agrees with our result (Figure 1).

In this study, the  $RDR_{15}$ , using the serum retinol concentration from the second blood sample as the denominator, was more sensitive than the  $RDR_0$ , which used the initial sample as denominator. The former procedure was used in the original RDR test on rats (Loerch et al. 1979). No comparison was offered to warrant using the second rather than the first value as the denominator, which would be more usual in calculating an increment. A study on women compared use of the first or the second serum retinol concentration as the denominator in calculating RDR and concluded that the  $RDR_0$  was more sensitive (Vaisman et al. 1992). We have re-examined the original data on rats (Loerch et al. 1979) and found that the  $RDR_5$ , corresponding to our  $RDR_{15}$ , was more sensitive than the  $RDR_0$ , as judged by the  $P$  values for differences between means. This finding was confirmed in the present study, in which the  $P$  values were lower in 9 of 11 comparisons of  $RDR_{15}$  to  $RDR_0$ .

The information yield of the two RDR tests was similar but slightly clearer with the  $RDR_{15}$  than with  $RDR_0$ . Less information about vitamin A depletion was obtained from serum retinol concentration. In the mares in January, for example, serum retinol indicated depletion in all 3 diet groups compared to the previous July, but no differences among diets (Figure 2, upper). The RDR indicated depletion from summer to winter and, in addition, revealed differences between diet groups (Figure 2, middle). In the weanlings, serum retinol concentration indicated no difference between groups (Figure 3, upper) but the RDR tests demonstrated a difference (Figure 3, middle).

Our results confirm and extend previous findings relating to season (McDowell 1989, NRC 1989). Although beta-carotene was not measured in the grass-clover pasture, it was presumably about 400 mg/kg dry matter or more, providing approximately 50 times

the equivalent of the vitamin A requirement from May to July for pregnant or lactating mares (NRC 1989). During this period, improved vitamin A status was shown by the RDR in PH and PHC mares (Figures 2, middle and lower) but by serum retinol in only the PH group (Figure 2, upper). Depletion of vitamin A during the fall and winter has been shown previously by declines in plasma or serum retinol concentration (Fonnesbeck and Symons 1967, Garton et al. 1964, Mäenpää et al. 1988a, 1988b). It was demonstrated more clearly in the present study by increases in RDR in the mares (Figures 2, middle and lower).

In contrast, the RDR revealed greater depletion in the PH group than in the PHC group of weanlings (Figures 3, middle and lower). Since body weight gains were the same in both groups, consumption of concentrate presumably reduced consumption of pasture, so the vitamin A status should be lower in the PHC than in the PH group. The contrary finding may be attributable to respiratory infection, which has been found to impair vitamin A metabolism in other species (West et al. 1989).

Reduced vitamin A status may increase the risk of respiratory infection (NRC 1989) and vice versa (West et al. 1989). In our case, we suspected that selenium deficiency (Greiwe-Crandell et al. 1992) made the PH group more vulnerable than the PHC group to viral infection. The higher serum IgG levels in the PH group in November might reflect respiratory infection during October and November, and the subsequent increase of IgG in the PHC group observed in January, might reflect infection in December.

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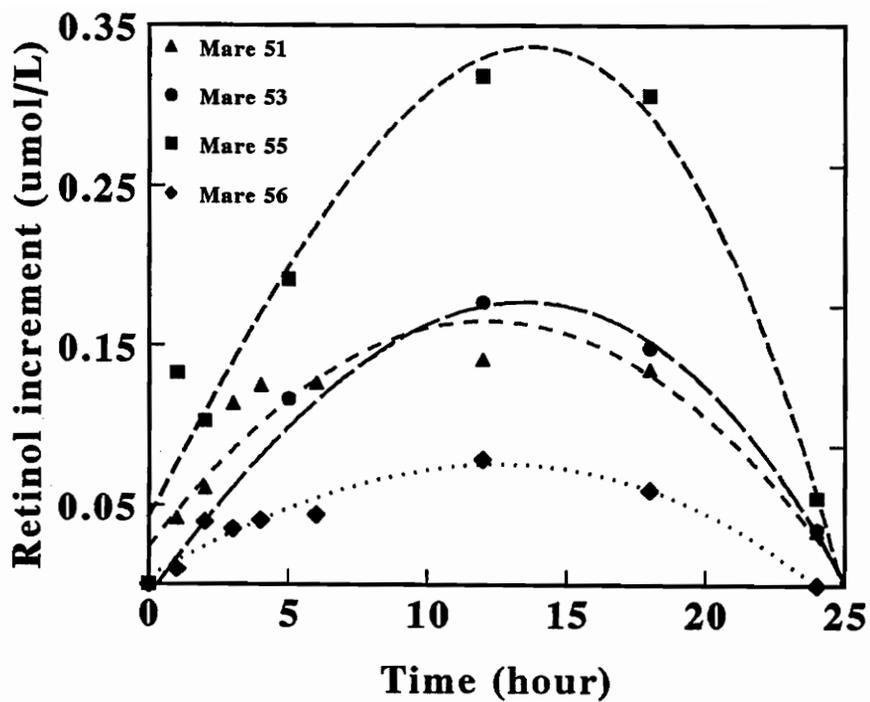
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**TABLE 1**  
*Composition of feed stuffs on a dry matter basis<sup>1</sup>*

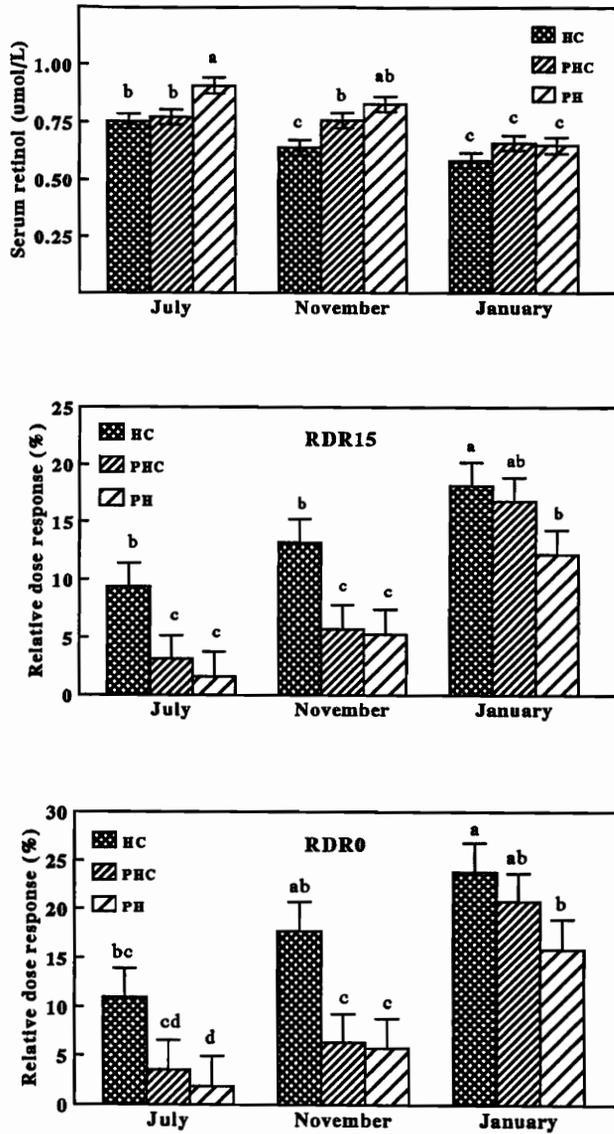
	<i>PASTURE</i>	<i>HAY</i>	<i>CONCENTRATE</i>
<b>Dry Matter (g/kg)</b>	352	911	901
<b>Crude Protein (g/kg)</b>	185	155	178
<b>ADF (g/kg)</b>	301	396	133
<b>TDN (g/kg)</b>	678	567	796
<b>Fat (g/kg)</b>	28	15	34
<b>Calcium (g/kg)</b>	4.5	5.7	7.6
<b>Phosphorus (g/kg)</b>	2.9	2.6	7.3
<b>Magnesium (g/kg)</b>	1.8	2.1	3.3
<b>Potassium (g/kg)</b>	12.4	13.2	7.4
<b>Zinc (mg/kg)</b>	24.6	23.3	186.2
<b>Copper (mg/kg)</b>	9.95	8.04	47.0
<b>Selenium (mg/kg)</b>	< 0.08	< 0.08	6.00
<b>β-Carotene (mg/kg)<sup>2</sup></b>	440	<9	0.02-0.44

<sup>1</sup>Values are means (pasture and hay, n = 20; concentrate, n = 4).

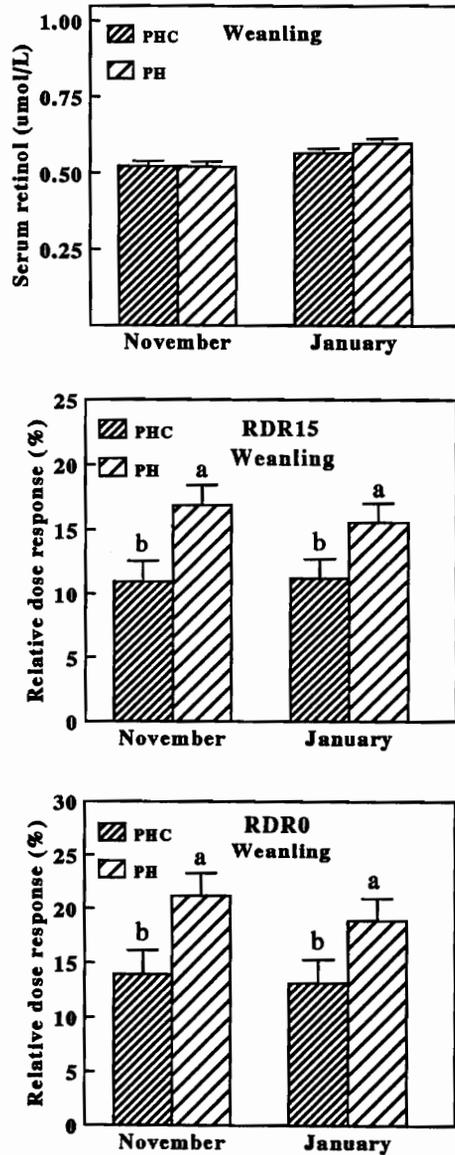
<sup>2</sup>Estimated from representative values (NRC 1989, McDowell 1989)



**Figure 1.** Increments in serum retinol concentration in 4 Thoroughbred mares during 24 hour period after an oral dose of retinol palmitate (123.5 mg/kg). Quadratic equations fit to the data (dashed and dotted lines) indicated peaks at 12 - 14 h.



**Figure 2.** Vitamin A status was evaluated in 3 groups of Thoroughbred mares fed hay and concentrate (HC, n = 15), pasture, hay and concentrate (PHC, n = 15) or pasture and hay (PH, n = 15) during July, November and January. Bars show means, flags show standard errors. Means with different letters are significantly different from one another ( $P < 0.05$ ). **Upper**, serum retinol concentrations were higher in the PH group than in the PHC and HC groups in July, lower in the HC group than in the PHC and PH groups in November, with no differences between groups in January. **Middle**, relative dose response (RDR) tests using serum retinol concentration at 15 hours as the denominator were higher in the HC group than in the PHC or PH groups in July and November, and higher in the HC group than in the PH group in January. **Lower**, the RDR test using serum retinol concentration just prior to the oral dose as the denominator were higher in the HC group than in the PHC or PH groups in July and November, and higher in the HC than in the PH group in January.



**Figure 3.** Vitamin A status was evaluated in 2 groups of Thoroughbred weanlings fed pasture, hay and concentrate (PHC, n = 11) or pasture and hay (PH, n = 12) during 2 sample times (November and January). Bars show means, flags show standard errors. Means with different letters are significantly different from one another ( $P < 0.05$ ).

**Upper,** serum retinol concentrations showed no differences between groups in November or January. **Middle,** relative dose response (RDR) tests with serum retinol concentration at 15 hours ( $RDR_{15}$ ) used as the denominator were higher in PH weanlings than in PHC weanlings in November and January. **Lower,** RDR tests with serum retinol concentration just prior to the oral dose ( $RDR_0$ ) used as the denominator gave a similar result.

(Footnotes)

<sup>1</sup>Part of this study was presented in a preliminary report (Greiwe-Crandell, K.M., Kronfeld, D.S., Morrow, G.A. and Teigs, W. (1993) Vitamin A depletion in Thoroughbreds: a comparison of pasture and non-pasture feeding regimes. Proc. of the 13th Equine Nutr. and Phys. Symp., Gainesville, FL pp. 1-2.).

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<sup>3</sup>The concentrate was donated by Purina Mills, StLouis, MO, courtesy of L. Breuer.

<sup>4</sup>The vitamin A palmitate was donated by Hoffman-LaRoche, Nutley, NJ, courtesy of T. Frye.

<sup>5</sup>The sick weanlings were attended by M.O. Furr, DVM, DACVIM, assistant professor of medicine in the Virginia-Maryland Regional College of Veterinary Medicine, Leesburg, VA.

<sup>6</sup>Abbreviations used in the text: ADF - acid detergent fiber; HC - diet group with unlimited access to hay and fed a specified amount of a vitamin A-free concentrate; PH - diet group with unlimited access to pasture and hay; PHC - diet group with unlimited access to pasture and hay and fed a specified amount of a vitamin A-free concentrate; RDR - relative dose response;  $RDR_0$  - relative dose response calculated using the 0h retinol concentration as denominator in equation,  $RDR_{15}$  - relative dose response calculated using the 15h retinol concentration as denominator in equation, TDN - total digestible nutrients.

## VITAMIN A AND $\beta$ -CAROTENE REPLETION IN MARES

### **Vitamin A Repletion in Thoroughbred Mares with Retinyl Palmitate or $\beta$ -Carotene<sup>1</sup>**

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## ABSTRACT

Forty-five Thoroughbred mares used on a 8 mo depletion study were kept for an additional 20 mo on the same 3 forage diets (15 mares each): hay and vitamin A-free concentrate (HC); pasture, hay and vitamin A free concentrate (PHC); or pasture and hay only (PH). Each diet group was divided into 3 supplement subgroups (5 mares each) and given either retinyl palmitate (A) at twice the recommended daily intake, the equivalent in the form of water dispersible  $\beta$ -carotene (B), or the vehicle as a placebo (C). Vitamin A status was monitored by both serum retinol (SR) and a relative dose response (RDR) test every 60 d. In the C subgroups, SR concentration was  $18.65 \pm .84 \mu\text{g/dL}$  (mean  $\pm$  SE) and the RDR was  $16.26 \pm 1.72 \%$  over the 20 mo. Both SR and RDR showed a seasonal fluctuation pattern regardless of supplementation. Vitamin A status was lower ( $P = .001$ ) in the non-pasture group (HC) than in the pasture groups (PH, PHC). Vitamin A status was improved by retinyl palmitate supplementation in all groups and by water dispersible  $\beta$ -carotene supplementation in the PH and PHC groups but not the HC group. Supplementation of non-pasture kept mares with vitamin A at 2 times the NRC recommended levels (HC-A) matched the SR but not the RDR of the pasture, control subgroups (PHC-C and PH-C).

