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Beta-carotene in the bovine corpus luteum

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BETA-CAROTENE IN THE BOVINE CORPUS LUTEUM

BY

STEPHEN G JUDD
B.S., University of New Hampshire, 1992

DISSERTATION

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ABSTRACT

BETA-CAROTENE IN THE BOVINE CORPUS LUTEUM

by

Stephen G Judd
University of New Hampshire, May, 1997

There have been conflicting reports which associate a low dietary intake or plasma concentration of beta-carotene (BC) with decreased fertility in dairy cows. The bovine corpus luteum (CL), a transient endocrine gland formed subsequent to ovulation, produces the steroid hormone progesterone which sustains pregnancy and is associated with fertility. Because of its high concentrations of the antioxidant nutrient beta-carotene, the CL was examined to identify an in vivo function for BC, other than that as a precursor of retinol. Five non-lactating Jersey cows were fed a basal ration containing a low BC concentration. Each cow received either 0, 1.6, 8.0, 16.0, or 80.0 mg/day of supplemental BC in the form of water-soluble beadlets incorporated into grain. Changes in plasma BC were measured weekly. After each of four feeding periods (7 weeks each), a mid-cycle CL (9-11 days following estrus) and plasma were harvested from each cow. Plasma and CL beta-carotene concentrations were directly related to the dietary BC level and, while on the BC deficient diet were similar to those seen in humans. Western immunoblotting was used to identify the steroidogenic enzymes adrenodoxin (Adx, ~14 kDa) and cholesterol side-chain cleavage cytochrome P-450 (P450scc, 49 kDa). The enzymes Adx and P-450scc catalyze the first and rate-limiting reaction in progesterone synthesis.

Antisera specific for Adx or P450scc, in addition to recognizing their respective proteins, recognized a protein of higher molecular weight (63 kDa). The 63 kDa protein band, believed to be P450scc and Adx chemically cross-linked in a one to one ratio, was present consistently in the lanes where CL beta-carotene concentrations were below ~11 nmol/g. On the other hand, the cross-linked protein was not apparent in lanes where the CL beta-carotene concentrations were above ~50 nmol/g. In lanes where the CL beta-carotene concentrations were between 11 and 50 nmol/g, the cross-linked protein was sometimes apparent, but not consistently. Thus, it appears that BC protects the mitochondrial enzymes Adx and P450scc against damage from free radicals produced during steroidogenesis. These observations may help explain studies which show that antioxidant vitamins enhance steroid production.

INTRODUCTION

In 1981, it was suggested that carotenoids, such as beta-carotene, may be protective against cancer (1). Since that time, many epidemiological surveys have been conducted to determine the relationship between dietary carotenoids and various diseases, including numerous cancers, heart disease, and macular degeneration. While there is an emerging consensus that consuming fruits and vegetables which are carotenoid-rich reduces the incidence of many chronic diseases, the beneficial effects of individual carotenoids remain in dispute. One reason for this, is that the mechanism of action whereby carotenoids might affect these diseases is unknown. Much of the research in this area has focused on one carotenoid, beta-carotene.

The structural characteristics of beta-carotene, its similarity to other carotenoids, and its relationship with Vitamin A compound the difficulties of identifying its critical functions. Beta-carotene imparts a yellow to orange color to those plants or tissues which contain it, though this color is often masked by the green of chlorophyll with which it colocalizes in photosynthetic tissue. When consumed by animals, beta-carotene may be utilized by the body to produce Vitamin A. In addition to this provitamin A function, beta-carotene may also act as an antioxidant, protecting the lipid with which it becomes associated from the damage of oxidants.

In addition to its relationship to chronic disease in humans, beta-carotene has been reported to affect fertility in cows. While field-based studies have shown

conflicting results, some suggest that feeding supplementary beta-carotene may enhance fertility, depending upon the beta-carotene status of the control group. However, as with human diseases, no mechanism of action for the relationship between beta-carotene and fertility has been elucidated.

One tissue which contains beta-carotene and also plays a role in fertility is the corpus luteum. The bovine corpus luteum, a metabolically active steroidogenic gland which is formed in the ovary of cows subsequent to ovulation, derives its name, "yellow body", from its coloration which is a result of its high beta-carotene concentration. After ovulation, the corpus luteum forms from the cells of the follicle and secretes the steroid progesterone. The high levels of circulating progesterone sustained by the corpus luteum, prepare the endometrium of the uterus for implantation of an embryo and help maintain pregnancy if successful implantation occurs. If the ovum is not fertilized, the corpus luteum undergoes regression, resulting in its functional and structural demise. A new corpus luteum is formed following each ovulation.

In addition to its possible implications for fertility, the bovine corpus luteum is an excellent model in which to examine beta-carotene metabolism and function because of its high concentration of beta-carotene, its high metabolic activity, and availability of the tissue. Since the corpus luteum is formed anew each estrous cycle, its beta-carotene concentration reflects changes in serum beta-carotene concentrations, and it does not retain a "memory" of past beta-carotene exposure. Additionally, the corpus luteum can be removed from the cow without harm to the ovary and without anesthesia

in a simple surgical procedure. This allows for the harvest of multiple corpora lutea over time from the same cow exposed to different dietary treatments.

The research described in this dissertation was undertaken in an attempt to characterize the accumulation, localization, and function of beta-carotene within the bovine corpus luteum. The goal was not only to elucidate a function of beta-carotene in the corpus luteum, but to establish a model in which beta-carotene metabolism within a tissue can be examined. The hypothesis being tested was that dietary beta-carotene affects plasma and corpus luteum beta-carotene concentrations and that high levels of corpus luteum beta-carotene inhibit cross-linking of steroidogenic enzymes.

Specific Aims:

1. Determine the relationship between dietary, plasma, lipoprotein, and corpus luteum concentrations of beta-carotene, and the variability in this relationship between cows.
2. Determine the subcellular distribution of beta-carotene within the bovine corpus luteum.
3. Determine the relationship between dietary, plasma, lipoprotein, or corpus luteum concentrations of beta-carotene and plasma progesterone concentration in the cow.
4. Determine the effect of varying dietary beta-carotene levels on the steroidogenic enzymes of the corpus luteum.

CHAPTER I

REVIEW OF THE LITERATURE

Beta-carotene

Structure and Function

Carotenoids are a class of hydrocarbons which are derived from lycopene (Ψ,Ψ -carotene), an acyclic $C_{40}H_{56}$ structure which has a central chain of conjugated double bonds and is formed by the condensation of isoprenoid subunits. The carotenoids are a diverse family of pigments which give rise to many of the yellow, orange and red colorations found in nature, though this coloration is masked by the intense green of chlorophyll in active photosynthetic tissue. While more than 600 carotenoids have been identified in nature (2), much of the research in this field has focused on beta-carotene (BC, ***Figure 1***) since it was first isolated from carrots by Wackenroder in 1831. Beta-carotene is a carotenoid derived from lycopene by cyclization at each end of the molecule, a process thought to occur only in bacteria, algae, plants, and fungi.

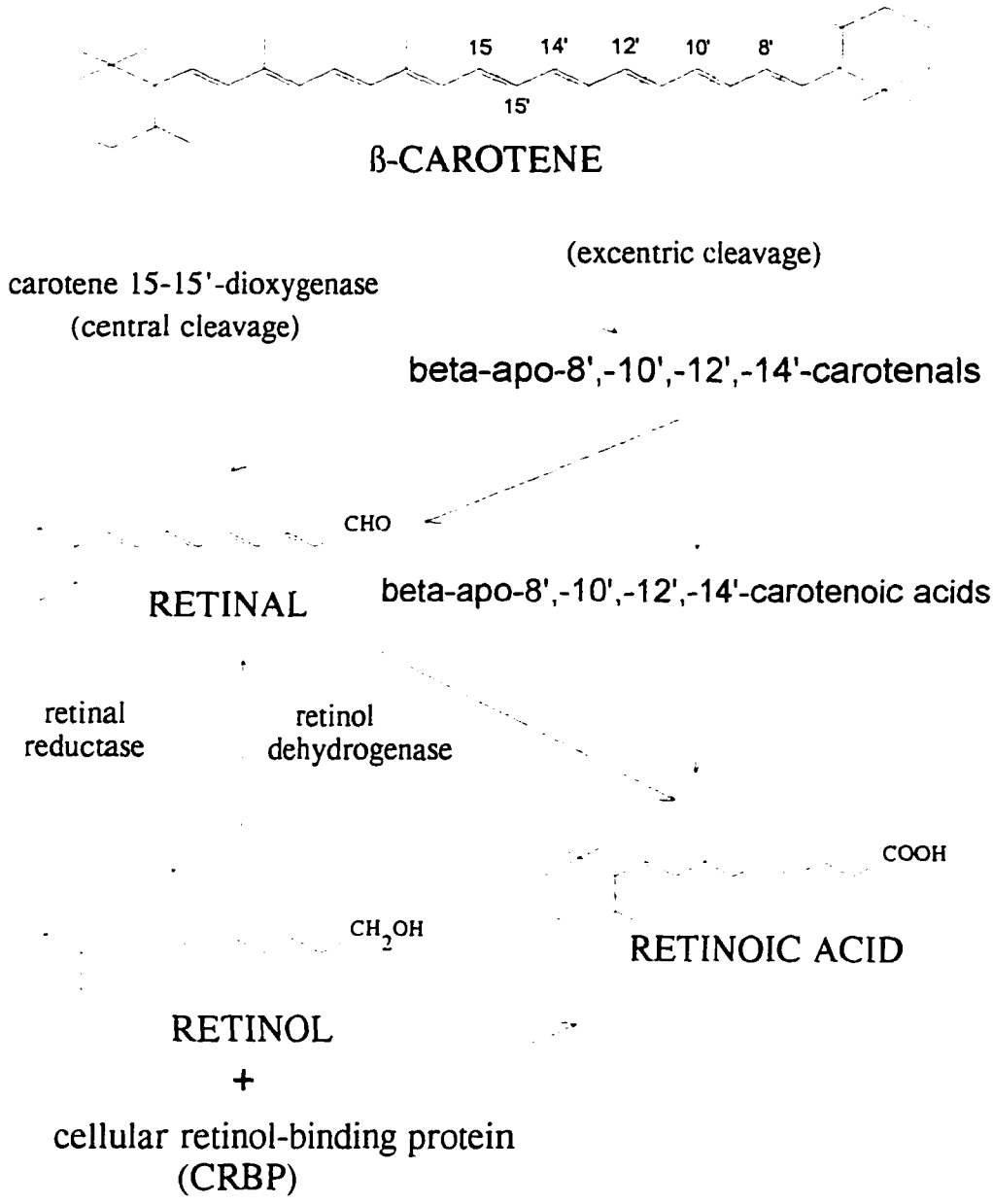

Figure 1 **Metabolic pathways of beta-carotene.** Beta-carotene may undergo central cleavage to yield retinal, which may then be converted to the active metabolites retinol or retinoic acid. Alternatively, beta-carotene may be cleaved at a non-central site (excentric cleavage) to yield a beta-apo-carotenal which can be subsequently converted to retinoic acid or retinol.

It has been proposed, by Olson (3), that carotenoids' biological effects in nature can be broken down into three categories: functions, actions and associations. Krinsky (4) grouped these effects of carotenoids as follows:

> 1. Functions: accessory pigments in photosynthesis, via singlet excited carotenoid; protection against photosensitization, via triplet excited carotenoid; provitamin A, via central and excentric cleavage.
> 2. Actions: antioxidant; immunoenhancement; inhibition of mutagenesis and transformation; inhibition of premalignant lesions; screening pigment in primate fovea.
> 3. Associations: decreased risk of macular degeneration and cataracts; decreased risk of some cancers; decreased risk of some cardiovascular events; nonphotochemical fluorescence quenching.

Functions are those effects of carotenoids which are "essential to the normal well-being of the organism in question." (3) In photosynthetic membranes, carotenoids perform two functions: 1) they collect light for photosynthesis and 2) protect the photosynthetic apparatus from the destructive effects of light and oxygen (5). Because of their chemical structures, carotenoids absorb light, with the maximal absorption being of blue-green light ($\sim$450 nm), however, most chlorophylls do not absorb blue-green light or are poor absorbers. In photosynthetic tissues, carotenoids absorb blue-green light and then transfer the resultant energy to chlorophyll, with which they are in a highly ordered relationship within their apoproteins, for photosynthesis. In addition to this role of transferring energy to chlorophyll, carotenoids also serve a protective function by accepting energy from chlorophyll. Under intense light conditions, chlorophyll can be excited into a triplet state which, if the energy is dissipated in an uncontrolled fashion, can lead to cellular damage. Carotenoids accept this excess energy from chlorophyll and dissipate it thermally, preventing damage to the organism. The energy from triplet state chlorophyll can also be transferred to oxygen yielding

singlet oxygen, which can also cause cellular damage. Carotenoids quench singlet oxygen, thereby protecting the organism.

Beta-carotene is one of the carotenoids which can be converted to retinol (Vitamin A), and therefore possesses what are known as provitamin A properties (***Figure 1***). This activity is considered a function because provitamin A carotenoids are able to alleviate the deficiency symptoms of animals on vitamin A-deficient diets. As a precursor to vitamin A, BC is cleaved in the intestinal mucosa by beta-carotene 15-15'-dioxygenase (EC 1.13.11.21) to retinal and then reduced by retinal reductase (EC 1.1.1.71) to form retinol. Beta-carotene, obtained from the diet, is the most nutritionally active carotenoid and accounts for 15-30% of total serum carotenoids in humans (6). The efficiency of this conversion, currently accepted to be 6:1 (BC to retinol on a weight-for-weight basis), and its impact on human nutriture has been reviewed (7). This 6:1 conversion efficiency is based on the assumptions that carotenoids are absorbed one half as well as retinol, and the average extent of conversion is 33%. However, these assumptions remain in question, and the extent to which dietary BC can substitute for preformed vitamin A is still being studied.

Retinol, preformed from dietary sources or formed in the intestinal mucosa from BC , is subsequently esterified, transported via chylomicrons through the lymph to the general circulation, and chylomicron remnants are formed in the capillaries. These chylomicron remnants are cleared primarily by the liver, and retinyl esters are stored or used as needed (8). Other tissues may also clear chylomicron remnants to some extent. Stored retinol may be released from the liver and delivered to target tissues bound to retinol binding protein and subsequently transported to active sites by

cell specific binding proteins. The functions of retinol in the body can be divided into visual and non-visual functions. In vision, retinol and its metabolites serve a vital role in the signal transduction process (9). The 11-cis-isomer of retinaldehyde, attached to opsin in rhodopsin, is isomerized to its all-trans configuration by light. This event triggers the nerve impulse to the brain which forms the perception of light. All-trans-retinaldehyde is released from the opsin and reduced to all-trans-retinol. In the dark, this is isomerized to 11-cis-retinol and oxidized to 11-cis-retinaldehyde, which recombines with opsin to re-form rhodopsin, completing the visual cycle. Deficiency of vitamin A reduces the available pool of retinol in the eye, and leads to night blindness, a classical vitamin A deficiency symptom. The non-visual functions of retinol include the maintenance of normal epithelial cell differentiation, normal bone growth, spermatogenesis, and embryonic development (9) .

The majority of the non-visual effects of retinoids are thought to be modulated by retinoic acid (10), however, it has been suggested that retinoic acid cannot substitute for all effects of retinol in growth regulation, and that other retinol metabolites may act as mediators. Retinoic acid exerts its effects through the regulation of gene transcription via retinoic acid nuclear receptors, which are members of the steroid hormone receptor superfamily (11). Retinoic acid is formed within target cells from retinol bound to cellular retinol-binding protein by cytosolic and microsomal retinol dehydrogenases (12,13). Additionally, retinoic acid can be formed directly from BC via, what is called, excentric cleavage (14). Excentric cleavage describes a process in which, rather than being cleaved centrally and producing two molecules of retinal, BC is cleaved nonspecifically at one of its non-central double bonds (***Figure 1***). This cleavage results

in the formation of beta-apocarotenals of various chain lengths, all of which can be converted either to retinal or oxidized to the corresponding beta-apocarotenoic acids, which may undergo a form of beta-oxidation to yield retinoic acid. The ability to convert BC to retinoic acid has been shown in intestinal mucosa, liver, kidney, lung, and testes (15). Thus, BC serves as a precursor to both retinol and directly for its active metabolite retinoic acid.