**Key Words:** Vitamin A,  $\beta$ -carotene, serum retinol, relative dose response, pasture, horse

## Introduction

Grazing horses derive vitamin A from provitamin A carotenoids present in the forages. Carotene contents of green forages, calculated as vitamin A, are well in excess of the requirements of horses (NRC, 1989). Dramatic losses of carotene content occur in forage during the winter or dry season and when forages are conserved, such as hay (McDowell, 1989). During the winter, vitamin A depletion occurred in grazing horses (Greiwe-Crandell et al., 1995) and in stabled horses fed conserved feeds (Mäenpää et al., 1988a). Vitamin A depletion has adverse effects on growth and reproduction in the horse (Donoghue et al., 1981; McDowell et al., 1995), and may increase the risk of infectious disease (Bondi and Sklan, 1984).

Concentrates for horses usually include vitamin A and(or)  $\beta$ -carotene to compensate for degradation of endogenous  $\beta$ -carotene in the forage. In the present experiment, we have determined the efficacy of supplementation with two synthetic sources of vitamin A, retinyl palmitate and water dispersible  $\beta$ -carotene, on the vitamin A status of previously depleted mares in pasture and non-pasture (dry lot) situations over two breeding seasons.

## Materials and Methods

*Diets.* Forty-five Thoroughbred mares depleted of vitamin A (Greiwe-Crandell et al., 1995) were kept in the same groups for an additional 20 mo, January 1992 to September 1993. Mares were weighed monthly and kept on a regular deworming and vaccination schedule. One group of 15 mares was maintained on pasture and allowed free choice access to hay during the winter months (PH); a second group of 15 mares was on

pasture with hay and given 4 kg/d of a vitamin A free concentrate (PHC); and a third group of 15 mares was kept on a dry lot (dirt paddock) and fed 2-yr old hay (to minimize the amount of  $\beta$ -carotene) and 5.5 kg/d of the concentrate (HC). The concentrate was made specially without vitamin A or yellow corn but was otherwise like a commercial mix (PurePride 200, Purina Mills, St. Louis, MO). The ingredients were: processed grain by-products, grain products, roughage products, molasses products, plant protein products, calcium carbonate, salt, choline chloride, riboflavin supplement, niacin supplement, vitamin E supplement, calcium pantothenate, vitamin B-12 supplement, vitamin D-3, animal fat preserved with ethoxyquin, ferrous carbonate, manganous oxide, zinc oxide, copper sulfate, magnesium oxide, calcium iodate, cobalt carbonate, and sodium selenite. Nutrient analysis is in Table 1.

*Vitamin treatments.* Five of the mares in each diet group (A) received 2 times the NRC requirement for pregnant mares of retinyl palmitate, 40 mg/d (Hoffman-LaRoche, Nutley, NJ); another 5 (B) received the nominal equivalent in  $\beta$ -carotene, 2.16 g/d of water dispersible 10% beadlets (Hoffman-LaRoche, Nutley, NJ); and the remaining 5 (C) received the vehicle as a placebo (control). The vehicle was 2 ml of corn oil mixed into 7.5 g brewers yeast. The mares were supplemented individually with the vitamins or placebo two times/wk. The amount of  $\beta$ -carotene was determined using the equivalents for pregnant equine (McDowell, 1989): 1g of 10% beadlet = 33,300 IU Vitamin A.

*Forages.* Pastures were approximately 80% bluegrass with 15% white clover the first year, but the clover increased dramatically the second year to 45%. Beta-carotene content of the fresh forage during the growing season was estimated at 350 to 500 mg/kg, which, when calculated as vitamin A, is well above the requirement (NRC, 1989). Hay was fed free choice: mixed grass hay round bales to the PH and PHC groups, and 2-yr old grass hay square bales to the HC group. Pasture, hay and concentrate were sampled at

regular intervals and submitted to the college's Forage Testing Laboratory, which uses standard procedures (AOAC, 1984) for proximate and mineral analysis (Table 1). Additional trace element analysis, using inductively coupled plasma spectrophotometry was conducted by the college's Soil Testing Laboratory. The pastures and hays were consistently deficient in selenium and low in phosphorus, so the PH group was given a 50:50 mix of trace mineral salts (NaCl, 98%; Fe, .5%; Mn, .2%; Zn, .2%; Cu, .04%; Co, .0007%; I, .002%) and a premix made especially for this study (P, 16%; Ca, 12%; Se, .0045%). Consumption averaged 84 g/(horse·d). Mares in the two groups fed the concentrate (PHC and HC) were given free choice trace mineral salt without the additional premix as Ca, P and Se were supplied in the concentrate. Diets were evaluated to ensure adequate nutrients intakes other than vitamin A or  $\beta$ -carotene (Table 2).

*Vitamin A status.* Vitamin A status was assessed by serum retinol concentration (SR) and a relative dose response (RDR) test every 60 d. Jugular blood samples were collected into two 10 mL tubes (Vacutainer, Becton Dickinson, Rutheford, NJ) and placed immediately in a dark closed container to minimize exposure to light. After centrifugation, serum was harvested and stored at -20°C until thawed for assay. Serum was analyzed for retinol by HPLC (Greiwe-Crandell et al., 1995). For the RDR, two blood samples were taken; the first at 1600 to 1700 (SR<sub>0</sub>) and the second sample 15 h later at 0700 to 0800 the following morning (SR<sub>15</sub>). An oral dose of 123.5 mg retinyl palmitate in 5 mL corn oil was given immediately after the first blood sample (SR<sub>0</sub>) was taken. Relative dose response (RDR, %) was calculated by the equation (Greiwe-Crandell et al., 1995):

$$\text{RDR} = 100 ( \text{SR}_{15} - \text{SR}_0 ) / \text{SR}_{15}$$

where  $SR_0$  is serum retinol concentration ( $\mu\text{g}/\text{dL}$ ) at the time of the oral dose, and  $SR_{15}$  is the concentration 15 h later. As liver stores of vitamin A are depleted in the animal, the RDR will increase. The serum retinol (SR) values reported are  $SR_0$ .

*Statistical analysis.* The data were summarized as least square means and standard errors. Analysis of variance with repeated measures was applied to SR and RDR with horse (diet\*treatment) as the error term. Orthogonal contrasts compared the mean of group HC against groups PHC and PH, the PHC against PH, also subgroups C against A and B, then A against B (SAS System for Windows (version 6.11), SAS Inst. Inc., Cary, NC, 27516). Unexpectedly,  $\beta$ -carotene supplementation (B subgroups) failed to improve vitamin A status, and this obscured obvious improvements with retinyl palmitate supplementation (A subgroups), so contrasts of subgroups A against C were added *a posteriori*. In addition, the retinyl palmitate supplemented non-pasture subgroup, HC-A, was compared to the unsupplemented pasture subgroups, PHC-C and PH-C.

## Results

*Baseline values.* In the vitamin A depleted mares (January, 1992), SR was  $18.1 \pm .5 \mu\text{g}/\text{dL}$  ( $.632 \pm .018 \mu\text{mol}/\text{L}$ ) with no difference ( $P = 0.39$ ) between diet groups. The RDR was  $16.1 \pm 1.4\%$  with no difference between diet groups ( $P = .21$ ). Corresponding values before depletion were  $23.49 \pm .79 \mu\text{g}/\text{dL}$  ( $.820 \pm .03 \mu\text{mol}/\text{L}$ ) for SR and  $4.02 \pm 1.62\%$  for the RDR test.

*Dietary effects.* During the repletion period, mean SR was  $17.4 \mu\text{g}/\text{dL}$  in the HC group. It was increased about 25% ( $P = .001$ ) in the pasture groups, PHC and PH (Figure 1). The mean RDR was 24.6% in the HC group. It was decreased by over 60% ( $P < .001$ ) in groups PHC and PH (Figure 1).

*Supplementation.* During the repletion test, mean SR was 18.6  $\mu\text{g/dL}$  in the control subgroup, C. It was increased ( $P = .057$ ) by 10% in the B subgroup and by 14% in the A subgroup (Figure 2). The mean RDR was 16.3% in subgroup C. It was decreased by 42% ( $P = .013$ ) in subgroup A but by only 5% ( $P = .75$ ) in subgroup B.

The effect of supplementation was most evident in the non-pasture group HC (Figure 3). The mean SR was 15.3  $\mu\text{g/dL}$  in the HC-C subgroup, and it increased by 31% ( $P = .002$ ) in the HC-A subgroup but by only 9% ( $P = .34$ ) in the HC-B subgroup. The mean RDR was 27.4% in the HC-C subgroup. It was decreased by 46% ( $P < .001$ ) in the HC-A subgroup. In contrast, the RDR was increased by 16% ( $P = .10$ ) in the HC-B subgroup, compared to the control (HC-C).

Retinyl palmitate supplementation of non-pasture kept mares was compared with no supplementation of pasture kept mares (Figure 4). No differences in SR was found between subgroups HC-A, PHC-C and PH-C. Mean RDR was about 30% higher ( $P = .079$ ) in the HC-A subgroup than in the PHC-C and PH-C subgroups (Figure 4).

*Seasonal change.* Monthly SR showed a quadratic response ( $P < .001$ ) or seasonal fluctuation pattern regardless of diet or supplement (Figures 5a and 5c). In the pasture groups, PH and PHC, mean SR was highest in September 1992 and 34% lower ( $P < .001$ ) in January 1993. In the HC group, mean SR was highest in July 1992 and 30% lower ( $P = .012$ ) in December 1992.

Using the RDR test, a quadratic response ( $P < .001$ ) or seasonal fluctuation was also observed (Figures 5b and 5d). In the diet groups, mean RDR was lower in September than January for the PH group ( $P < .001$ ), the PHC group ( $P < .001$ ) and the HC group ( $P < .001$ ). It was consistently higher in the HC group than in the PH and PHC groups (Figure 5b), and reached a peak in the HC group in January 1993. In the supplement groups (Figure 5d), mean RDR was 36% lower ( $P < .001$ ) in subgroup A than

subgroups B or C in September 1992 and remained lower through the rest of the experimental period except in January, 1993. The HC-C subgroup showed steady depletion through January, 1993, then from March to September there was no change seen in RDR (Figure 7).

*Temporal change.* After 20 mo of supplementation, SR was increased in both pasture groups, PH and PHC, regardless of supplement subgroup, A, B and C (Figure 6a). In the non-pasture group, HC, an increase in SR was observed only in the A subgroup.

The RDR was decreased in only one pasture group, PHC, regardless of supplement subgroup (Figure 6b). Supplementation with  $\beta$ -carotene reduced RDR in the PHC group only. Supplementation with retinyl palmitate reduced RDR in all three groups. In the non-pasture group, HC, the RDR was greatly increased in the C subgroup.

## Discussion

*Repletion.* Vitamin A depletion was consistently more evident in non-pasture kept mares than in those with access to pasture. Vitamin A repletion occurred seasonally in pasture kept mares and concurrently in response to supplementation with retinyl palmitate. The supplementation of an external source of vitamin A at twice the current recommendation (NRC, 1989) in addition to the massive amounts of provitamin carotenes in the pasture during the spring and summer, did not result in excessive circulating or stored retinol. The Vitamin A status of horses receiving abundant carotenes in the pasture was considered optimal. The vitamin A status of retinyl palmitate supplemented non-pasture kept mares failed, however, to match that of non-supplemented pasture kept mares according to the RDR (Figure 4), thus remained suboptimal.

Vitamin A status was improved by  $\beta$ -carotene supplementation in pasture kept mares (Figure 6) but not non-pasture kept mares (Figure 3). The natural oil soluble form of  $\beta$ -carotene or other carotenes in pasture may have facilitated the absorption of the water dispersible form. According to the RDR,  $\beta$ -carotene supplementation appeared to have had a negative effect on liver stores of vitamin A in the HC group. Beta-carotene concentrations in serum were measured and found to be below detectable limits in almost all of the HC mares (data not shown).

Similar difficulties with supplementation of the same form of  $\beta$ -carotene in Thoroughbred and pony mares have been reported (Watson et al., 1995). Daily supplementation had no effect on plasma retinol or  $\beta$ -carotene concentrations and up to 36% of the  $\beta$ -carotene supplement was found in the feces. Little if any of the water dispersible, synthetic  $\beta$ -carotene appeared to be absorbed. Low efficiencies of absorption and(or) conversion may have developed as a protective adaptation to avoid hypercarotenosis or hypervitaminosis A when horses are consuming abundant quantities of carotenes in the forage (Diamond, 1991). The bioavailability of  $\beta$ -carotene may be over estimated by chemical analysis because of the presence or absence of other substances in the diet which may influence efficiency of absorption and metabolism; and there are significant differences in  $\beta$ -carotene utilization within a species (Ullrey, 1972). The efficiency of conversion of carotenes to vitamin A is relatively poor in horses (Fonnesbeck and Symons, 1967). Lower conversion efficiencies were observed with higher  $\beta$ -carotene intake (Wellenreiter et al., 1969). Most of the work on bioavailability of  $\beta$ -carotene in animals has been with natural rather than synthetic sources.

*Seasonal fluctuations.* Regardless of supplementation, there was a seasonal pattern in SR and RDR in pasture horses (Figure 5). Seasonal fluctuations have been demonstrated with low vitamin A status in the winter months and higher during the

summer in horses kept year round on pastures (Garton et al., 1964). In Finland where horses were kept on pasture during spring, summer and fall and in stalls during the winter, serum retinol levels in pregnant and lactating mares observed in June were much higher than those with supplementation of 22 mg/d vitamin A in winter (Mäenpää et al., 1988b). No differences in serum retinol were found between supplemented and unsupplemented mares during the winter.

In Virginia, vitamin A status was examined in grazing horses on pasture all year round (Greiwe-Crandell et al., 1995). Serum vitamin A was found to be 20% lower in the winter than in the summer. The lower serum retinol levels in the winter were most likely due to decreased intakes of  $\beta$ -carotene from declining content in pasture and hays. However, because a seasonal fluctuation was observed in the non-pasture group (fed 2-yr old hay year round), the decrease in vitamin A status may also have been influenced by the temperature. An influence of temperature in the availability of carotenes to the animal from pasture and hay has been found in cattle (Wing, 1969).

In the present study, vitamin A status fell in the pasture groups in July, 1992: serum retinol concentration decreased and RDR increased (Figure 5a and 5b). This change in vitamin A status was not observed in the HC group. Rainfall during July 1992 was abnormally high (15 cm) and the forage had an average moisture contents of 75% (data not shown) compared with the normal average of 65% (Table 1). The high water content of the forage may have reduced its intake and affected absorption of vitamin A. Digestibilities of carotenes in cattle were correlated with the dry matter content of the plant (Wing, 1969). Structural changes from the all-trans form of vitamin A to the cis forms are promoted by excessive moisture, resulting in a marked loss of vitamin A potency (McDowell, 1989). The influence of the other factors (carotene content, temperature, moisture content) in the horse had a greater impact on vitamin A status than

that of vitamin supplementation, as evident in the failure of retinyl palmitate supplementation to erase the seasonal fluctuations.

An adaptive response to very low levels of carotene intake was seen in the HC - C subgroup (Figure 7). In March, 1993, after 22 mo of no external source of vitamin A (8 mo on a depletion study (Greiwe-Crandell et al., 1995), plus 14 mo on the current study), the RDR appears to go into a steady state (no further change in status) until the end of the experiment in September. More efficient metabolism of vitamin A was found in rats with very low vitamin A status (Lewis et al., 1990). In a tracer study with rats, when vitamin A stores were depleted and intake was inadequate to maintain normal plasma retinol concentration, then degradative utilization decreased whereas functional utilization was maintained until plasma retinol fell below some critical level (Green and Green, 1994). Because no gross signs of vitamin A depletion were observed during our study, we believe that these animals never dropped below the critical level.

*The RDR advantage.* The RDR test has proven effective in pinpointing problems with vitamin A status that were not apparent in SR concentration (Loerch et al, 1979; Flores et al., 1984; Vaisman et al., 1992; Greiwe-Crandell et al., 1995). A test for vitamin A status that is more sensitive is important when investigating optimal nutrition rather than of minimal requirements (Underwood, 1990). The following are examples of where the RDR was found to be more sensitive than SR in this study. 1) The sensitivity of the RDR test was clearly illustrated in Figure 5, a versus b, where the differences in vitamin A status were more definitive between the non-pasture group (HC) and the pasture groups (PH and PHC). 2) No change in SR concentration of the HC-C subgroup was evident over the 20 mo period (Figure 6a). In contrast, the RDR indicated that there was a progressive deterioration in vitamin A status in the HC-C subgroup (20% less vitamin A stores) over the experimental period (Figure 6b). 3) A negative effect of  $\beta$ -carotene supplementation

in the HC group was revealed by the RDR but not the SR (Table 3). 4) The RDR indicated that the HC-A subgroup was lower in vitamin A status than even the control subgroups of the mares on pasture (Figure 4).

If the vitamin A status of horses consuming green pasture is considered optimal, then supplementation in this study of retinyl palmitate at 40 mg/d (72,000 IU/d) to mares not consuming green forage was barely sufficient to maintain optimum levels. Several criteria indicated that a daily intake of 1.5 to 5 times the recommendation levels (NRC, 1978) represented an optimal range for growing horses (Donoghue et al., 1981). These two studies suggest that current recommendations for vitamin A requirement may be lower than optimal for growth in young horses and adult mares with no access to pasture.

### **Implications**

Vitamin A status in depleted mares was improved by supplementation with retinyl palmitate. The water dispersible form of  $\beta$ -carotene only improved vitamin A status in mares on pasture. Retinyl palmitate supplementation at twice the currently recommended dietary level of vitamin A for pregnant mares failed to eliminate seasonal fluctuations in vitamin A status in the mares on pasture. In mares with no access to pasture, supplementation with twice the currently recommended daily intake of vitamin A barely matched the vitamin A status of unsupplemented mares on green pasture.

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Table 1. Composition of feedstuffs (dry matter basis)<sup>a</sup>

Ingredients	Bluegrass & clover pasture	Grass & alfalfa hay	2-yr old hay	Pelleted concentrate
DM, %	35.2	93.3	89.1	90.1
CP, %	18.5	13.4	9.5	17.8
ADF, %	30.1	36.7	43.0	13.3
TDN, %	67.8	58.5	53.7	79.6
DE, Mcal/kg	2.98	2.57	2.36	3.50
Fat, %	2.80	1.40	1.60	3.40
Calcium, %	.45	.84	.45	.76
Phosphorus, %	.29	.29	.22	.73
Magnesium, %	.18	.25	.24	.33
Potassium, %	1.24	1.47	1.09	.74
Zinc, mg/kg	24.6	36.7	16.7	186.2
Copper, mg/kg	9.95	8.67	7.33	47.0
Selenium, mg/kg	< .08	< .08	< .08	6.00
$\beta$ -carotene, mg/kg <sup>b</sup>	440	38.0	< 4	< 1

<sup>a</sup>Values are means (bluegrass & clover pasture, n = 18; grass & alfalfa hay, n = 7; 2 yr. old hay, n = 12; pelleted concentrate, n = 4).

<sup>b</sup>Estimated from representative values (NRC, 1989; McDowell, 1989).

Table 2. Estimated nutrient contents of diets (dry matter basis)<sup>a</sup>

Ingredient	PH <sup>b</sup>	PHC	HC	NRC <sup>c</sup>
DM, %	47.8	58.1	89.5	---
CP, %	16.5	16.7	13.0	10.6
DE, Mcal/kg	2.83	2.88	2.54	2.40
Calcium, %	.65	.67	.58	.45
Phosphorus, %	.36	.43	.44	.34
Magnesium, %	.21	.24	.28	.11
Potassium, %	1.34	1.17	1.02	.38
Zinc, mg/kg	37.2	80.2	90.1	40.0
Copper, mg/kg	10.4	21.5	24.5	10.0
Selenium, mg/kg	.08	.19	.28	.10
Vitamin A equivalents, mg/kg <sup>d</sup>	35.0	22.5	< .10	2.00

<sup>a</sup>Mean intakes of concentrate and hay were known, pasture intake was estimated by difference assuming that total intake was 2% of body weight (NRC, 1989).

<sup>b</sup>Diet groups are PH = pasture and hay, PHC = pasture, hay and concentrate, HC = hay and concentrate .

<sup>c</sup>Dietary recommendations for pregnant mares (NRC, 1989).

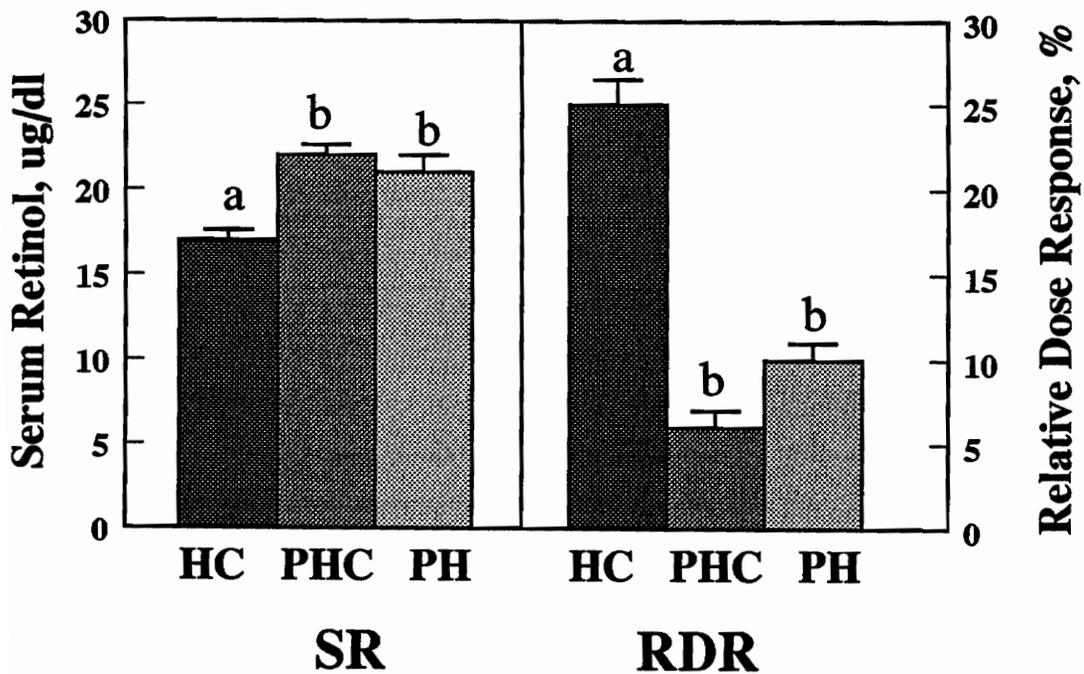
<sup>d</sup>Estimated from representative values (NRC, 1989; McDowell, 1989).

Table 3. Means and SE of serum retinol concentration ( $\mu\text{g/dL}$ ) and relative dose response (%) of the three diet groups receiving the three different vitamin supplements

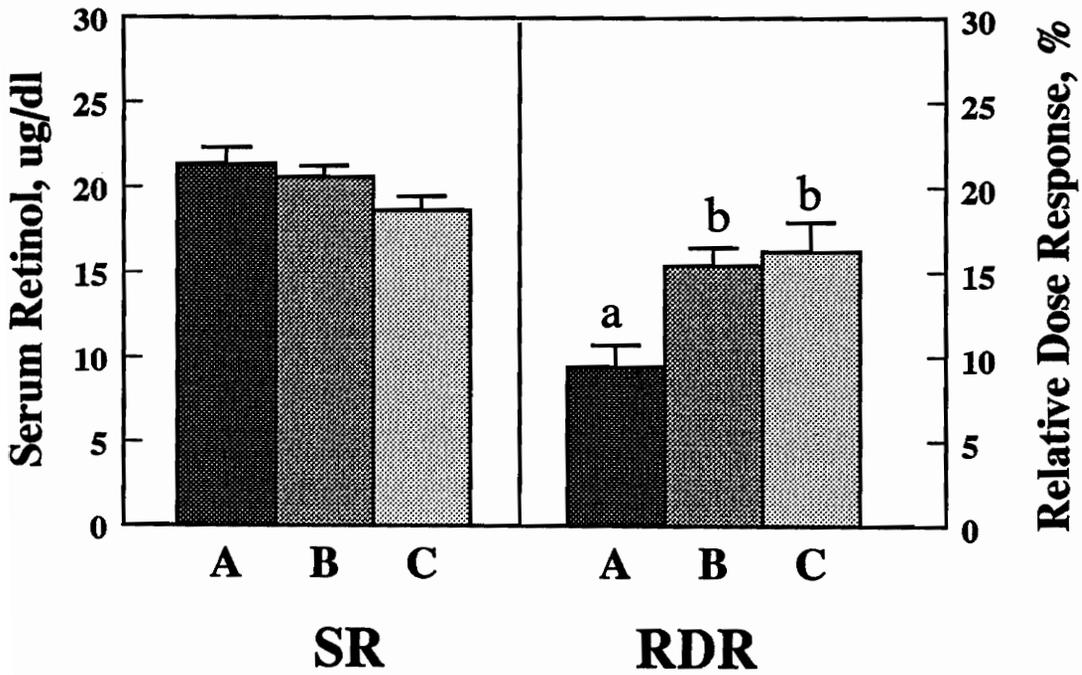
Supplement subgroups <sup>b</sup>	Diet groups <sup>a</sup>			All
	PH	PHC	HC	
<b>Serum retinol (SR)</b>				
A	21.87 $\pm$ 1.40	22.10 $\pm$ 1.04	20.09 $\pm$ .88	21.35 $\pm$ .97
B	21.44 $\pm$ 1.01	23.66 $\pm$ .75	16.66 $\pm$ .94	20.59 $\pm$ .63
C	19.93 $\pm$ 1.41	20.69 $\pm$ .75	15.33 $\pm$ 1.07	18.65 $\pm$ .84
All	21.08 $\pm$ 1.21	22.15 $\pm$ .81	17.36 $\pm$ .86	20.20 $\pm$ .62
<b>Relative Dose Response (RDR)</b>				
A	7.88 $\pm$ 1.40	5.70 $\pm$ 1.12	14.70 $\pm$ 1.90	9.43 $\pm$ 1.32
B	8.93 $\pm$ 1.40	5.65 $\pm$ 1.04	31.71 $\pm$ 2.65	15.43 $\pm$ 1.04
C	12.72 $\pm$ 1.73	8.68 $\pm$ 1.28	27.38 $\pm$ 3.41	16.26 $\pm$ 1.72
All	9.85 $\pm$ 1.18	6.68 $\pm$ .93	24.60 $\pm$ 2.02	13.71 $\pm$ 2.10

<sup>a</sup> Diet groups are PH = pasture and hay, PHC = pasture, hay and concentrate, HC = hay and concentrate .

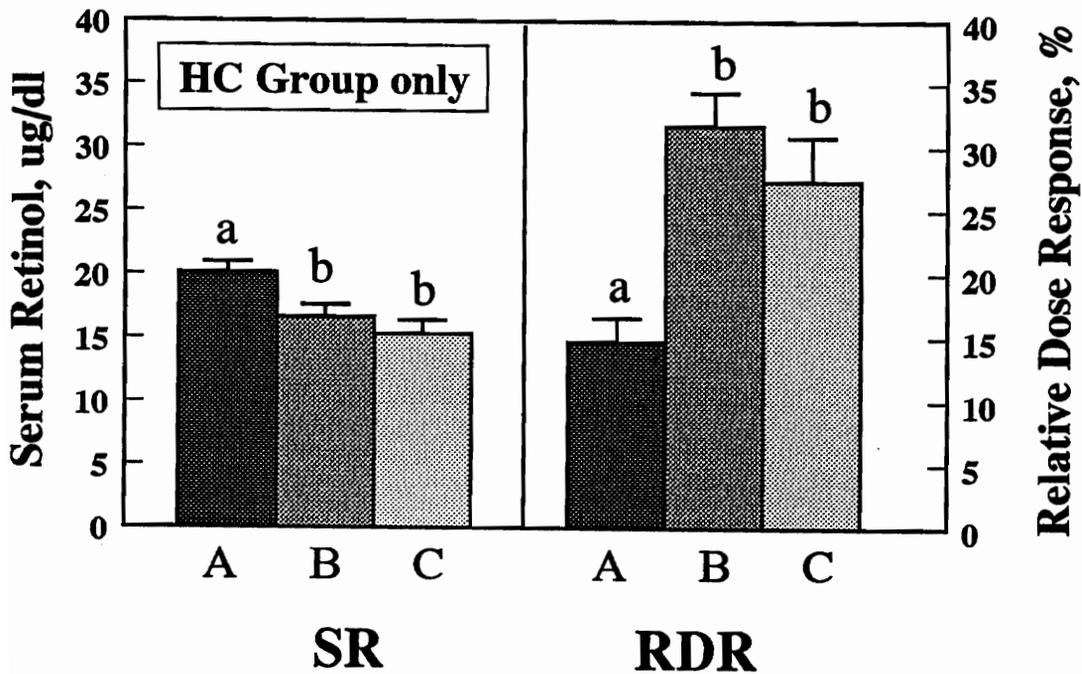
<sup>b</sup> Supplement subgroups are A = Retinyl palmitate, B =  $\beta$ -carotene, C = Control (placebo).