Carotenoids' actions have been described as physiological or pharmacological responses to the administration of carotenoids (3) and may be related to the intact carotenoid molecule, as opposed to a metabolite. The antioxidant actions of BC are due to its extended system of conjugated double bonds (see ***Figure 1***). While the exact mechanism of beta-carotene/radical reactions is not clear, it is hypothesized that beta-carotene may react directly with a peroxyl radical to form a resonance-stabilized radical (16). This is supported by the fact that most of the products of the interaction of beta-carotene and radicals are carbonyl derivatives of beta-carotene (17). In plants, where BC and other carotenoids are found in close association with chlorophyll, BC protects the chloroplast from singlet oxygen, a free-radical produced during conditions of high light intensity (2).

The ability of BC to act as an antioxidant in vitro has been shown by Jialal et al. (18), who demonstrated that beta-carotene inhibits the oxidation of isolated low density lipoprotein (LDL); the oxidative modification of LDL is believed to be the necessary first-step in the development of atherosclerosis (19).

In vivo inhibition of oxidation has also been demonstrated. Allard and colleagues (20) have shown that supplementation of smokers with 20 mg/day BC for

four weeks significantly reduced breath-pentane output (BPO), a measure of in vivo lipid peroxidation. No effect on BPO was seen in non-smokers, though they showed similar increases in serum BC concentration. It is not clear whether this measure of lipid peroxidation has any significance for the health of the subjects, or where the effect of BC is exerted.

The cellular transformation inhibiting effects of provitamin A and non-provitamin A carotenoids have also been demonstrated in vitro (21,22). Bertram et al. (23) showed that treatment of a murine fibroblast cell-line with either BC or canthaxanthin, a non-provitamin A carotenoid, inhibited the 3-methylcholanthrene-induced transformation of the cells. Since both a provitamin A and non-provitamin A carotenoid exerted the same effect, they concluded that this transformation inhibiting effect may be due to their lipid antioxidant properties. This same group subsequently showed that this inhibition was independent of the carotenoids' antioxidant status, but was due to the carotenoids' ability to up-regulate expression of the Connexin43 gene which encodes for a major gap junctional protein (24). Schwartz and colleagues (25) demonstrated that BC, when added to cultured cells, inhibits human epidermal carcinoma cell proliferation, but not normal keratinocyte proliferation. This inhibition was associated with the appearance of a unique heat-shock protein, presumably induced by the BC. Thus, it appears that carotenoids may have the ability to regulate gene expression unrelated to their provitamin A or antioxidant activity.

Another well-defined action of carotenoids is the prevention of photosensitivity in patients with erythropoietic protoporphyria when given large doses of oral beta-carotene. This action is believed to be mediated by the quenching of singlet oxygen in

the skin, in a mechanism analogous to that which occurs in photosynthetic tissues (26). Pharmacological doses of BC (~ 180 mg/day) are given to these patients, and it is not clear if a similar protective process occurs in people without photosensitivity who have a much lower BC intake.

The interest in the actions and functions of BC have been sparked, largely, by the proposition that dietary BC may materially reduce human cancer rates, put forth in 1981 by Peto et al. (1). Since that time, there have been numerous reports of associations between increased consumption of carotenoid-rich foods and a decreased incidence of chronic diseases such as cataract, macular degeneration, cancers of the oral cavity and cervix, and coronary heart disease (27). While the evidence strongly indicates that increased consumption of fruits and vegetables is associated with a decreased incidence of chronic disease, intervention studies which have supplemented populations with BC have returned conflicting results.

The Linxian China study (28) provided supplementation of BC, vitamin E, and selenium to a marginally malnourished population, and found decreased mortality due to gastric and total cancer. The Physicians' Health Study (29), on the other hand, was a twelve year study in which male physicians were provided BC supplementation. This study showed that BC had neither a positive or negative effect on the incidence of cancer or heart disease. A third study, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention study (30), conducted in Finland suggests that supplementary BC increased the risk of lung cancer in high-risk groups. Thus, it is not clear whether BC affords any protection against the onset of chronic disease or if it may be harmful in some

situations. Additionally, BC may simply serves as a biomarker for some other protective agent or habits.

While many of the associations between carotenoids and decreased disease incidence have been interpreted to mean that carotenoids are protective, the possibility remains that these are only associations, which may not be causally related. Krinsky (4) reviewed the antioxidant effects and actions of carotenoids associated with inhibition of mutagenesis and malignant transformation, and concluded that while there is ample evidence that carotenoids can act as an antioxidant in vitro, there is a need for clear-cut studies indicating whether or not carotenoids behave as antioxidants in vivo.

Absorption, Transport, and Tissue Accumulation

Beta-carotene is a normal constituent of the blood and other tissues of humans, cows, and ferrets, but not most rodents. Indeed, one of the challenges of BC research is in finding a suitable animal model for studies where humans cannot be used. Many animals either do not absorb BC or are extremely efficient in converting it to retinol, resulting in little or no intact BC being found in the plasma or tissue. The absorption and transport of BC has been studied extensively in humans, while there is limited information about tissue accumulation because of the difficulty in obtaining tissues for analysis. Animal models for human BC metabolism have been generally limited to cows, particularly preruminant calves which have digestive systems more like humans than do mature cows which are ruminants, and ferrets. Both of these species have been shown to absorb and accumulate intact BC (31,32). Other animals, such as rabbits, respond to dietary BC with increased serum retinoic acid levels, but do not absorb or accumulate detectable levels of BC (33).

Human. Because it is an extremely lipophilic molecule, BC is associated with lipoproteins in plasma and the lipid portions of cells, including membranes (both cell and organelle) and lipid droplets in adipose and steroidogenic tissues. The absorption of BC is, therefore, dependent on the presence of dietary fat reflecting its transport into the intestinal mucosa via micelles. Prince and Frisoli (34) showed that a single dose of BC (50 mg) consumed in the absence of dietary fat resulted in no appreciable change in serum BC. However, the same dose, when given with 200 g of fat, resulted in a 2.5-fold increase in serum BC concentration. They also demonstrated, via remittance spectroscopy, that BC accumulated in the skin of subjects on chronic supplementation, though the effect was delayed by two weeks as compared to the serum response.

Once absorbed by the intestine, BC is secreted in chylomicrons into the lymph. Absorbed BC is first detectable in the triglyceride-rich lipoprotein fraction which is made up predominantly of chylomicrons, their remnants, and very low-density lipoproteins (VLDL) secreted by the liver (35). Beta-carotene associated with high-density lipoproteins (HDL) likely comes from chylomicrons during the transfer of surface components from chylomicrons to HDL and as a result of catabolism of the triglyceride-rich lipoproteins (36). Ultimately, BC accumulates in the tissues, primarily adipose, liver, and muscle, with the highest tissue concentrations occurring in the corpus luteum and adrenal glands (6).

One difficulty with studies of BC absorption, has been the reliance on serum appearance of intact BC as an indicator of absorption. Some BC may be converted to retinol in the intestinal mucosa, and secreted in the form of retinyl esters as described above. Indeed, large inter-subject variability has been observed in the serum response

to supplementation with oral BC (37-40). Whether this variability is due to differences in the efficiency of conversion of BC to retinol, or to differences in the absorption of BC into the intestinal mucosa remains in dispute (41).

The use of labeled BC may help answer the question of just how much of a dose of BC is absorbed intact, and how much is converted to retinol after absorption. In a study of one individual consuming a single dose of beta-carotene-d8, a stable deuterated BC isotope, Novotny et al. (42) were able to construct a compartmental model of BC metabolism. They concluded that 22% of a 40 mg BC dose was absorbed: 17.8% as intact BC and 4.2% as retinoid. In agreement with the previous studies, they saw an initial increase in chylomicron BC concentration followed by an increase in the other lipoprotein fractions. The residence time of BC in the body was estimated to be 51 days, reflecting a stable tissue reserve of BC which requires time to deplete.