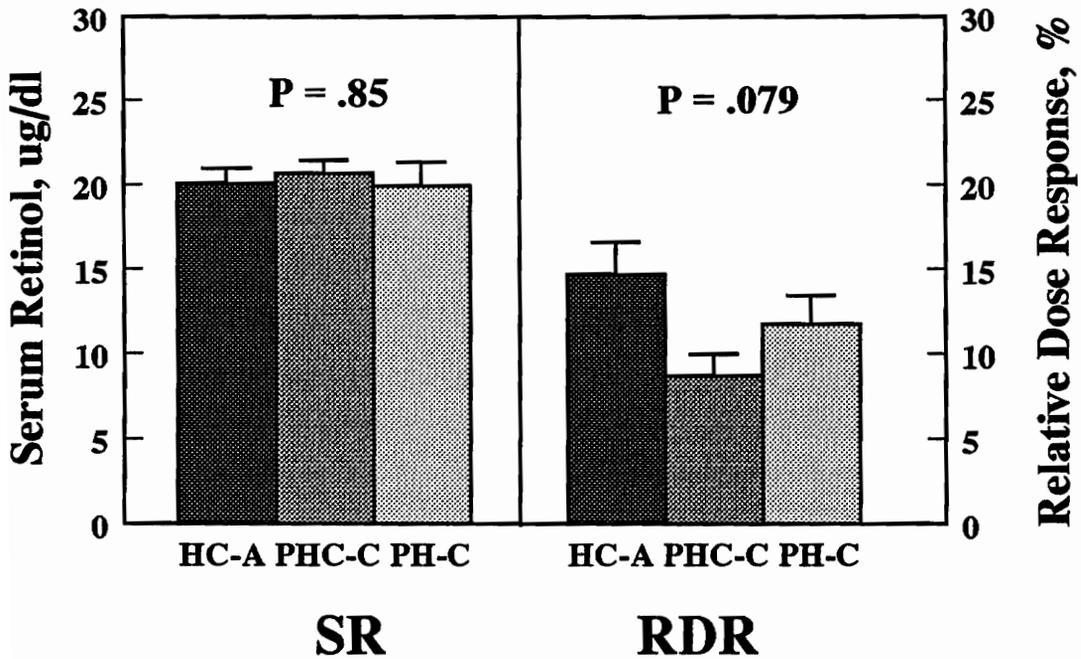
**Figure 1.** Diet affected vitamin A status. Serum retinol concentration (SR) was lower (a versus b,  $P = .001$ ) in the HC (pasture and hay) group than in the pasture groups, PHC (pasture, hay and concentrate) and PH (hay and concentrate). Also, the RDR (relative dose response) was higher (a versus b,  $P < .001$ ) in the HC group than in PHC and PH groups. Means are over 20 mo, bars are SE.



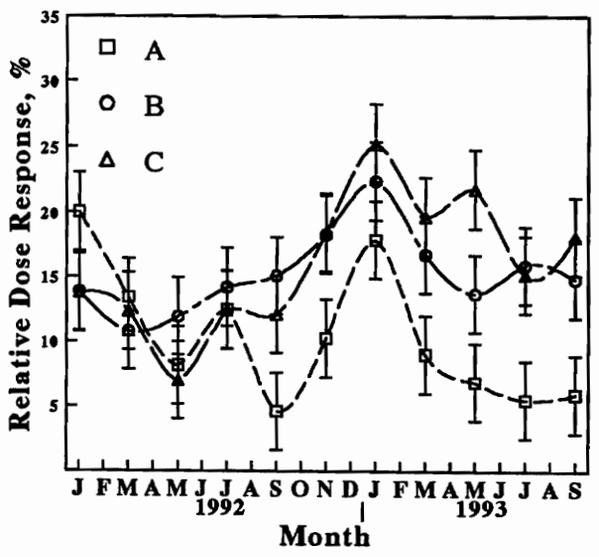
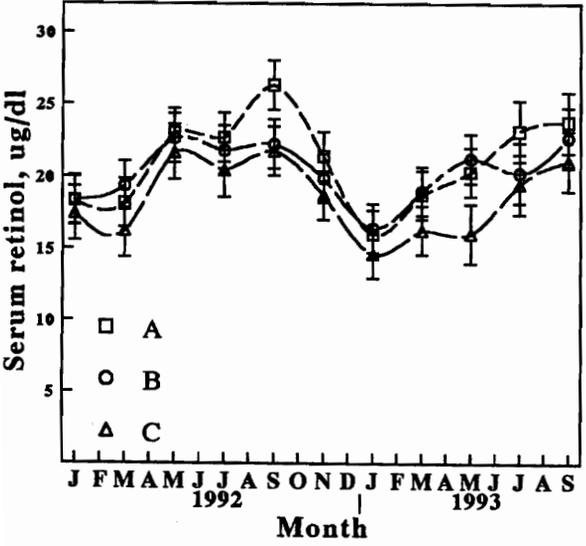
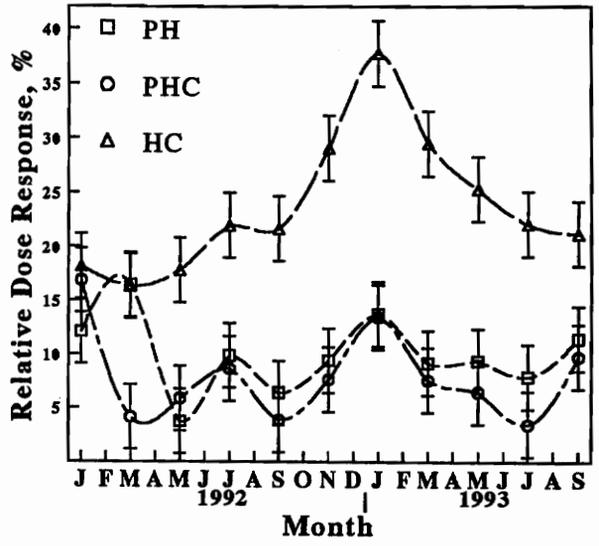
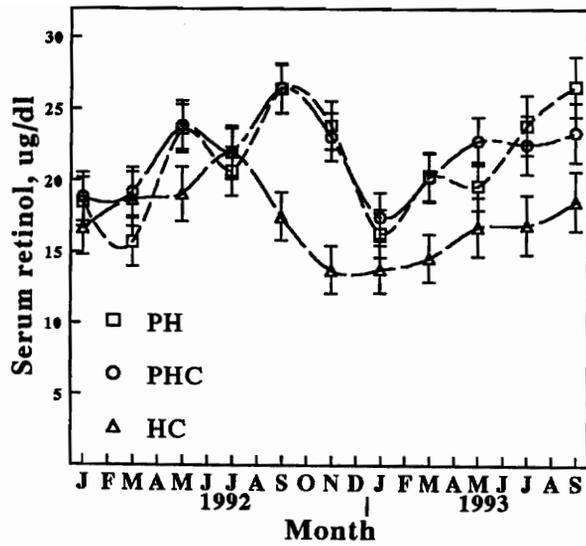
**Figure 2.** Supplementation affected vitamin A status. Serum retinol concentration (SR) tended to be lower ( $P = .057$ ) in the control (C) subgroup than in the subgroup supplemented with retinyl palmitate (A). The relative dose response (RDR) was lower (a versus b) in subgroup A than in subgroup B ( $\beta$ -carotene supplemented) ( $P = .027$ ) or subgroup C ( $P = .013$ ). Means are over 20 mo, bars are SE.



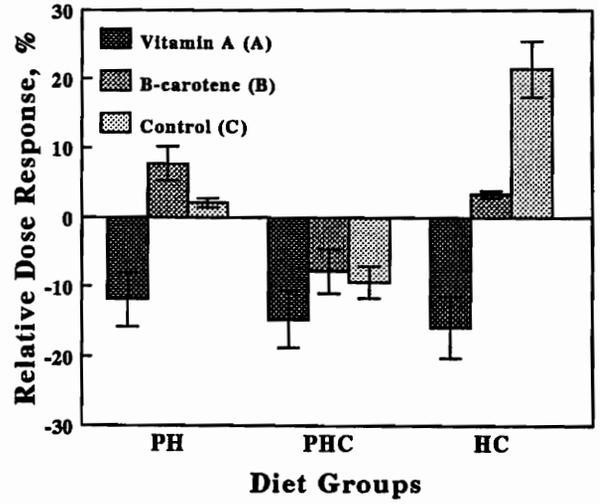
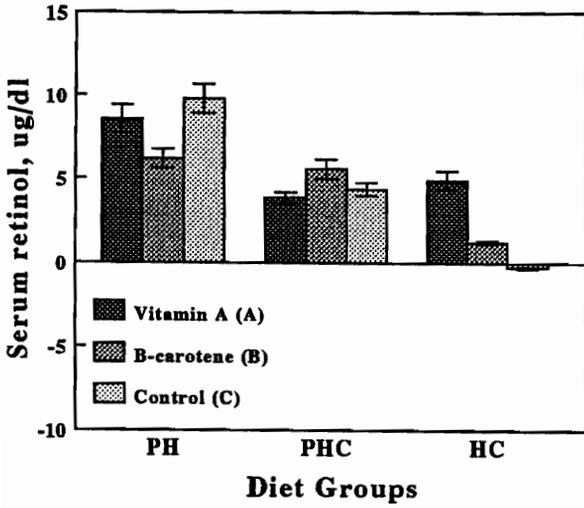
**Figure 3.** Supplementation with retinyl palmitate but not  $\beta$ -carotene improved vitamin A status in the non-pasture group, HC (hay and concentrate). Mean serum retinol concentration (SR) was higher (a versus b,  $P \leq .019$ ) in the subgroup supplemented with retinyl palmitate (A) than the  $\beta$ -carotene (B) or control (C) subgroups. The mean relative dose response (RDR) was lower (a versus b,  $P < .001$ ) in subgroup A than in subgroup B or C. Means are over 20 mo, bars are SE.



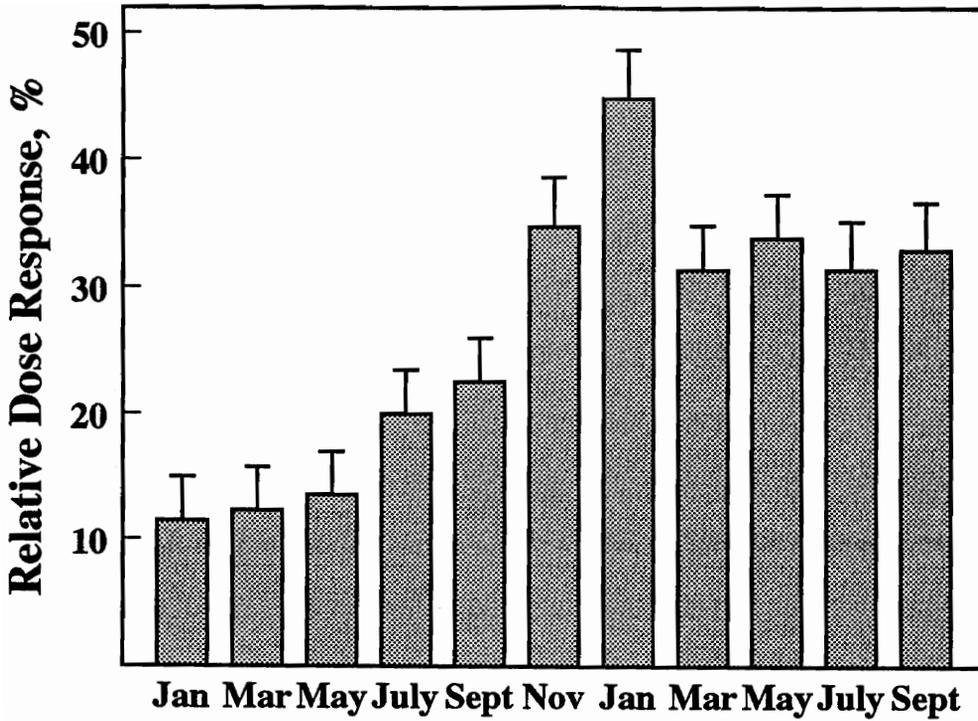
**Figure 4.** Supplementation of non-pasture kept mares with retinyl palmitate (subgroup HC-A) at twice the currently recommended level resulted in similar serum retinol (SR) concentrations but not relative dose responses (RDR) comparable to unsupplemented, pasture kept mares (subgroups PHC-C and PH-C). Means are over 20 mo, bars are SE.



**Figure 5.** Seasonal change in diet (upper, a and b) and supplement (lower, c and d) groups as measured by serum retinol (left, a and c) and RDR (right, b and d). Diet groups are: PH = pasture and hay; PHC = pasture, hay and concentrate; HC = hay and concentrate. Supplement groups are: A = retinyl palmitate; B = water dispersible  $\beta$ -carotene; and C = placebo (control). Means are 15 horses per group for each sampling date, bars are SE.



**Figure 6.** Change in serum retinol levels (a, left) and RDR (b, right) values calculated the difference between January 1992 and September 1993. Diet groups are: PH = pasture and hay; PHC = pasture, hay and concentrate; HC = hay and concentrate. Means of 5 horses/treatment, bars are SD.



**Figure 7.** Vitamin A status over 20 mo experiment as measured every two mo by the relative dose response (RDR) in non-pasture kept mares receiving no vitamin A supplementation (HC-C). Bars are means with SE, N = 5 horses.

## **Daily $\beta$ -Carotene Supplementation of Vitamin A Depleted Mares**

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## ABSTRACT

Supplementation twice a week with a water dispersible form of  $\beta$ -carotene failed to reverse the depletion of vitamin A status in a previous study of mares fed no other significant source of vitamin A. This experiment tested the effectiveness of daily supplementation. Six vitamin A depleted Thoroughbred mares were fed the water dispersible  $\beta$ -carotene in a daily dose equivalent to 4 times the current requirement for vitamin A for 14 days. Vitamin A status was assessed by serum retinol concentration and a relative dose response test before and after the 14 days. Serum was analyzed for serum retinol and  $\beta$ -carotene. Daily  $\beta$ -carotene supplementation failed to increase serum concentration of retinol and  $\beta$ -carotene or to significantly improve vitamin A status.

Key words:  $\beta$ -carotene, relative dose response, serum retinol, horses

## **Introduction**

Beta-carotene is a precursor of vitamin A that is found in abundance in green forages. Seasonal fluctuations in the vitamin A status of horses may reflect a decrease in  $\beta$ -carotene in pastures during the fall and winter (Greiwe-Crandell et al., 1995). The process of conserving forages destroys more than 80% of the  $\beta$ -carotene present, and biological oxidation continues at a rate of 6 to 7% per month during storage (McDowell, 1989). Horses fed mainly conserved forages, such as hay or silage, may become depleted in vitamin A status (Fonnesbeck and Symons, 1967; Greiwe-Crandell et al., 1995). Once depleted, if horses are allowed access to green grass, serum retinol concentration has been found to improve dramatically in 2 weeks (Mäenpää et al., 1988).

In a previous study (Greiwe-Crandell et al., submitted for publication), vitamin A depletion was found in mares kept in a drylot, fed 2 yr old hay and supplemented with a water dispersible form of  $\beta$ -carotene given individually twice a week for 2 years. The purpose of this study was to assess whether supplementation daily with this form of  $\beta$ -carotene would be effective in improving vitamin A status.

## **Materials and Methods**

Six pregnant Thoroughbred mares were used. They had been housed on a drylot (no access to pasture) for over 2 years and were vitamin A depleted. Four of the mares had been on  $\beta$ -carotene supplement for 20 months and 2 of the mares had received a placebo during the same period. They weighed  $563.6 \pm 49$  kg (mean  $\pm$  SD).

The mares were fed 2 year old grass hay free choice and 5.5 kg/d of a commercial concentrate made without vitamin A (Purina Pure Pride 200, Purina Mills, St. Louis, MO). The mares were supplemented individually and once daily with 4.3g of 10% water dispersible  $\beta$ -carotene beadlet (Rovimix, Hoffman LaRoche, Nutley, NJ), which was nominally equivalent to 4 times (144,000 IU/d) the daily requirement (36,000 IU/d) for vitamin A (NRC, 1989) using the conversion factor of 1 mg  $\beta$ -carotene for 333 IU of vitamin A activity (McDowell, 1989). The  $\beta$ -carotene beadlet was mixed into 4.3g of brewers yeast and 2 ml corn oil to facilitate administration.

Vitamin A status was assessed by serum retinol concentration and by a retinol dose response (RDR) test just before and after 2 weeks of supplementation. A 20 ml sample of jugular blood was drawn into 10 ml tubes (Vacutainer, Becton Dickinson, Rutheford, NJ) and immediately followed by an oral dose of 250,000 IU retinyl palmitate in 5 cc corn oil squirted into the back of the mouth to avoid any loss. A second jugular blood sample was taken 15 hours later. Serum was analyzed for  $\beta$ -carotene and retinol by HPLC (Greiwe-Crandell et al, 1995). The retinol dose response (RDR, %) was calculated by the equation:

$$\text{RDR} = 100 (\text{SR}_{15} - \text{SR}_0) / \text{SR}_{15}$$

Where  $\text{SR}_0$  is serum retinol concentration before administration of the vitamin A palmitate and  $\text{SR}_{15}$  is serum retinol 15 hours later. The data on serum retinol and RDR were summarized as means and standard errors. Differences between means before and after supplementation were compared by the paired  $t$ -test. Significance was inferred when  $P < 0.05$ .

## Results and Discussion

Serum concentration of  $\beta$ -carotene was less than 5  $\mu\text{g}/\text{dl}$  in all horses. The usual range was 5 to 160  $\mu\text{g}/\text{dl}$  (mean, 36  $\mu\text{g}/\text{dl}$ ) in mares grazing our bluegrass/clover pastures (data not shown). These results were consistent with little if any  $\beta$ -carotene being absorbed in the supplemented mares, confirming the observation of Watson et al., (1996).

Initial serum retinol concentrations and RDR tests were consistent with depleted vitamin A status (Greiwe-Crandell et al., 1995). After 2 weeks of daily supplementation with  $\beta$ -carotene, mean serum retinol concentration increased by 14% ( $P = .332$ ) and the RDR test decreased by 25% ( $P = .182$ ), indicating a slight but not significant improvement in vitamin A status (Figure 1). The slight increase in vitamin A status was probably mainly due to the carryover effect of the large dose of retinyl palmitate given at the beginning of the two week study. This effect has confounded other studies on repeatability of the RDR in humans (Solomons et al., 1990).

A previous study showed that serum retinol concentration increased substantially (nearly 50%) when vitamin A depleted mares were placed on green pasture for 2 weeks (Mäenpää et al., 1988). Thus, the period of 2 weeks should have been sufficient to reveal an improvement of vitamin A status in our mares if supplementation with this form of  $\beta$ -carotene had been effective. The RDR test reflects the availability of retinol binding protein in the liver, hence vitamin A reserves (Loerch et al., 1979). We conclude that little vitamin A was available to the mares from this water dispersible form of  $\beta$ -carotene.

A similar conclusion was reached by Watson et al. (1996), who reported no increase in plasma  $\beta$ -carotene or retinol in pony mares from supplementation of the same form of water dispersible  $\beta$ -carotene beadlet for 8 weeks. Up to a third of the total intake of the  $\beta$ -carotene could be accounted for in the feces (the horses were also noted to have

had orange feces), indicating that much of the  $\beta$ -carotene was passing through the alimentary tract without being absorbed. Plant  $\beta$ -carotene is associated with a lipid carrier whereas the synthetic  $\beta$ -carotene is hydrophilic and this may account for the differential uptake. Provision of a water dispersible form of  $\beta$ -carotene may circumvent the transport mechanisms involved in absorption.

Another factor interfering with absorption may have been interaction of  $\beta$ -carotene with fiber in the diet. It has been shown that  $\beta$ -carotene utilization in horses is affected by the type of forage fed (Fonnesbeck and Symons, 1967). Therefore, the nature of the basal diet fed the horses may have reduced the availability of vitamin A from water dispersible  $\beta$ -carotene.

The biopotency of carotenes may be over estimated by chemical analysis because of the presence or absence of other substances in the diet which may influence efficiency with which  $\beta$ -carotene is absorbed and metabolized; and the significant differences in vitamin utilization between and within differing species (Ullrey, 1972). The bioavailability of corn carotene in pigs, for example, was found to be half the chemically determined concentration (Wellenreiter et al., 1969).

A single large daily dose of  $\beta$ -carotene may also not be the ideal method of administration. Beta-carotene has a relatively slow rate of cleavage to vitamin A in the intestine (Olson, 1989). The comparative efficiency of absorption of  $\beta$ -carotene for horses is roughly 20 to 33% as efficient as the rat (the standard) (McDowell, 1989), and lower efficiencies have been observed with higher  $\beta$ -carotene intakes (Wellenreiter et al., 1969). A poor efficiency of utilization of  $\beta$ -carotene may have confounded our previous study in which a high dose was given twice a week (Greiwe-Crandell et al., submitted for publication).

Supplementation with  $\beta$ -carotene remains an appealing alternative to use of vitamin A esters for several reasons:  $\beta$ -carotene is non-toxic, in contrast to vitamin A (Bendich, 1988); absorbed  $\beta$ -carotene may have additional, independent benefits on reproductive functions (Ferraro and Cote, 1984; van der Holst et al., 1984; Arbeiter and Lorin, 1986; Schubert and Hennig, 1986) and as an antioxidant (Di Mascio et al., 1991; Krinsky, 1991; Canfield et al., 1992). Further work is needed to establish the bioavailability of different synthetic forms of  $\beta$ -carotene.

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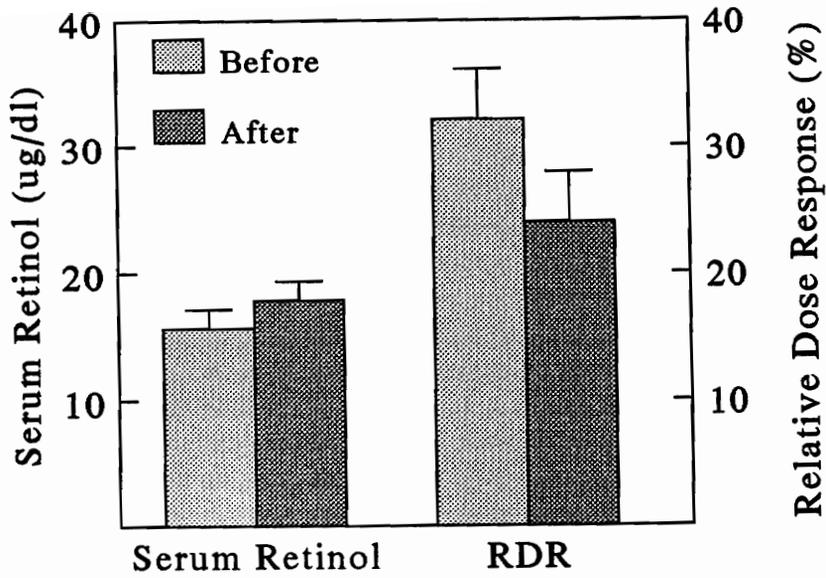


Figure 1. Serum retinol (left) and RDR (right) before and after 2 weeks of daily supplementation of  $\beta$ -carotene. Bars are means and standard errors. Changes are not significant in serum retinol ( $P = 0.332$ ) or the RDR ( $P = 0.182$ ).

# **Reproductive Efficiency of Thoroughbred Mares on Different Forage Regimens with Supplementation of Retinyl Palmitate and $\beta$ -Carotene**

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## ABSTRACT

Forty-five Thoroughbred mares were depleted of vitamin A in an 8 month study then repleted in a 20 month study. They were maintained on one of 3 forage diets (15 mares each group): hay and vitamin A-free concentrate (HC); pasture, hay and vitamin A free concentrate (PHC); or pasture and hay only (PH). During the repletion phase, each diet group was divided into 3 supplement subgroups (5 mares each) and given either retinyl palmitate (A) at twice the currently recommended intake, the nominal equivalent in water dispersible  $\beta$ -carotene (B) beadlet, or the vehicle as a placebo (C). Foals were weighed at birth and monthly thereafter. Reproductive rates were calculated for the depletion and repletion phases. Pregnancy rate (PR), foaling rate (FR) and pregnancy loss (PL) were not affected during the depletion of vitamin A. Increased frequencies of retained placenta and contracted tendons were observed in vitamin A depleted mares and foals, respectively. Foals born to mares in the HC group had lower birth weights and slower growth rates than foals born to mares in the PH and PHC groups. During the repletion phase, the HC group had higher pregnancy loss than either pasture group (PH and PHC). The supplementation of  $\beta$ -carotene had a negative effect on FR and PL as compared to the retinyl palmitate supplementation. Mares and foals appeared to benefit from access to pasture or from supplementation with vitamin A but not from supplementation with water dispersible  $\beta$ -carotene.

Key words: mare, vitamin A,  $\beta$ -carotene, reproductive rates, birth weights, pasture

## **Introduction**

Reproductive efficiency in the mare is the poorest among domestic livestock species with the mean US foaling rate at 65% (Evans et al., 1990). Horses have been selected for speed, athletic ability or conformation rather than for reproductive efficiency. Reproduction is known to be influenced by nutrition in the horse (Henneke et al., 1981; Potter et al., 1985). Vitamin A is essential for the maintenance of reproductive function and fetal development (Chew, 1993). A clinical trial suggested vitamin A supplementation improved fertility in barren mares (Stowe, 1967). Higher pregnancy rates and lower embryonic mortality in the mare have been observed with the supplementation of  $\beta$ -carotene (Ferraro and Cote, 1984; Schubert and Hennig, 1986; van der Holst, 1984; Arbeiter and Lorin, 1986; Enbergs and Klemm, 1987). Other studies have not confirmed any reproductive benefits of  $\beta$ -carotene supplementation (Eitzer and Rapp, 1985; Watson et al., 1996). Observations on the reproductive efficiency of mares, on foal weights, and on general health during a study of vitamin A depletion and repletion are reported in this paper.

## **Materials and Methods**

Forty-five Thoroughbred mares were used in a 8 month vitamin A depletion study (Greiwe-Crandell et al., 1995) and an additional 20 month repletion study (Greiwe-Crandell et al., 1996). The mares were matched by age, parity, and reproductive history and allotted evenly to diet/supplement subgroups. One group of 15 mares was kept on bluegrass/clover pasture and given mixed grass hay free choice during the winter months (PH); a second group of 15 was kept on similar pasture, given similar hay and fed 4 kg/d

of a vitamin A free concentrate (PHC); and a third group of 15 was kept on a drylot (non-pasture) and fed 2 year old hay (to minimize  $\beta$ -carotene intake) and 5.5 kg/d of the vitamin A free concentrate (HC). The concentrate was like a commercial mix but made for this experiment without vitamin A (PurePride 200, Purina Mills, St. Louis, MO). After the 8 month depletion phase, each diet group was subdivided into 3 supplementation subgroups for the repletion phase. Five of the mares in each group received 2 times the requirement for 600 kg pregnant mares (NRC, 1989) of retinyl palmitate, 40 mg/d (72,000 IU/d) (Hoffman-LaRoche, Nutley, NJ); another 5 received the equivalent in  $\beta$ -carotene, 2.16 g/d water-dispersible 10% beadlets (Hoffman-LaRoche, Nutley, NJ); and the last 5 received the vehicle as a placebo (control subgroup). The amount of  $\beta$ -carotene given was determined using the equivalents for pregnancy given in McDowell (1989): 1 g of 10% beadlet = 33,300 IU Vitamin A. The vitamin supplements were given in 2 ml of corn oil mixed into 7.5 g of brewers yeast and fed individually 2 times per week.

Diets are summarized in Table 1. Pastures were mainly bluegrass and white clover. Hay fed to PHC and PH mares in addition to pasture during the winter was mixed grass, hay fed to the HC mares all year round was 2-yr old grass hay and practically devoid of  $\beta$ -carotene. Pasture, hay and concentrate were sampled at regular intervals and submitted to the college's Forage Testing Laboratory, which uses standard procedures (AOAC, 1984) for proximate and mineral analysis. Further information of the diets can be found in Greiwe-Crandell (1996).

Mares were weighed monthly, foals were weighed at birth and then monthly until sold as yearlings. They were examined carefully for general health at these times. Mares and foals were maintained on a regular deworming and vaccination schedule.

During the experimental period, five stallions were used for breeding purposes. The mares were field bred. They were exposed to the stallions starting April 1 and ending

on July 15. If it was necessary to use a stallion for more than one group at a time, the stallion was rotated every other day between groups. No significant differences in pregnancy rates of mares were found between the different stallions. All mares were pregnancy checked at 60 days. The foals were born in the field and kept with the dam until 6 months of age. Pregnancy rate (PR, %) and foaling rate (FR, %) for the diet and supplement groups were calculated by the following equations (Ginther, 1979):

$$PR = \frac{\text{mares pregnant at 60 days}}{\text{mares bred}} \times 100$$

$$FR = \frac{\text{mares foaling}}{\text{mares bred}} \times 100$$

Pregnancy loss (PL, %), which included loss due to twinning, was calculated by:

$$PL = \frac{\text{mares pregnant} - \text{mares foaling}}{\text{mares pregnant}} \times 100$$

The data was evaluated by Fishers Exact Probability Test using a statistical software program (Statistix 4.1, 1994). One-tailed tests were used for the effects of vitamin A depletion on reproduction; two-tailed tests for the effects of supplementation. Body weights were compared by the unpaired  $t$ -test.

## Results and Discussion

### Reproductive Efficiency

*Reproductive rates.* The totals for all mares over the two breeding seasons was 80% PR and 67% FR . Means of 75% PR and 66% FR were reported for 5 Thoroughbred farms in Virginia (Hutton and Meacham, 1968). Therefore, our rates were typical.

Details of rates are summarized in Tables 2 and 3. Overall PR was 86% in 1991 and 74% in 1992 ( $P = .093$ ). The decline in PR from 1991 to 1992 may have been associated with deterioration of vitamin A status (Greiwe-Crandell et al., 1995). The PR was about 50% higher in the retinyl palmitate supplemented subgroup than in the  $\beta$ -carotene supplemented subgroup, with values for the control subgroup in between (Table 3). Overall PR was 82% in 1993 (data not shown) after an additional 12 months of supplementation of retinyl palmitate in 15 mares and  $\beta$ -carotene in another 15.