Cows. Many studies have focused on the suitability of the pre-ruminant calf as an animal model for studying carotenoid metabolism. Adult cows, like all ruminants, have a four-compartmented stomach which is quite different than monogastric animals such as humans. However, the immature calf's digestive tract is not fully developed and they are considered functional monogastrics. While adult cows do absorb intact BC, the preruminant calf is a better model for studying BC absorption. Poor et al. (43) demonstrated that calves given a single-dose BC supplement showed serum response peaks at 12 and 30 hours post-dosing, similar to what is seen in humans. Additionally, serum data from the calves could be fitted to a two-compartment model, yielding an elimination constant similar to reported human values. They also showed that intact BC accumulated in adrenal, liver, spleen, lung, adipose, and kidney tissues. While

there are many similarities between the humans and preruminant calves, their lipoprotein profiles are very different. Bovine HDL is easily distinguished from human HDL because of the elevated proportion of cholesteryl ester found in the cow. Bovine HDL accounts for 80% of total lipoproteins, while in humans, HDL accounts for only about 30% of total lipoproteins (44).

Because of these differences, approximately 60% of the BC in bovine serum is associated with HDL (45), while in humans only ~23% of serum BC is associated with HDL (36). As expected, because of their compositional similarity to human LDL, bovine HDL of larger size and higher lipid content have a higher BC concentration (46).

The accumulation of BC within the tissues of the bovine has been demonstrated in several studies. Poor et al. (43) showed that adrenal tissue showed increases in the BC concentration 24 hours after a single oral dose (20mg) of BC and remained elevated 11 days later. Liver, spleen, and lung BC concentrations were also elevated at 24 hours post-dosing, and then rapidly declined. Adipose and kidney BC concentrations peaked at 72 hours and 144 hours post-dose, respectively.

Chew et al. (45) studied the uptake of BC by blood cells, plasma, and lipoproteins in preruminant calves given either single or multiple oral doses of BC. They found that the relative increase in BC associated with the lipoproteins was greater for LDL than for HDL or VLDL. Orally administered BC also resulted in an increase in the BC concentration of lymphocytes, but not neutrophils or erythrocytes. The increase in the BC concentration of the lymphocytes was largely due to increases in the BC content of the mitochondrial and nuclear fractions of the lymphocytes.

Hoppe et al. (47) also demonstrated an increase in plasma and tissue BC concentrations and liver vitamin A as a result of supplementing preruminant calves with orally administered BC. A dose-dependent accumulation of BC was found for liver, heart, lungs, adrenals, and adipose tissue.

Thus, the functionally monogastric calf may be a useful model for studying the absorption, transport, and tissue accumulation of carotenoids and particularly BC. While the mature cow is not monogastric, it does absorb and accumulate intact carotenoids, including BC, making it a potential model for examining the functions of BC in tissues. Indeed, because of its high consumption of plant matter, the cow has extremely high BC concentrations, with the highest being found in the adrenals and corpus luteum.

Corpus Luteum

The corpus luteum (CL), first described by de Graaf, is a transient steroidogenic tissue which is formed in the mammalian ovary subsequent to ovulation. In non-fertile cycles it begins to regress prior to the next ovulation, while in fertile cycles it continues to produce pregnancy-sustaining progesterone.

The CL is essentially a differentiated follicle in which the theca and granulosa cells of the follicle give rise to the small and large steroidogenic luteal cells, respectively, after ovulation. In addition to these steroidogenic luteal cells, which make up ~70% of the volume of the CL, the CL contains macrophages, fibroblasts, and endothelial cells. While large luteal cells account for only 3% of the luteal cells, they make up ~40% of the volume of the CL, and are responsible for most of the basal secretion of progesterone. Small luteal cells also produce progesterone and are

highly responsive to luteinizing hormone (LH), the major luteotropin in cattle (48). The ratio of small to large luteal cells decreases as the CL ages as small luteal cells grow in size (49).

The process of luteinization, the differentiation of the follicle into the CL, is associated with the surge in LH concentration just prior to ovulation and results in the morphological and functional changes to the follicular cells. These changes result in the structural reorganization of the cells and the production of large quantities of the steroid hormone progesterone.

Several studies have demonstrated the relationship between the CL, progesterone, and successful pregnancy. Staples and Hansel, in 1961, examined the effects of oxytocin injection, which inhibits CL development, on embryo survival 15 days after insemination in 70 Holstein heifers. They determined that the threshold of CL progesterone necessary for embryo survival at fifteen days is 100 μg (50).

In a 1967 study, Estergreen et al. (51) performed ovariectomy of forty-one cows at 48-268 days of pregnancy. Prior to 165-180 days of pregnancy, the corpus luteum was essential to maintaining the pregnancy. Thereafter, a viable fetus was sometimes maintained after removal of the CL, but the CL was necessary to prevent shortened gestations.

Progesterone supplementation after insemination was shown to increase pregnancy rate to 60% compared to 30% in untreated controls and it also increased plasma progesterone concentrations (52). Lamming et al. (53) found that cows which fail to maintain early embryos have a small but measurable depression in progesterone output, though not sufficient to be the sole explanation.

Progesterone production by the bovine CL is regulated primarily by LH. Luteinizing hormone, a peptide hormone originating in the anterior pituitary, binds to LH receptors on the cells of the CL and leads to an increase in progesterone secretion. Binding of LH to its receptors activates adenylate cyclase thereby increasing intracellular cAMP concentration and activating protein kinase A. The active protein kinase leads to increased protein synthesis, increased cholesterol esterase activity, increased activity of the cholesterol side-chain cleavage complex, increased transport of cholesterol into the mitochondria, and ultimately increased progesterone synthesis and secretion (54).

When fertilization and embryo formation does not occur, the uterus releases prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) which inhibits the secretion of progesterone by the CL and causes the loss of luteal tissue (luteolysis). The anti-steroidogenic effects of $PGF_{2\alpha}$ are believed to be mediated by the inhibition of cholesterol transport to the steroidogenic enzymes. Luteolysis is manifested by a decrease in luteal blood flow and apoptotic death of the cells of the CL (55).

Steroidogenesis. The principle function of the CL is the production and secretion of the steroid hormone progesterone which inhibits release of gonadotropic hormones by the anterior pituitary and prepares the uterus for implantation of an embryo. Because of the need to produce large quantities of progesterone, the CL is a metabolically active tissue characterized by a high blood flow and luteal cells which contain large numbers of mitochondria, lipid droplets, and smooth endoplasmic reticulum.

Like all steroidogenic tissues, the CL relies on a supply of cholesterol as its precursor for steroid synthesis. In the rat, the CL is dependent on cholesterol supplied by plasma lipoproteins, primarily HDL (56,57). Similarly, bovine luteal cell grown in culture have been shown to utilize cholesterol from both LDL and HDL to increase progesterone production (58). In a review of the subject, Grummer and Carroll (59) concluded that lipoproteins provide the majority of cholesterol for steroid biosynthesis, and that trophic hormones increase lipoprotein binding, internalization, degradation, and conversion of lipoprotein-derived sterol to steroids.

Cholesterol is extracted from the circulating lipoproteins or entire lipoproteins are internalized and stored intracellularly. Cholesterol is then transported into the mitochondria to undergo the first committed and rate-limiting step of steroidogenesis, side-chain cleavage. The transport of cholesterol into the mitochondria is highly regulated and is responsible for the rapid increase or decrease in steroid synthesis in response to hormones (60). McLean et al. (61) showed that in the rat CL, estradiol stimulates progesterone synthesis by increasing the mitochondrial content of sterol carrier protein-2 without affecting other steroidogenic enzymes. Thus, the increase in progesterone synthesis was due to an increase in the availability of cholesterol to the mitochondria.

In the cow, a protein, steroid acute regulatory protein (StAR), transfers cholesterol from the outer to the inner mitochondrial membrane to initiate steroidogenesis. Corpora lutea from cows treated with $PGF_{2\alpha}$ exhibited a 50% decline in StAR mRNA over 12 hours, while other steroidogenic enzyme mRNA showed no change. The decline in StAR mRNA was paralleled by a decline in progesterone,

indicating a regulation of progesterone synthesis by control of the cholesterol supply (62).

Once in the mitochondria, cholesterol side-chain cleavage cytochrome P-450 enzyme (P450scc) (63) catalyzes the conversion of cholesterol to pregnenolone. This reaction, three successive monooxygenations, requires electrons which are transferred from NADPH to adrenodoxin reductase and to P450scc by adrenodoxin (Adx). Adrenodoxin acts as a shuttle transferring single electrons from adrenodoxin reductase to P450scc (64). The average relative concentrations of adrenodoxin reductase, Adx, and P450scc in the CL is 1:2.5:3, indicating that Adx and P450scc are present in approximately equimolar quantities (65).

While it appears that the primary acute regulation of steroidogenesis is at the level of substrate availability (i.e., StAR regulation), changes in the concentrations of specific steroidogenic enzymes occur during the ovarian cycle. These changes in specific contents are reflected in the changes in the types of steroids produced. Specifically, by the mid-luteal phase, concentrations of Adx and P-450scc have increased 15 and 12-fold, respectively, compared to follicular and early-luteal levels. These changes accompany the dramatic increase in progesterone production (66). It appears that the acute regulation of steroidogenesis is accomplished by controlling the transport of cholesterol from the outer to inner mitochondrial membrane, and that the long-term steroidogenic capacity of a cell is determined by the concentration of the steroidogenic enzymes (63).

The process of electron transfer has been shown to leak electrons and generate oxygen free radicals under certain conditions in vitro. Hanukoglu et al. (67) suggested

that it was possible that the high levels of antioxidants found in steroidogenic tissue protected against damage from such radicals.

Numerous studies have examined the role of oxygen radicals and antioxidants in controlling steroidogenesis in the corpus luteum of rats. Riley and Behrman observed that the generation of hydrogen peroxide (H_2O_2) in rat luteal tissue is an early and sustained component of CL regression (68) and that H_2O_2 inhibits steroidogenesis by blocking intracellular transport of cholesterol to mitochondria or translocation of cholesterol across the outer mitochondrial membrane (69). It appears that this inhibition of cholesterol transport is mediated by the inhibition of rapidly inducible proteins analogous to StAR (70).

Beta-carotene and fertility

Corpus luteum is Latin for "yellow body"; a name derived from the yellow/orange appearance of the bovine CL. This coloration is due to a high concentration of carotenoids, mainly beta-carotene, found in the bovine and human CL. This has led to studies attempting to elucidate the metabolic fate of beta-carotene in the CL and its possible relationship to CL function.

The bovine CL has been shown to have relatively high concentrations of carotenoids and retinal (71-73). It has also been shown that the bovine corpus luteum possesses the ability to convert beta-carotene into retinal and retinol (72,74,75).

Gawienowski et al. (72) demonstrated in 1974 that bovine corpus luteum slices are able to convert radiolabeled beta-carotene to retinal. In 1983, Sklan (75) demonstrated carotene cleavage using homogenized CL. This study compared corpus rubicans (early CL), mid-ovulation CL (7-12d post ovulation) and regressing CL (<3d

prior to complete regression). The data suggest that the mid-ovulation CL has greater carotene cleavage activity than either of the other two CL types. Because only two corpora rubicans and two regressing CL vs. 12 midovulation CL were used, the results were not statistically significant, however the results do suggest that BC metabolism may be associated with the functioning of the CL.

Talavera and Chew (76) demonstrated that retinol, retinoic acid and beta-carotene, added to culture medium, each stimulate progesterone secretion by porcine luteal cells *in vitro*. Beta-carotene showed the most marked increase, stimulating progesterone secretion 10-fold. While each of the three stimulated progesterone secretion, the fact that beta-carotene had the largest effect led the authors to conclude that beta-carotene may play a very important role in regulating luteal cell function.

Yokoe and Takenaka (77) reported that the concentrations of carotenoids and Vitamin E in the human CL increase with CL development, peaking at the mid-luteal phase. During in vitro experiments, they determined that Vitamin E may play a role in protecting microsomal membranes from superoxide radical produced during the aromatization process of steroidogenesis. They additionally postulated that carotenoids may protect cytoplasmic membrane from singlet oxygen generated by steroid-hydroxylation.

Based on this information, it is clear that CL contains high concentrations of beta-carotene, much of which is contained in the mitochondria and microsomes, centers of steroidogenic activity (73). Additionally, the CL has the capability to metabolize the BC. Beta-carotene, retinol and retinoic acid have been shown to influence progesterone production in vitro and retinol has long been considered to have a role in reproduction.

Taken together, this suggests that the accumulation, distribution and metabolism of beta-carotene and the retinoids may be regulated to meet some specific purpose. Indeed, Aten and colleagues (78) concluded that in the rat the antioxidant reserve of the ovary is in a dynamic state and may be endocrine-regulated.

While the hypothesis that BC may have a functional role in the CL is intriguing, studies which have examined the relationship between BC and fertility in cows have produced contradictory results (79-81). In a review of the subject in 1982, Hemken and Bremel reported that a number of studies from Germany had demonstrated increased fertility in cows supplemented with beta-carotene, even though the diets were adequate in Vitamin A. However, a similar study from Israel found no beneficial effects (81).

Graves-Hoagland et al. (80) measured plasma BC and vitamin A in 39 cows and correlated these values with in vivo progesterone production following gonadotropin-releasing-hormone-induced LH release. They found that progesterone production was positively correlated with beta-carotene concentration and negatively correlated with vitamin A.

Jackson (82) fed three groups of cows low BC diets for varying periods (5, 4, or 2 weeks) and then synchronized their estrus and inseminated them. Plasma BC levels declined in all groups, and was directly correlated with length of time to conception. Cows with the highest BC levels conceived on the first insemination, while those cows with the lowest BC levels required repeated services or did not conceive. The number of cows in the study was small (15), but the results did suggest a role for BC in fertility.

Ascarelli et al. (81) fed cows supplemental beta-carotene (>500 mg/day) and compared reproductive parameters of this group to an unsupplemented control group receiving a ration of low BC content. Their results are inconclusive in that they saw an increase in conception rates with supplementation, but only in younger cows during the first half of the experiment. It is not clear what factors (seasonal, etc.) may have influenced their results.

Folman et al. (83) reviewed the case for beta-carotene's enhancement of fertility in cows and found that in some experiments, where the basal BC levels were below 0.5 μg/ml. BC supplementation increased fertility. However, there was no case in which fertility was affected when plasma BC exceeded 1.5 μg/ml. This suggests some minimum requirement for BC, above which no effects of supplementation will be apparent.

Given the oxidative nature of steroidogenesis and the antioxidant properties of BC, it is reasonable to examine the effects of BC on steroidogenesis. Covalent cross-linking between P450scc and adrenodoxin has been shown to occur when bovine luteal cells are cultured (84). In the study, Western immunoblotting after gel electrophoresis under denaturing conditions was used to probe cultured cells for P450scc and adrenodoxin proteins. In addition to a reactive band at the appropriate relative molecular mass (M_r), 49 kDa and 14 kDa, for their respective proteins, both antibodies reacted with a protein of higher M_r (63 kDa). This corresponds to the weight obtained when P450scc and adrenodoxin are chemically cross-linked. The degree of cross-linking increased with time in culture, while the level of BC in the cells decreased with time in culture. Supplementation of the medium with BC, but not alpha-tocopherol or

ascorbic acid, inhibited the degree of cross-linking. The authors thus conclude that BC's role in the corpus luteum is to inhibit the cross-linking of the two enzymes, thereby retaining steroidogenic activity. This cross-linking has also been observed *in vivo*, in cows fed relatively high amounts of concentrates (i.e., grain) (R. Rodgers, personal communication). [Concentrates tend to have a lower carotenoid concentration than do forages, such as hay and grass silage.] This study is the first that suggests a specific mechanism by which beta-carotene might be related to progesterone synthesis.