Overall FR was 73% in 1992 and 62% in 1993 ( $P = .18$ ). The FR was 100% higher in the retinyl palmitate supplemented subgroup than in the  $\beta$ -carotene supplemented subgroup with values for the control subgroup in between (Table 3). The PL was approximately 16% in 1991-2 and 1992-3. Comparing years suggests that vitamin A depletion had a slight effect on PR but not FR or PL (Tables 2 and 3).

In 1992-3 (Table 3), the PL was higher in the HC group than in the 2 pasture groups combined ( $P = .095$ ). The HC group had a lower vitamin A status than the PH or PHC group, which had the benefit of natural carotenes from the grass (Greiwe-Crandell , 1996).

During repletion, the PL was higher in the  $\beta$ -carotene supplemented subgroup (Table 3) than in the control subgroup ( $P = .054$ ) or the retinyl palmitate supplemented

subgroup ( $P = .044$ ). Thus the water-dispersible form of  $\beta$ -carotene, in contrast to natural provitamins in pasture, had a negative effect on PL.

Adverse effects of  $\beta$ -carotene supplementation on the fertility (conception rates, days open, number of inseminations per conception, and pregnancy rates) were found in dairy cows, in comparison with retinyl acetate supplementation (Folman et al., 1987). No effect on fertility in mares (length of estrus cycle, pregnancy rate, and early embryonic death) following  $\beta$ -carotene supplementation was observed in other studies (Eitzer and Rapp, 1985; Enbergs and Klemm, 1987; Watson et al., 1996). In contrast,  $\beta$ -carotene supplementation improved pregnancy rates (Ferraro and Cote, 1984; van der Holst, 1984), even using the water dispersible form (Schubert and Hennig, 1986; Arbeiter and Lorin, 1986). Beta-carotene supplementation also was found to increase the intensity of heat shown by mares, to decrease early embryonic loss (Ferraro and Cote, 1984; van der Holst, 1984) and to improve induction of estrus (Ahlswede and Konemann, 1980). The findings of other studies on  $\beta$ -carotene supplementation in mares are summarized in Table 4 and compared with the results of the current study.

*Foal weights.* Body weights at birth, weaning and 14 months are summarized (Table 5). Foals born to the HC group were lighter than the PHC ( $P = 0.021$ ) and PH group foals ( $P = 0.0004$ ). The HC group foals were still lighter than the PHC group ( $P = 0.077$ ) and the PH group ( $P = 0.057$ ) foals at weaning. They remained lighter even at 14 months (PHC,  $P = 0.0098$ ; PH,  $P = 0.063$ ) of age when they were sold (Table 5). The HC group foals after weaning were fed alfalfa/grass hay and a concentrate with vitamin A. In April, 1993, they were taken off of the drylot and turned out on pasture with the other two groups of yearlings. Thus, vitamin A depletion was not a limiting factor in their growth rates after weaning, but detrimental effects of prior depletion may have persisted. Recent findings in humans suggest that fetuses adapted to a limited supply of nutrients

may permanently adapt their physiology and metabolism (Barker, 1996). Low birth weight infants may reach the same adult height a normal infant despite a slower growth rate.

*Retained placenta.* Other possible effects of the vitamin A depletion observed during the 1992 foaling season (March - July) even though supplementation began in February, were retained placenta and contracted tendons. The equine placenta is expelled 30 minutes to 3 hours after parturition; if still present after 3 hours it is considered a retained placenta (Vandeplassche, 1971). In our study, there was a high percentage of retained placentas: 20% (6 of the 30 mares), compared to 3% (1 of 30 mares) the previous year ( $P = .051$ ). The percentage of broodmares that retain their placentas is believed to range from 2 to 10% (Vandeplassche, 1971). Vitamin A affects all epithelial tissues; a deficiency can alter the epithelial lining of the reproductive tract and may result in retained placenta (McDowell, 1989).

*Contracted tendons.* Also observed in our study was a high percentage of congenital flexure limb deformities (contracted tendons); 30% (9 of 30), in newborns from mares depleted of vitamin A for two trimesters, compared to 7% (2 of 30) in newborns of undepleted mares the previous year ( $P = .021$ ). Disturbed fetal growth during the embryonic state of pregnancy has been implicated in congenital contracted tendons (Hintz, 1996). Poor growth, knuckling of the fetlock, enlarged joints and weak and crooked legs have been documented in vitamin A deficient foals (Naviaux, 1985). Although these abnormalities gradually abated without intervention in a few weeks, and all foals appeared normal by 12 months of age, the effects on performance later in life are not known as these individuals were sold as yearlings.

## **Implications**

Vitamin A depletion may impair reproductive efficiency in mares and increase the risk of retained placenta. It also may reduce birth weights of foals and increase risk of contracted tendons. These manifestations of vitamin A depletion may be improved by access to green pasture and retinyl palmitate supplementation but not water dispersible  $\beta$ -carotene.

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Table 1. Estimated nutrient contents of diets (dry matter basis)<sup>a</sup>

Ingredients	PH <sup>b</sup>	PHC	HC	NRC <sup>c</sup>
DM %	47.8	58.1	89.5	---
CP %	16.5	16.7	13.0	10.6
DE Mcal/kg	2.83	2.88	2.54	2.40
Calcium %	0.65	0.67	0.58	0.45
Phosphorus %	0.36	0.43	0.44	0.34
Magnesium %	0.21	0.24	0.28	0.11
Potassium %	1.34	1.17	1.02	0.38
Zinc mg/kg	37.2	80.2	90.1	40.0
Copper mg/kg	10.4	21.5	24.5	10.0
Selenium mg/kg	0.08	0.19	0.28	0.10
Vitamin A equivalents IU/kg <sup>d</sup>	64,000	41,000	< 10	3650

<sup>a</sup>Mean intakes of concentrate and hay were known, pasture intake was estimated by difference assuming that total intake was 2% of body weight (NRC, 1989).

<sup>b</sup>Diet groups are PH = pasture and hay, PHC = pasture, hay and concentrate, HC = hay and concentrate.

<sup>c</sup>Dietary recommendations for late pregnancy mares (NRC, 1989).

<sup>d</sup>Estimated from representative values (NRC, 1989; McDowell, 1989)

Table 2. Reproductive rates for the depletion phase (1991-1992)

Diet groups	Pregnancy rate % 1991	Foaling rate % 1992	Pregnancy loss % 1991-1992
HCa	80.0	66.7	16.6
PHC	86.7	73.3	15.5
PH	92.9	78.6	15.4
Total	86.4	72.7	15.7

<sup>a</sup> Diet groups are HC = hay and concentrate, PHC = pasture, hay and concentrate, PH = pasture and hay.

Table 3. Reproductive rates for the repletion phase (1992-1993)

Diet groups & Supplement subgroups	Pregnancy rate % 1992	Foaling rate % 1993	Pregnancy loss % 1992-1993
HC <sup>a</sup>	71.4	50.0	30.0 <sup>g</sup>
PHC	73.3	66.7	9.1 <sup>h</sup>
PH	76.9	69.2	10.0 <sup>h</sup>
A <sup>b</sup>	86.7 <sup>c</sup>	80.0 <sup>e</sup>	7.7 <sup>i</sup>
B	57.1 <sup>d</sup>	35.7 <sup>f</sup>	37.5 <sup>j</sup>
C	76.9	69.2	10.0 <sup>k</sup>
Total	73.8	61.9	16.1

<sup>a</sup> Diet groups are HC = hay and concentrate, PHC = pasture, hay and concentrate, PH = pasture and hay.

<sup>b</sup> Supplement subgroups are A = retinyl palmitate, B =  $\beta$ -carotene, C = placebo.

<sup>c,d</sup>  $P = 0.108$ ; <sup>e,f</sup>  $P = 0.025$ , <sup>g,h</sup>  $P = 0.095$ ; <sup>i,j</sup>  $P = 0.044$ ; <sup>j,k</sup>  $P = 0.054$ .

Table 4: Summary of published information on the effects of  $\beta$ -carotene supplementation in mares. Adapted from Watson et al., 1996.

$\beta$ -carotene (mg/day)	Measurements	Effect	Authors
500	Induction of estrus	+	Ahlsvede and Konermann (1980)
	Plasma $\beta$ -carotene	+	
100	Pregnancy rate	+	Ferraro and Cote (1984)
230 - 700	Pregnancy rate	+	Schubert and Hennig (1986)*
	Pregnancy rate	+	
	Plasma $\beta$ -carotene	+	
400	Intensity of estrus	+	van der Holst (1984)
	Pregnancy rate	+	
	Early embryonic death	+	
	Plasma $\beta$ -carotene	+	
400	Pregnancy rate	0	Eitzer and Rapp (1985)
	Early embryonic death	0	
	Plasma $\beta$ -carotene	+	
500	Pregnancy rate	+	Arbeiter and Lorin (1986)*
	Plasma $\beta$ -carotene	+	
1000	Pregnancy rate	0	Enbergs and Klemt (1987)
	Plasma $\beta$ -carotene	+	
810	Cyclical ovarian activity	0	Watson et al. (1996)*
	Plasma $\beta$ -carotene	0	
734	Plasma $\beta$ -carotene	0	
3670	Plasma $\beta$ -carotene	0	
2160	Pregnancy rate	0	This study*
	Foaling rate	0	
	Pregnancy loss	-	
	Plasma $\beta$ -carotene	0	

+ = positive effect; 0 = no effect; - = negative effect.

Note: Synthetic  $\beta$ -carotene was used by all authors although the source was not always acknowledged. In those cases where the source was given (\*) it was Rovimix.

Table 5. Mean weights (kg  $\pm$  SD) of foals born in 1992 at birth, weaning and 14 months

Diet groups <sup>a</sup>	Birth	Weaning	14 months
HC	44.9 $\pm$ 6.6 <sup>b</sup>	213.4 $\pm$ 43.6 <sup>e</sup>	356.2 $\pm$ 40.7 <sup>h</sup>
PHC	52.6 $\pm$ 6.7 <sup>c</sup>	247.4 $\pm$ 33.9 <sup>f</sup>	406.6 $\pm$ 33.8 <sup>i</sup>
PH	56.5 $\pm$ 5.0 <sup>d</sup>	249.3 $\pm$ 33.3 <sup>g</sup>	388.8 $\pm$ 30.7 <sup>j</sup>

<sup>a</sup> Diet groups are PH = pasture and hay, PHC = pasture, hay and concentrate, HC = hay and concentrate. <sup>b,c</sup>  $P = 0.021$ ; <sup>b,d</sup>  $P = 0.0004$ ; <sup>e,f</sup>  $P = 0.077$ ; <sup>e,g</sup>  $P = 0.057$ ; <sup>h,i</sup>  $P = 0.0098$ ; <sup>h,j</sup>  $P = 0.063$ .

## **Vitamin A Status of Neonatal Foals Assessed by Serum Retinol Concentration and a Relative Dose Response Test**

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## SUMMARY

The objective of the study was to evaluate the vitamin A status of the newborn foal. Serum retinol and the relative dose response (RDR) test between 12 and 24 hours postpartum were used to assess vitamin A status in 28 Thoroughbred foals born to mares in 3 different feed groups being supplemented with vitamin A palmitate,  $\beta$ -carotene or a placebo. A correlation was found between foal and mare serum. Serum retinol levels were lower and RDRs were higher in the newborns than in the mares. No differences were found between foals born of mares with different vitamin A supplementation.

**Key Words:** vitamin A, equine neonate, serum retinol, relative dose response

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### **Der Vitamin-A-Status neugeborener Fohlen, überprüft durch Bestimmung der Serum-Retinol-Konzentrationen und einen Dosis-Response-Test**

In der vorliegenden Untersuchung sollte der Vitamin A Status neugeborener Fohlen überprüft werden. Dazu wurden der Serum-Retinol-Spiegel und der relative Dosis-Response-Test 12 und 24 Stunden post partum bei 28 Vollblutfohlen, deren Mütter in drei unterschiedlichen Fütterungsgruppen mit Vitamin A Palmitat,  $\beta$ -Carotin oder einem Placebo supplementiert wurden, verwendet. Die Serumwerte von Fohlen und Mütter korrelierten miteinander. Die Serum-Retinol-Spiegel waren bei den Fohlen niedriger, die Reaktion auf die Retinolgabe stärker als bei den Stuten. Zwischen Fohlen, deren Mütter unterschiedliche Vitamin A Gaben erhalten hatten, bestand kein Unterschied.

**Schlüsselwörter:** VitaminA, neugeborenes Fohlen, Serum-Retinol, relativer Dosis-Response-Test

## INTRODUCTION

Serum retinol concentration is not usually regarded as a sensitive indicator of vitamin A status. The blood concentration of retinol is regulated mainly by the rate of its secretion from the liver in the form of retinol bound to retinol binding protein. Hepatic stores are usually capable of maintaining a constant level of serum retinol despite variations in dietary supply (Blomhoff et al. 1991). The relative dose response (RDR) test, based on the response of serum retinol to an oral dose of retinol palmitate, was developed in rats as a more sensitive test of vitamin A depletion (Loerch et al. 1979). An RDR test has been used to demonstrate depleted vitamin A status in mature horses kept in stalls (Jarrett and Schurg 1987). Seasonal depletion of vitamin A has been demonstrated using the RDR test in grazing horses (Greiwe-Crandell et al. 1995a,b).

Evaluation of the vitamin A status in the newborn foal using the RDR test has not been investigated to our knowledge. Newborn and older foal vitamin A serum values have been found to be lower than values of their dams (Stowe 1982; Mäenpää et al. 1988), and liver concentration of vitamin A in neonatal foals was similar to that found previously in deficient adult horses (Irwin et al. 1991).

The objective of this study was to compare vitamin A status in mares and newborn foals using the RDR test.

## MATERIALS AND METHODS

*Horses.* Forty-five Thoroughbred mares were divided into 3 groups and kept on different forage systems for 30 months. The first forage system was a dry lot where mares were fed 2 year old grass hay and a concentrate made from ingredients low in  $\beta$ -carotene

and without added vitamin A (HC). The second and third systems were bluegrass/clover pastures supplemented with alfalfa/grass hay during the winter (PH) where one of the groups was also fed the vitamin A free concentrate (PHC). After 9 months of vitamin A depletion, each group of 15 mares was divided into 3 subgroups: 5 mares were supplemented with vitamin A palmitate at twice the NRC requirement (NRC 1989) (A); 5 mares were given  $\beta$ -carotene at the equivalent of twice the NRC requirement of vitamin A (B); and 5 mares were given a placebo (C). Supplements were given 2 times per week orally and individually. Every 2 months vitamin A status was assessed by RDR test as well as serum retinol concentration. During March - July (1991) all of the mares were exposed to a stallion (pasture bred), and of the 45 mares 39 were found pregnant (by ultrasound) at 30 days, 34 foaled (1992) to produce 30 live foals.

*Sampling.* Twenty eight of these foals were used for the study. Jugular blood samples were taken between 12 and 24 hours postpartum after the foal was walking and suckling. Samples were collected into 10 mL tubes (Vacutainer, Becton Dickinson, Rutheford NJ) and placed immediately in a light excluding container. They were centrifuged within 30 min., and the serum stored at  $-20^{\circ}\text{C}$  until thawed for assay.