Present Study

There is continued interest in the role of BC within tissues, in order to understand the possible mechanisms underlying the reported associations between BC and disease incidence in humans. For ethical and practical reasons, the study of BC metabolism in human tissues is very limited. It is therefore necessary to develop an animal model which absorbs and metabolizes BC in a manner similar to humans. The cow is one such model.

In addition to providing a model for human BC metabolism, the reported associations between BC and fertility in the cow are deserving of further study. Fertility, like the diseases in humans reported to be associated with BC, is a multifactorial process. This means that BC may play only a small role in one aspect of fertility, and its effects may only be apparent when other conditions are just right. This may explain the discrepancies between the various studies examining this issue.

Because of BC's ability to act as an antioxidant, the oxidative nature of steroidogenesis, the high concentration of BC in the CL, and the known role of the CL in fertility, the CL may prove to be an excellent model in which to study BC.

Specific Aims

With this in mind, the following study was designed to develop the bovine CL as a model for the study of tissue metabolism and function of BC and to elucidate a mechanism whereby BC might influence progesterone production or fertility by addressing the following specific aims:

1. Determine the relationship between dietary, plasma, lipoprotein, and corpus luteum concentrations of beta-carotene, and the variability in this relationship between cows.
2. Determine the subcellular distribution of beta-carotene within the bovine corpus luteum.
3. Determine the relationship between dietary, plasma, lipoprotein, or corpus luteum concentrations of beta-carotene and plasma progesterone concentration in the cow.
4. Determine the effect of varying dietary beta-carotene levels on the cross-linking of adrenodoxin and cytochrome P450 side-chain cleavage, two steroidogenic enzymes, in the corpus luteum.

CHAPTER II

MATERIALS AND METHODS

Experimental Design

Preliminary experiments

Preliminary experiments were conducted to determine the optimum method for CL beta-carotene quantitation, the intra- and inter-cow variability in plasma and CL beta-carotene concentration, and the relationship between plasma and CL beta-carotene concentrations. Five non-lactating Jersey cows were housed at the University of New Hampshire Dairy Teaching and Research Center in one pen of a bedded-pack barn. The cows were allowed ad libitum access to feed (typical dry cow total mixed ration containing haylage, corn silage, corn meal, soybean meal, and minerals) and water. Corpora lutea and plasma were collected ten days after cows exhibited standing heat.

Supplementation experiment

A supplementation experiment was conducted to determine the relationship between dietary and plasma and CL beta-carotene, the subcellular localization of beta-carotene within the CL, and the effect of dietary beta-carotene on the cross-linking of adrenodoxin and cytochrome P-450scc. Five non-lactating Jersey cows (different from those used in preliminary experiments) were housed at the University of New Hampshire Dairy Teaching and Research Center in one pen of a bedded-pack barn. All

procedures involving animals were approved by The University of New Hampshire Animal Care and Use Committee. Cows were fed once daily in individual feed bins. Access to feed was regulated by headgates (American Calan, Inc.) to individual feed bins which were keyed to open only when triggered by the transponder worn by the assigned cow. This assured that each cow was able to eat only that feed intended for her.

Cows received daily approximately sixteen pounds (as fed) of a basal ration consisting of 12 pounds chopped wheat straw, 3 pounds corn silage, 0.6 pounds soybean meal, and 0.6 pounds Sunshine Plus® pellets (Blue Seal Feeds, Londonderry, NH) mixed together. This was top-dressed with 2.6 pounds of Coarse 20® (Blue Seal Feeds), a molasses-coated grain. This ration was formulated to meet all essential requirements of the National Research Council (1989), while providing a minimal amount of beta-carotene.

This experiment lasted for twenty-eight weeks, and was divided into four blocks of seven weeks each. Top-dressed grain was supplemented with beta-carotene, in the form of water-soluble beadlets (kindly donated by Roche, Nutley, NJ), to create five treatment levels of 0.0, 1.6, 8.0, 16.0, or 80.0 mg of supplemented beta-carotene per day. Each cow was randomly assigned to receive one of these treatments for each seven-week block, such that, only one cow received any one concentration during each block and each cow received four of the five different treatments over the course of the experiment. This is a Latin Square design with one row missing (***Table 1***)(85); the sums of squares can be partitioned into four terms: block, cow, treatment, and error.

	Cow 1	Cow 2	Cow 3	Cow 4	Cow 5
Block 1	80	1.6	16	8	0
Block 2	16	0	8	1.6	80
Block 3	0	8	80	16	1.6
Block 4	1.6	16	0	80	8

Table 1 Incomplete Latin Square design showing supplemental dietary beta-carotene (mg/d) fed to cows during the supplementation experiment.

Sample Collection

Weekly blood samples were collected via jugular or tail venipuncture into vacutainers containing heparin. Plasma was separated from red blood cells by centrifugation at 2500 rpm, 4°C for 15 minutes, and was aliquotted and stored at -70°C for subsequent beta-carotene analysis.

Each cow was injected, intramuscularly, with 5 mL Lutalyse® (The Upjohn Company, Kalamazoo, MI) providing 25 mg dinoprost tromethamine (Prostaglandin F_2 alpha) approximately fourteen and twenty-five days prior to harvest of the corpora lutea (CL). These injections ensured that all five cows would be at the same stage of the estrous cycle, so that CL harvest could take place within a three-day window. Corpora lutea were obtained via enucleation per vaginum during the mid-luteal phase (9-12 days after ovulation) of the estrous cycle. Blood samples were collected from the jugular vein into vacutainers containing heparin just prior to CL harvest, and plasma was isolated and stored as described above.

Immediately after harvest, the papilla was removed from the CL, the CL was bisected, drained of any fluid and weighed. Portions of the tissue were flash frozen in liquid nitrogen for subsequent immunoblotting or placed in fixative, 10% neutral

buffered formalin or glutaraldehyde (3%) in cacodylate buffer (0.1M), for subsequent histological or electron microscopical examination, respectively. The remaining tissue was placed in ice-cold medium (Ham's F-12 with L-glutamine and without phenol red, Life Technologies, Grand Island, NY) containing gentamicin (3 mg / 100 ml, Life Technologies) and transported back to the lab.

Tissue Culture

Approximately 1.0 g of CL tissue was placed in a petri dish, connective tissue was removed, and the CL tissue was weighed. The CL tissue was then minced with two sterile scalpels to pieces smaller than 1.0 mm^3. The minced tissue was placed in a conical centrifuge tube containing 25.0 ml medium with gentamicin and 0.5% bovine serum albumin (BSA, Sigma Chemical, St. Louis, MO) and agitated in an orbital shaker bath (2500 rpm, 37° C) for two minutes. After allowing the tissue to settle, the medium was aspirated and replaced with fresh medium. To this, collagenase (Type I, Worthington Biochemical,) was added according to the following formula:

(2000)(weight of CL in grams) / (activity of collagenase) = mg of collagenase

The tube was then agitated in an orbital shaker bath (2500 rpm, 37° C). Every fifteen minutes during the digestion, tissue was further dissociated by pipetting up and down with a small bore pipet. After 60 minutes, remaining tissue pieces were allowed to settle, and the cloudy medium was transferred to a clean tube and centrifuged at 600 x *g* for 8 minutes. The supernatant was aspirated, the cell pellet gently resuspended in 10 ml of Ham's F-12 medium with gentamicin, and centrifuged 600 x *g* for 8 minutes. This process was repeated, typically two or three times, until the supernatant appeared clear. The supernatant was aspirated, and the cell pellet gently resuspended in 5.0 ml

of medium with gentamicin and 10% fetal bovine serum (FBS, Sigma Chemical, St. Louis, MO). Cells were counted using a hemocytometer and viability determined by trypan blue exclusion. Flasks (25 cm^2, Corning) were seeded with at least one million viable cells and brought to a final volume of 5.0 ml with medium containing gentamicin and 10% FBS (58). Flasks were incubated at 37° C in a 5% CO_2 atmosphere with 95% humidity. Approximately 16-18 hours later, non-attached cells were removed by aspiration, the monolayer was rinsed with medium containing gentamicin, and fresh medium (with gentamicin and 10% FBS) was applied. Forty-eight hours after the initial seeding, the medium was aspirated, the cell monolayer rinsed with Hank's Balanced Salt Solution without Ca^{2+} and Mg^{2+} (HBSS modified), and the cells harvested via trypsinization. The cell suspension was centrifuged and the resulting cell pellet was resuspended in 1.0 ml of lysing buffer (0.01 M phosphate buffered saline, 0.1% sodium dodecyl sulfate, 1% sodium cholate) and the lysate stored at -70°C for subsequent immunoblotting.