All samples were prepared in duplicate. Protein in serum was precipitated with ethanol and retinol was extracted with hexane after addition of retinol acetate as an internal standard (Bieri et al. 1979, Miller and Yang 1985), then samples were injected into a HPLC (Waters  $\mu$ Bondapak Reverse Phase C18 Column [3.9x300 mm] with Waters 484 Tunable Absorbance Detector set at 325 nm and Waters 600E Controller, Millipore, Milford, MA). Solvent was 98% methanol and 2% water, with a flow rate of 1 mL/min for 7 min with retention time for retinol and retinyl acetate eluding at 4-5 and 5-6 min respectively.

The RDR test consisted of taking a blood sample and dosing orally with 50,000 IU vitamin A palmitate in corn oil, then taking the second sample 5 hours later. The RDR was calculated by the following equation:

$$\text{RDR} = 100(T_5 - T_0)/T_5\%$$

where  $T_0$  and  $T_5$  are the first and second serum retinol concentrations, respectively. The higher the percent RDR, the more depleted the animal (Loerch et al. 1979).

*Statistics.* The RDR and serum retinol data were summarized as least-squares means and standard errors and examined by ANOVA using the GLM procedures of SAS (SAS/STAT Version 6, SAS Institute, Cary, NC). Linear regressions of foal data on corresponding mare data were analyzed (SlideWrite Plus, Version 3.00, Advanced Graphics Software, Carlsbad, CA).

## RESULTS AND DISCUSSION

Vitamin A status as assessed by serum retinol and the RDR was lower in the foals than in the mares (Figure 1). Mean foal serum levels were 26% lower than in the mare ( $P = 0.0001$ ). Mean foal RDR percentages were 59% higher than in the mares ( $P = 0.0001$ ). As the homeostatic mechanism of vitamin A metabolism maintains a constant level in the blood, one cannot ascertain whether the lower serum levels alone in the foal are a physiologic norm or a depleted level. The fact that the RDRs (which are an indirect measurement of hepatic stores) are higher in the foals suggests that the lower serum retinol levels are in fact a reflection of a relative depletion in the foals at birth. Irwin et al (1991) analyzed liver samples of newborn foals before suckling and found the hepatic

vitamin A levels much lower than the levels of mature horses. This low liver vitamin A content is consistent with the high RDR in newborn foals (Figure 1), both indicating low hepatic reserves.

The results of this study suggest that the vitamin A status of newborn foals was low. Similar results have been found with serum vitamin A activity in newborn foals (Stowe 1982) and with RDR tests in newborn lambs (Kronfeld et al. 1990). Vitamin A status improved rapidly with consumption of colostrum in both foals and lambs. Mares were found to increase the amount of vitamin A in the colostrum at least 2-fold during the first 24 hours after birth (Stowe, 1982). Improvement of immune competence by colostrum consumption involves the transfer of immunoglobulins and, in the light of these studies, perhaps also the transfer of vitamin A. Public health studies have revealed the powerful influence of vitamin A status on susceptibility to infectious disease (West et al. 1989).

The effect of diet of the dam on the newborn was not very clear. The PH group of newborns appeared to be influenced the most by vitamin A status of the mare. The mean serum retinol for the PH group was higher than in the HC ( $P = 0.034$ ) and the PHC ( $P = 0.086$ ) groups of foals (Figure 2, upper). While the mean foal RDR for the HC group was higher than PHC or PH groups, it was not significant (Figure 2, lower). Dietary effect on vitamin A status is more clearly seen in the mares. Serum retinol in the mares was higher in the PHC ( $P = 0.006$ ) and PH ( $P = 0.006$ ) groups than the HC group (Figure 2, upper). The same pattern was also seen with the RDR test, the HC group of mares was higher than PH ( $P = 0.033$ ) and PHC ( $P = 0.1$ ) groups (Figure 2, lower). Both PHC and PH groups had access to abundant carotene in the green grass of the pasture during this time of year which may account for the replete vitamin A status.

Supplementation with either vitamin A palmitate or  $\beta$ -carotene in the dam had a moderate influence on newborn foal serum levels (Figure 3, upper) or RDR tests (Figure 3, lower). Although mean serum retinol concentration was slightly higher in the B group of mares than in the A group, the RDR test revealed that the B group was more depleted than the A group ( $P = 0.099$ ). Supplementation of this form of water soluble  $\beta$ -carotene was found to be an ineffective source of vitamin A in the mares as reported in Greiwe-Crandell et al (1995b).

Correlations were found between foal and mare serum retinol levels ( $R_{val} = 0.363$ ,  $P = 0.058$ ) and RDRs ( $R_{val} = 0.379$ ,  $P = 0.047$ ). The linear regression estimated foal values from the mares with the following prediction equations for serum retinol and for RDR:

$$SR_f = 9.21 + 0.29sr_m \quad (1)$$

where  $Sr_f$  = serum retinol of foal ( $\mu\text{g/dl}$ ),  $Sr_m$  = serum retinol of mare ( $\mu\text{g/dl}$ ), and

$$RDR_f = 18.25 + 0.47RDR_m \quad (2)$$

where  $RDR_f$  = relative dose response of foal (%), and  $RDR_m$  = relative dose response of mare (%).

In conclusion, the RDR test has elucidated the trend seen previously in the serum retinol levels and has demonstrated a low vitamin A status in the newborn foal.

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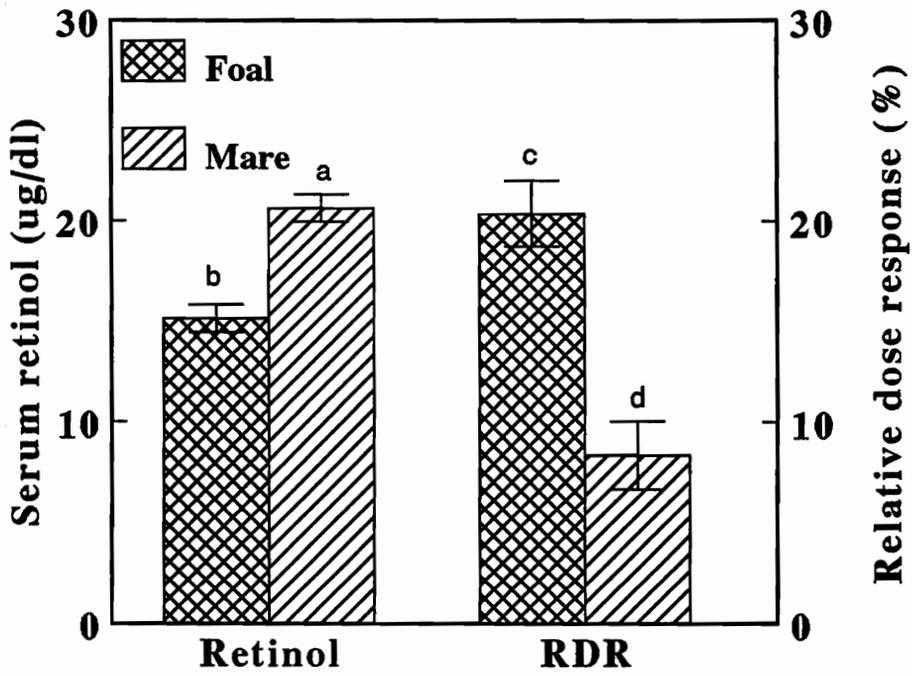


Figure 1. Mean serum retinol and relative dose response (RDR) for newborn foals and mares. <sup>ab</sup> $P = 0.0001$  and <sup>cd</sup> $P = 0.0001$ .

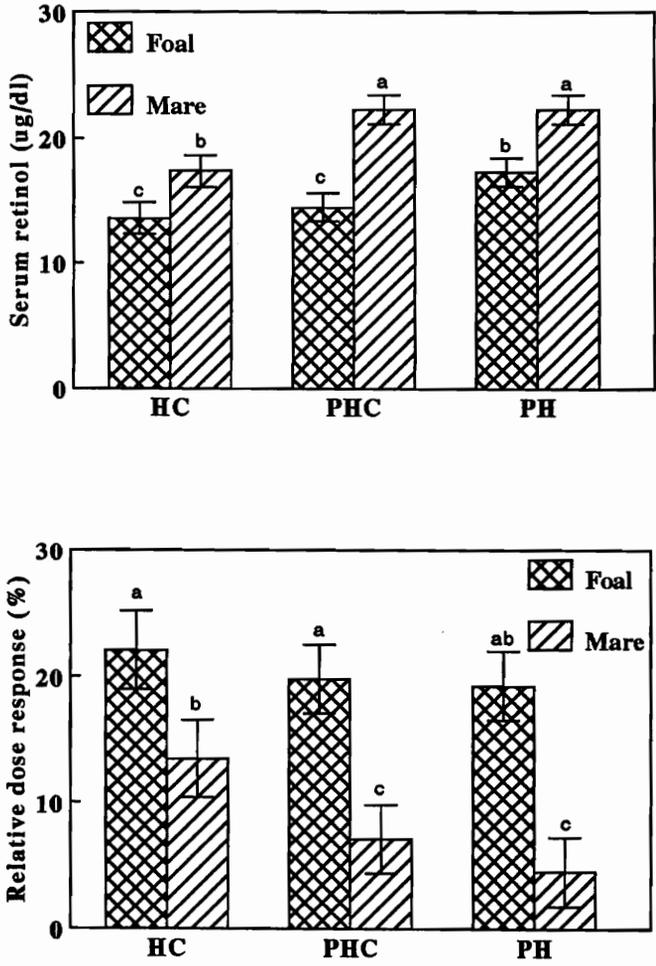
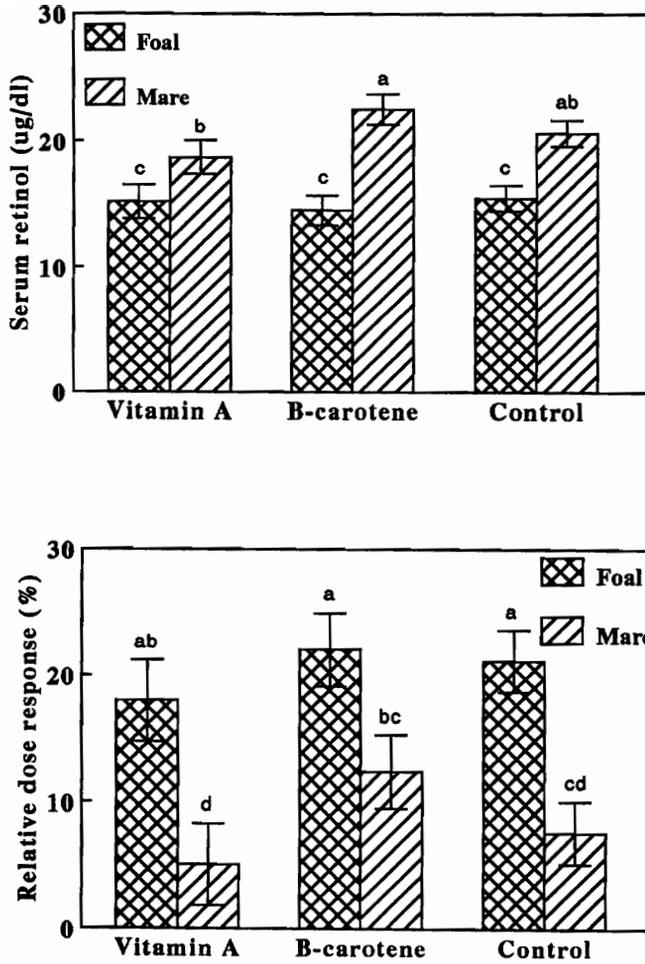


Figure 2. Mean serum retinol (upper) and relative dose response (RDR) (lower) in newborn foals and mares for the 3 diet groups: hay & concentrate (HC), pasture, hay & concentrate (PHC), pasture & hay (PH). Upper:  $^{ab}P \leq 0.0057$ ,  $^{ac}P < 0.0001$ ,  $^{bc}P < 0.0856$ . Lower:  $^{ab}P \leq 0.12$ ,  $^{ac}P \leq 0.0028$ ,  $^{bc}P \leq 0.1$ .



*Figure 3.* Mean serum retinol (upper) and relative dose response (RDR) (lower) in newborn foals and mares for the 3 supplement groups: vitamin A,  $\beta$ -carotene, control (placebo). Upper:  $^{ab}P \leq 0.0009$ , Lower:  $^{ab}P \leq 0.025$ ,  $^{ac}P \leq 0.0003$ ,  $^{ad}P \leq 0.0003$ ,  $^{bc}P \leq 0.0143$ ,  $^{bd}P \leq 0.0071$ ,  $^{cd}P \leq 0.0986$ .

## DISCUSSION

### **The Utility of the Relative Dose Response (RDR) Test**

#### *The adaptation of the RDR to horses.*

An important aspect of this research has been the adaptation of the RDR test to the horse. The RDR is potentially a useful tool to assess vitamin A status of a horse or group of horses, especially in the field. It has been found sensitive enough to detect minor changes in vitamin A status that were not manifested in serum concentrations (Papers 1 and 2). During the process of adapting the methods to the horse some interesting questions about vitamin A metabolism in the horse arose (Papers 1 and 5).

*Serum Retinol Concentration Peak.* In order for the RDR to be a simple and practical method of vitamin A assessment, there need to be only 2 blood samples taken, one before the dose of vitamin A and another when retinol concentration peaks in the blood. The timing of when serum retinol concentration peaks is different in the horse than in other species. The full extent to which the timing was investigated here is not presented in paper 1 (J. Nutr. article), only the results of the third experiment.

Our first study was with 2 horses not fasted before starting the experiment. After catheterizing, we gave a large oral dose of retinyl palmitate (123.5 mg) and took samples hourly for 12 hours and then one at 24 hours. Upon analysis of the samples, serum retinol appeared to be still on the rise at 12 hours but back to baseline at 24 hours. A second study with 4 horses (fasted for 12 hours) involved blood samples every three hours for 24 hours. Peak serum retinol was found between 12 and 18 hours. A third study was done to investigate the effects of fasting versus feeding before the initial blood sample of the RDR. Again using 4 horses, two were fasted 12 hours before the dose and then fed, and the

other two were not fasted. Samples were taken every hour until six hours and then every three hours until 24 hours. Here we found the peak to be at 12.5 hours. The results of this final study are reported in Paper 2 (J. Nutr. article).

If we had not repeated the experiment 3 times with similar results, we would have been skeptical of our results in view of the results of Jarrett (1987). The differences between her study and ours are discussed in Paper 1. Jarrett found a sharp peak in total plasma vitamin A at 4 hours. In analyzing for total plasma vitamin A, the vitamin A esters as well as retinol are measured. The esters appearing in the plasma at that time are those recently absorbed and not yet taken up by the liver. The basis of the RDR is to measure retinol (attached to RBP) after release from the liver. By sampling at 4 hours postdosing and analyzing for total vitamin A, the actual dose response of retinol from the liver was not measured.

The curves observed in each of our studies were gradual without a sharp peak. It was therefore concluded that the second blood sample could be taken anywhere between 12 and 16 hours to evaluate the response. Because the experiment was a field trial and there were limitations of daylight, it was decided to take the second blood sample at 15 hours for the RDR in mares and weanling/yearlings.