Progesterone

Plasma

Plasma progesterone concentrations were determined utilizing the Milenia Progesterone enzyme immunoassay (Diagnostic Products Corporation, Los Angeles, CA). Data reduction was performed using 4-parameter logistic regression in SigmaStat (Jandel Corporation, San Rafael, CA).

Corpus Luteum

Corpus luteum progesterone content was determined by radioimmunoassay (RIA)(86). Corpus luteum tissue (50-200 mg) was homogenized in 3.5 ml PBS with

0.1% gelatin and 0.1% sodium azide (PBSG) using a Polytron homogenizer. Subsequently, 100 µl of the homogenate was extracted twice with 2.0 ml petroleum ether, the petroleum ether was evaporated, and the residue resuspended in 500 µl PBSG. This was diluted (100x) and 100 µl was analyzed in duplicate by RIA using tritiated progesterone (0.01 µCi / assay tube) and anti-progesterone antibody (10,000x final dilution).

Subcellular Fractionation

Crude subcellular fractions were prepared as previously described (45,73). Approximately one gram of CL was homogenized in 5 volumes of 0.25 M sucrose containing 10 mM HEPES and 1 mM EDTA. The resulting homogenate was centrifuged (600 x *g* for 10 min. at 4°C) and the crude nuclear pellet was separated from the supernatant. The nuclear fraction was further purified by homogenizing in 1.0 ml of 0.8 M sucrose containing 10 mM HEPES, 1 mM EDTA, and 1 mM $MgCl_2$ and centrifugation (67,000 x *g* for 30 min. at 4°C). The postnuclear supernatant obtained earlier was centrifuged (17,300 x *g* for 20 min. at 4°C) to obtain the mitochondrial pellet. The postmitochondrial supernatant was centrifuged (102,000 x *g* for 60 min. at 4°C) to separate the floating lipid fraction, cytosolic fraction, and microsomal pellet.

Beta-carotene Extraction

Plasma

The procedure used to extract and quantify plasma beta-carotene is based on methods previously described (87). Four hundred µl of plasma was precipitated with

200 µl of 100% ethanol and 200 µl of an ethanolic solution of retinyl acetate and tocopherol acetate as an internal standard, and then extracted twice with 2.5 ml of hexane. The hexane layer was collected in an amber glass vial with a teflon-lined cap (Scientific Specialties, Randallstown, MD) and evaporated under a stream of nitrogen (N_2) at 30°C. The sample was reconstituted in 200 µl of 100% ethanol, thus concentrating the sample twice.

High-density Lipoprotein

To 500 µl of plasma, was added 50 µl of HDL Cholesterol Reagent (dextran sulfate (10g/L) and Mg ions (0.5 mol/L), Sigma) to precipitate the low- and very low-density lipoproteins (88). After vortexing and incubating for five minutes at room temperature (RT), the solution was centrifuged (1500 x *g*, 10 min, RT). Four hundred µl of the supernatant (HDL containing solution) was removed and extracted as described for plasma above.

Tissue

Approximately 0.1 to 0.3 g of CL tissue was minced, weighed, and placed into a glass homogenization pestle with 1.0 ml of 0.5% collagenase (Type Ia, Sigma) in phosphate buffered saline (PBS) and 25 µl of 25% ascorbate (89). Digestion was allowed to take place for one hour in an orbital shaker bath (2500 rpm, 37°C). The solution was homogenized with a drill-mounted Teflon mortar (three strokes, 1500 rpm) and 1.0 ml of 100% ethanol was added to precipitate the proteins. The resulting solution was extracted three times with 2.5 ml of hexane, and the hexane extract was pooled in an amber vial. The hexane was subsequently evaporated at 30°C under N_2,

and the resulting residue was resuspended in ethanol (1.0 ml per gram of tissue digested) for analysis by HPLC.

Subcellular Fractions

Subcellular fractions were precipitated with the ethanolic internal standard and extracted twice with 2.5 ml of hexane. The hexane was subsequently evaporated at 30° C under N_2, and the resulting residue was resuspended in ethanol for analysis by HPLC.

Beta-carotene Analysis

The HPLC (high performance liquid chromatography) system (Beckman 338) was a reverse-phase HPLC with a C18 Baker Bond wide pore (5 μm) column (J.T. Baker, Phillipsburg, NJ), a variable wavelength spectrophotometer (Beckman 406), and a Beckman 167 Scanning Detector Module (Beckman Instruments, Wakefield, MA). The mobile phase was a gradient changing from solvent A (methanol and 0.1% ammonium acetate) to solvent B (methanol: methylene chloride (80:20) and 0.1% ammonium acetate) over the first four minutes of the run. The wavelengths were set at 292, 325, and 452 nm for detection of tocopherol acetate, retinyl acetate, and beta-carotene, respectively. The flow rate was 1.5 ml per minute. Twenty μl of sample was injected for each sample. Signal acquisition, integration, and beta-carotene quantitation were performed with an HP 3365 ChemStation (Hewlett-Packard, Burlington, MA) based on a standard curve. The internal standard was used to correct for any volume lost during preparation of the sample. Throughout the research period, the laboratory participated in the micronutrients measurement quality assurance

program sponsored by the National Institute of Standards and Technology, Gaithersburg, MD.

Gel Electrophoresis

Frozen CL tissue was thawed and homogenized in 1.0 ml lysing buffer in a glass on glass homogenizing pestle. The homogenate was centrifuged (10000 x g, 10 minutes) to remove particulate matter and the protein concentration was determined by the method of Markwell (90). Homogenates were mixed with buffer (91) containing 1% SDS, 0.2 M β-mercaptoethanol, and 1 mM EDTA (Bio-Rad Laboratories, Hercules, CA) to give 5.0 or 50.0 μg protein per 25 μl solution for separation of adrenodoxin or cytochrome P-450scc, respectively. Samples were boiled for 10 min prior to electrophoresis on polyacrylamide gels (4% stacking gel and 10 or 15% separation gel for Adx or cytochrome P-450scc, respectively). Samples, pre-stained molecular weight markers (Broad range, Bio-Rad Laboratories), and protein standards (Adx purified according to protocol below) were electrophoresed for 2 hours at 100 volts using a Bio-Rad Mini-Protean II electrophoresis system.

Electroblotting

Separated proteins were transferred from gels to nitrocellulose membranes (0.2 or 0.45 μm pore size for Adx or cytochrome P-450scc, respectively). Transfer was accomplished using a Bio-Rad Mini-Protean II electroblotting system with 20% methanol, 10 mM Tris, 150 mM glycine solution, 4°C, 75mA for 16 hours (92).

Western Immunoblotting

Membranes were incubated in milk block solution (Kirkegaard-Perry Laboratories, Gaithersburg, MD) for 1 hour at RT and then with specific antibody (Adx antibody raised as described below and anti-rat cytochrome P-450scc from American Research Products, Belmont, MA) for two hours at RT. Membranes were washed three times, for 15 min, with wash solution (Kirkegaard-Perry Laboratories) and incubated with secondary antibody (alkaline phosphatase-labeled goat anti-rabbit IgG, Kirkegaard-Perry Laboratories) for 1 hour at RT. Membranes were washed three times, for 15 min, with wash solution and incubated with 5-bromo-4-chloro-3-indoyl-phosphate/nitroblue tetrazolium substrate (BCIP/NBT, Kirkegaard-Perry Laboratories) for approximately 15 min. or until color development was apparent.