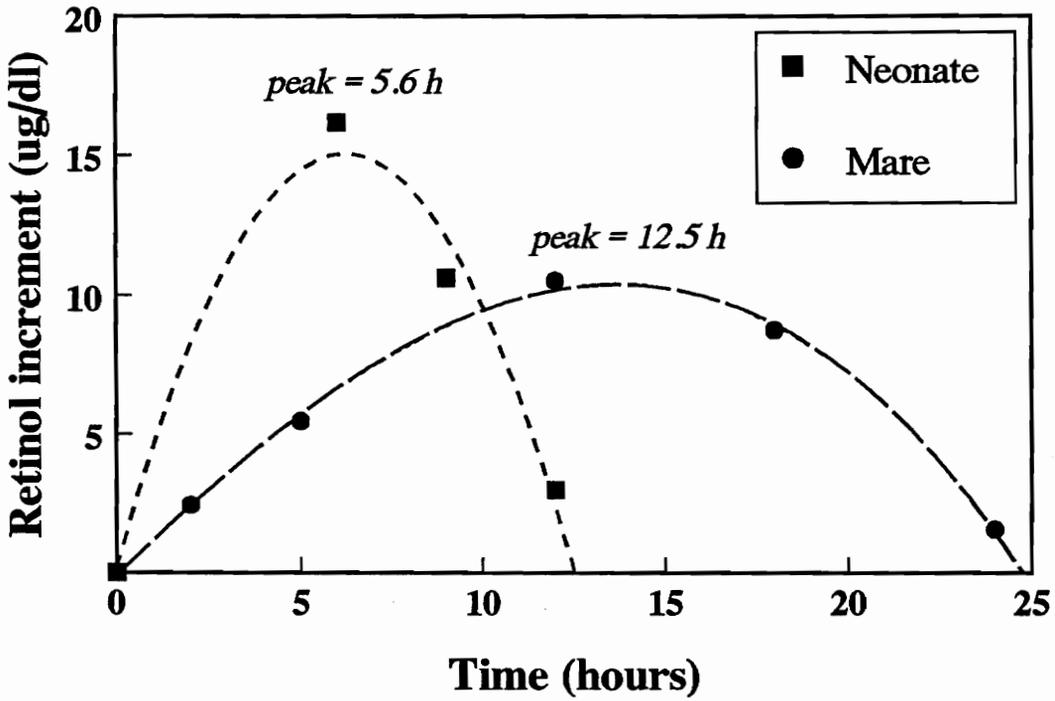
*Age.* The three RDR experiments indicated that serum retinol peaked at 12 to 15 hours post dosing. In studies with humans and rats, serum retinol peaked at 5 hours (Loerch et al., 1979; Flores et al., 1984) Why does it take longer for serum retinol to peak in the horse than in other species? There appears to be something unique about lipid (including fat soluble vitamins) absorption and transport in the horse.

No evidence of chylomicrons was found in the adult pony (Watson, 1991). Other blood lipids, such as LDL, VLDL and HDL were measurable, but at half the values usually found in the human. The low fat content of the diet and the manner in which the ration is

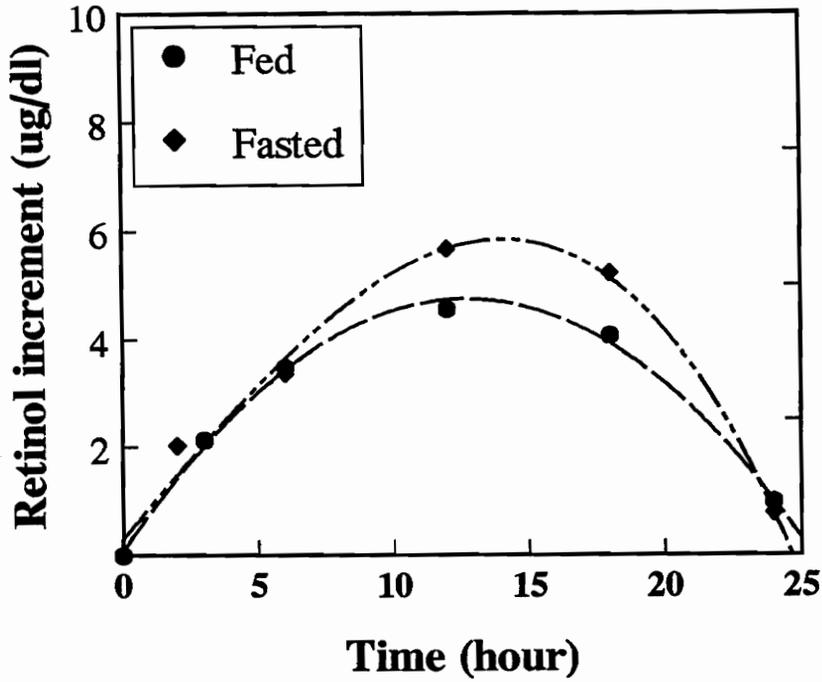
ingested (several small meals instead of few large meals) may have subdued the appearance of chylomicrons. The retinyl palmitate ester formed in the mucosal cell, in the human, is transported in the center of the chylomicron until it reaches the liver where it is de-esterified and then either bound to RBP and released from the liver to target tissues or re-esterified and stored in the stellate cells until it is needed. In the horse, the lipid carrier for vitamin A esters to the liver is not known, only that the absorption and transport of vitamin A esters from the intestine is slow, and(or) the release of retinol from the liver may be slow as compared to the human and the rat (Watson, 1991).

In contrast, serum retinol concentration in the equine neonate peaked in the blood around 5 hours, similar to rats and humans (Figure 1). This difference between mare and foal was not discussed in paper 5 but needs to be addressed. Once again the difference may be in fat utilization. The neonate was found to have a lipid profile very similar to humans in chylomicron structure and transport of fats (Watson, 1991). The foals absorb and transport fats differently from adult horses because of the high fat content of their diet from the milk. There is also a possibility that the vitamin A associated with the lipid fraction in the milk may be more readily available for absorption, thus a more rapid release of retinol from the liver.

*Fasted versus Fed.* No difference in serum retinol response curves to the oral dose of retinyl palmitate was found between fasted or fed states (Figure 2). Fasting a horse for 12 hours is mildly stressful and not always practical. Similar problems were found with children awaiting a RDR test. In a study on Guatemalan children it was concluded that a fed state did not affect the result of the RDR as long as the meal was not more than 4 hours before the initial blood sample (Mejia et al., 1984). However, horses have been observed to graze 12 to 18 hours a day (Gudmundsson and Dyrmondsson, 1994). This would provide almost a constant influx of nutrients to the small intestine,



**Figure 1.** The peak in serum retinol over a 24 hour period after a dose of retinyl palmitate is different for adults (12.5 hr) and neonates (5.6 hr). The data were fit by a quadratic equation ( $u = a + bx - cx^2$ ), and the peak was calculated from the equation (peak  $x = -b/2c$ ).



**Figure 2.** Serum retinol concentrations over a 24 hour period indicate there is no difference in response to an oral dose of retinyl palmitate in 2 fed horses or 2 fasted before the dose. Respective peaks were 13 and 14 hours for fed and fasted horses respectively.

which may explain the unique lipid profile (Watson, 1991). With a constant supply of nutrients, the fluctuations normally seen in meal feeders on the lipoprotein system were not observed in the horse (Watson, 1991). Therefore, the response to an oral dose of vitamin A should not be affected by the absence or presence of feed in the gut. It was important in this study that the horses did not have to be removed from the pastures to be fasted in order for the RDR to work.

*Ranges in RDR values.* In order to set normal limits for the RDR in rats, liver samples were taken immediately following a RDR test (Loerch et al., 1979). This is an invasive procedure and was not practical with the number of animals we were dealing with. No reports have been found that define a normal range of RDR in the horse. In the present studies, the 95% confidence interval of the RDR was 0 to 14% in mares on summer pasture before depletion (paper 1), and 0 to 15% in mares on summer pasture replenished with retinyl palmitate (paper 2). Consequently, an RDR of > 15% is likely to be found in only one of 40 adult mares with a replete vitamin A status, and this upper limit may be a useful diagnostic indicator of a low vitamin A status in an adult horse.

### ***Sensitivity of the RDR***

Outright nutrient deficiencies are uncommon today in our domestic species because of research in the last 100 years. Current interest has turned to nutritional needs for optimal performance. It does not take a severe vitamin A deficiency to affect normal body function, even slight deficiency may set awry systems dependent on the vitamin (Underwood, 1994). It would be advantageous to be able to identify the beginnings of vitamin A deficiency for optimization of nutrient needs. In this study we have tested the sensitivity of the RDR to detect a decrease in vitamin A status before a decrease in serum retinol. We found that serum retinol was more sensitive to vitamin A status than

previously expected, but in certain circumstances, the RDR was more discriminating. The RDR detected the depleting status of nonpasture kept mares sooner than serum retinol; yearlings who were selenium deficient had higher RDR but similar serum levels; the difference between pasture kept mares and nonpasture kept mares over the repletion phase was pronounced with the RDR; the RDR demonstrated the severity of depletion in the nonpasture, nonsupplemented mares not seen in the serum levels; and the RDR demonstrated that the vitamin A supplemented nonpasture kept mares were indeed lower in vitamin A status than the control mares on pasture.

## Conclusions

The results of these studies lead to several conclusions:

1. The vitamin A status of horses may be assessed by the combination of SR and RDR. Discrimination was better with RDR than with SR in mares and yearlings but not neonates.
2. Seasonal fluctuations in vitamin A status of mares reflected changes in availability of  $\beta$ -carotene in pastures. The RDR test indicated vitamin A depletion, thus supplementation of vitamin A is needed for mares maintained on pasture during late fall and winter.
3. Mares with no access to pasture had low vitamin A status that was not fully corrected by supplementation with retinyl palmitate at twice the currently recommended minimum requirement for pregnant mares. Thus this minimum requirement should be increased for pregnant mares.
4. A water dispersible form of  $\beta$ -carotene improved the vitamin A status of depleted mares maintained on pasture. It failed to do so, however, in nonpasture mares kept on a drylot. Thus the natural, oil soluble form of the provitamin in pasture may improve the availability of the water dispersible form. The water dispersible form of  $\beta$ -carotene should not be recommended for horses that are not kept on pasture as the sole source of vitamin A.
5. Infection with herpesvirus-4, manifested by respiratory disease, was associated with reduced vitamin A status of yearlings. Thus vitamin A supplementation should be recommended for the supportive care of young horses that are treated for upper respiratory infections.

6. Neonatal foals had a low vitamin A status as indicated by comparison with normal ranges of serum retinol and RDR in adult mares. Thus, these neonates need supplies of vitamin A from colostrum and milk.

6. Several conditions involving low vitamin A status were essentially subclinical, and the lack of clinical signs or lesions accentuated the value of measuring serum retinol and RDR. Nevertheless, health effects of vitamin A deficiency may have been manifested in the following conditions:

a. Birth and weaning weights were lower in the foals born to mares in the drylot (nonpasture), despite the nutritional needs of these foals and their dams exceeding current recommendations in all respects (NRC, 1989). These subnormal body weights may reflect a subclinical vitamin A deficiency that was not fully corrected by supplementation with retinyl palmitate at twice the current recommended minimum requirement during late pregnancy.

b. Pregnancy loss tended to be higher in the nonpasture mares than in pasture mares. Also in the nonpasture mares, pregnancy loss was exacerbated by supplementation with water dispersible  $\beta$ -carotene.

c. An increase in frequency of retained placentas and of foals born with contracted tendons was seen in vitamin A depleted mares.

### ***Implications***

1. This research has developed tests of vitamin A status in horses that are directly applicable in the field. The RDR should be used in conjunction with serum retinol levels to provide more information about vitamin A status.

2. Vitamin A should be supplemented to horses maintained on pasture during the fall and winter, and preferably at least 2 times the current recommendations at all times to pregnant mares that have no access to pasture.

3. A water dispersible  $\beta$ -carotene is not recommended for nonpasture kept horses as a sole source of vitamin A.

### ***The advantages or differences over previous research in this field***

The only previous attempt to apply the RDR test to the horse was confined to stalls and mistakenly used total vitamin A (included esters) instead of serum retinol. The present research is the first effective application of the RDR to a grazing animal. Many previous experiments on horses ran for one year but not close to three years as in the present studies. Statistically, the horses were supplemented individually so the horse was used as the unit, which gave more degrees of freedom than in other studies in which the animals were group fed. The mares in each supplement group were together eating the same diets and living the same schedule so there was no environmental factor to differentiate between vitamin supplement groups.

### ***Constraints of the research***

Although the number of horses was sufficient to show effects on vitamin A status in the horse, it was not quite enough to demonstrate convincingly certain effects of vitamin A supplementation on reproductive efficiency. Another constraint was the need for intervals between RDR tests to avoid being confounded by the carryover effects of the oral dose given for the test. The 8 weeks between RDR tests was sufficient to eliminate any carryover effect (Papers 1 and 2), but the 2 week interval probably was not (Paper 3).

Supplementation twice a week rather than daily was justifiable for vitamin A but perhaps not  $\beta$ -carotene, hence the need for the study of daily supplementation (Paper 3).

### ***Direction of further research***

1. More research needs to be done on the effects of temperature and humidity on vitamin A status. Winter and early spring are critical times for the broodmare because of the developing fetus and onset of lactation. Two important questions need to be answered: Is there a higher rate of utilization of vitamin A with the drop in temperature? Would supplementation of more than 2 times the current recommendation be advisable during that period?

2. An interesting aspect would be to study the effects of low vitamin A status on immune function in the horse, especially the performance horse that is exposed to various external stresses and young horses exposed to respiratory diseases.

3. The question of whether there are additional benefits of  $\beta$ -carotene supplementation above those of its provitamin function in the horse has still not been resolved. The water dispersible  $\beta$ -carotene is apparently not the most appropriate form for use in the horse. Other oil dispersible forms should be investigated in horses with adequate vitamin A in the diet in order to separate provitamin from other effects.

4. A study with larger numbers of mares would be needed to effectively test whether vitamin A and  $\beta$ -carotene affect reproductive efficiency.

5. More research is needed on the influence of vitamin A content in colostrum and milk on foal nutrition.

6. The RDR test may have clinical application, such as assessing the risk of herpesvirus-4 infections or the risk of developmental orthopedic diseases.

7. The differences in the time at which retinol peaks in the blood after an oral dose between adults and neonates is intriguing. Further research is needed on lipid metabolism in the horse.

### ***Closing remarks***

Vitamin A nutrition is clearly important in the horse. The common assumption that the horse can obtain everything it needs from grass can be misleading. With domestication of the horse we have added many new stresses to the animal that were not encountered during its evolution. To obtain maximal performance out of our animals we need to provide optimal nutrition. In order to define optimal in the horse we need more sensitive measures of nutrient status. This study has taken us one step closer.

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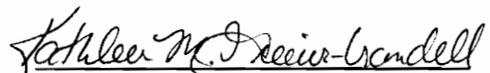
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