Histology

Paraffin-embedded sections were prepared for histological examination by the Veterinary Diagnostic Laboratory at the University of New Hampshire. Sections were stained with either hematoxylin and eosin (H+E) or Masson's trichrome (93), and examined using an Olympus Model BHS System Microscope (Olympus Corporation, Lake Success, NY)

Transmission Electron Microscopy

Tissues were fixed in glutaraldehyde (3%, Polysciences, Inc., Warrington, PA) in cacodylate buffer (0.1M, pH 7.3) with $CaCl_2$ (1 drop of 1% per 10 ml fixative) for four hours at RT. Tissue was rinsed four times with cacodylate-sucrose buffer (~300

mOsm) and postfixed in 1% osmium tetroxide (OsO_4, Polysciences, Inc.) in 0.1M cacodylate buffer (pH 7.2) for 1 hr at 4°C. The tissues were rinsed four times with cacodylate-sucrose buffer and dehydrated in a graded series of ethanol dilutions (35%, 50%, 70%, 95%, and twice at 100%) for 15 min each and then placed in a transitional solvent, propylene oxide, for 15 min at RT. Tissue was then immersed in a mixture of complete resin (Epon 812 with 40% dodecenylsuccinic anhydride, 60% nadic methyl anhydride, and 0.14 ml / 10 ml of 2,4,6 tri-(dimethylaminomethyl) phenol) and propylene oxide (1:1 v/v) for 1 hour, followed by overnight infiltration with complete resin (94). Tissue was then embedded with fresh complete resin in (00) BEEM capsules (Polysciences, Inc.), placed in an oven at 60°C for 72 hrs to allow the resin to polymerize, and the BEEM capsules were removed from the solidified resin. Thin sections of 90-150 nm were cut using a Reichert-Jung Ultracut E (Cambridge Instruments, Inc., Deerfield, IL) with a diamond knife (Edgecraft Corporation, Avondale, PA) and mounted on 200 square mesh copper grids (Ernest F. Fullam, Inc., Latham, NY). The sections were stained with 2% aqueous uranyl acetate for 15 min at RT, rinsed in double distilled H_20, followed by staining with aqueous lead citrate for 30 min at RT in the presence of sodium hydroxide pellets to prevent reaction with atmospheric CO_2 (95). All stained tissues were examined and photographed with a JEOL 100S transmission electron microscope (JEOL, Inc., Peabody, MA).

Adrenodoxin Isolation and Antibody Production

Adrenodoxin was isolated from bovine adrenals (PelFreez Biologics, Rogers, AR) by the method of Orme-Johnson and Beinert (96). Whole bovine adrenals (500 g) were homogenized in 1 liter of buffer containing 0.05 M KCl with a Waring

blender. The homogenate was centrifuged for 15 min at 1600 x *g*, and the pellet was resuspended in 500 ml of buffer and centrifuged again. The supernatants were combined, chilled to 4°C, and 10 g diethylaminoethyl cellulose (DEAE-cellulose, Sigma) were added while stirring. The solution was allowed to stir overnight at 4°C. The DEAE-cellulose, with adsorbed protein, was recovered by centrifugation at 1600 x *g*, 4°C for 10 min. The DEAE-cellulose was washed twice with buffer (0.1 M KCl) by resuspension and centrifugation. The DEAE-cellulose was transferred to a Buchner funnel, and the adsorbed protein was removed with three 100 ml washes of buffer (0.5 M KCl). The resulting solution was diluted 5-fold with buffer (0.05 M KCl), any turbidity removed by centrifugation (14000 x *g*, 4°C, 30 min), and then passed through a column (2.5 x 15 cm) of DEAE-cellulose equilibrated with buffer (0.05 M KCl). The column was washed with buffer containing 0.17 M KCl (500 ml) and the brown protein was eluted with buffer containing 0.33 M KCl (30 ml). This solution was passed through a Sephadex G-100 (Sigma) column (2.5 x 50 cm) equilibrated with buffer containing 0.1 M KCl and 0.05 M Tris, pH 8.1. The brown protein was eluted with equilibrating buffer, collected in approximately 30 ml, and passed through a column (1.5 x 8 cm) of DEAE-cellulose equilibrated with buffer (0.05 M KCl). The column was washed with equilibrating buffer and the brown protein was eluted with ~ 4 ml of buffer containing 0.5 M KCl. The resulting solution was dialyzed overnight at 4°C against 500 volumes of 0.1 M Tris, pH 8.1, under nitrogen. The resulting solution was assessed at The University of New Hampshire Protein Sequencing Facility, using a PI 2090 Integrated Micro-Sequencing System (Porton Instruments) to verify the presence of adrenodoxin.

Antibodies to bovine adrenodoxin were prepared by polyclonal antibody production (97). Two New Zealand White rabbits, approximately four months old were immunized by subcutaneous injections with 2 mg bovine adrenodoxin, purified as stated above. The protein was emulsified in 1 mL of complete Freund's adjuvant (Sigma) and 1 mL phosphate-buffered saline for the primary immunization. Two mL of the emulsion was injected at ~ 20 sites (0.1 mL per injection site). The immunizations were repeated four times at two week intervals with incomplete Freund's adjuvant. Blood samples (5-30 mL) were drawn with a 20 gauge needle from the central ear artery, and tested for the presence of antibodies reactive with adrenodoxin, by enzyme-linked immunoassay (ELISA), at one week intervals beginning 5-6 weeks after the initial immunization. Acepromazine maleate (0.1 mL, 10 mg/mL) was injected into the marginal ear vein for vasodilation. The rabbits were exsanguinated by cardiac puncture eleven weeks after the initial immunization and antibody was purified by affinity chromatography.

Statistical Analysis

Data were analyzed using the PROC GLM module of SAS (SAS Institute Inc., Cary, NC, Proprietary Software Release 6.11 Licensed to University Of New Hampshire, Site 0004324001.) and linear orthogonal contrasts were determined (85). Data were grouped according to the amount of dietary BC fed to the cow during each block of the supplementation experiment. Values are reported as least squares means ± SEM.

CHAPTER III

RESULTS

Extraction and Quantification

The extraction and quantification of BC from tissue is generally more complicated than extraction from plasma, because of the various matrix components in tissue. In plasma, BC is associated with the lipoproteins and may be extracted with hexane following precipitation of the plasma proteins with ethanol. In other matrices, there is the problem of ensuring that the BC is free to partition into the hexane layer. Therefore, three different protocols were tested for the extraction of BC from CL tissue: direct extraction of homogenized tissue, saponification (the digestion of tissue with 10 N KOH prior to hexane extraction), and collagenase digest. Direct extraction proved impractical, because the hexane used in the extraction formed a gel with some component of the homogenized tissue. Saponification resulted in the splitting of various peaks in the chromatogram, making it difficult to quantify BC, and was therefore not used (data not shown). The method chosen was the collagenase digest detailed in the material and methods section. Representative chromatograms show the baseline separation of those analytes identified and quantified (**Figure 2**). While not quantified, there was no apparent change in the shape of the beta-carotene peak which might be indicative of isomerization.

To determine the number of extractions needed to recover the BC from the CL, tissue was digested with collagenase, homogenized, and precipitated with ethanol, and the resultant homogenate was extracted nine times with 2.5 ml of hexane. Each extract was then analyzed separately by HPLC to determine the BC concentration. The BC content of the fourth through ninth extractions was below the limit of detection when analyzed separately, so these extractions were pooled. By dividing the BC content of each fraction by the total BC content of all fractions, a percent of total BC recovered per fraction was obtained. The recovery was 58, 29, 8, and 5 % for the first, second, third, and fourth-ninth extractions, respectively (values are means of three trials). Subsequently, all CL homogenates were extracted three times with 2.5 ml of hexane.

To establish the validity and estimate the analytical variability of this method, BC was quantified ten times each (each analysis performed on a different day) in three corpora lutea, which had been minced fresh, and frozen in individual vials ($\sim$0.1 g). The analytical variability in assessing the CL beta-carotene concentration was determined to be 4.4% (**Table 2**), so the method was accepted as valid. Once the protocol was accepted, all tissue was analyzed in duplicate (i.e., duplicate extractions). The variation in alpha-tocopherol and BC between duplicate extractions has remained below 5% (data not shown).

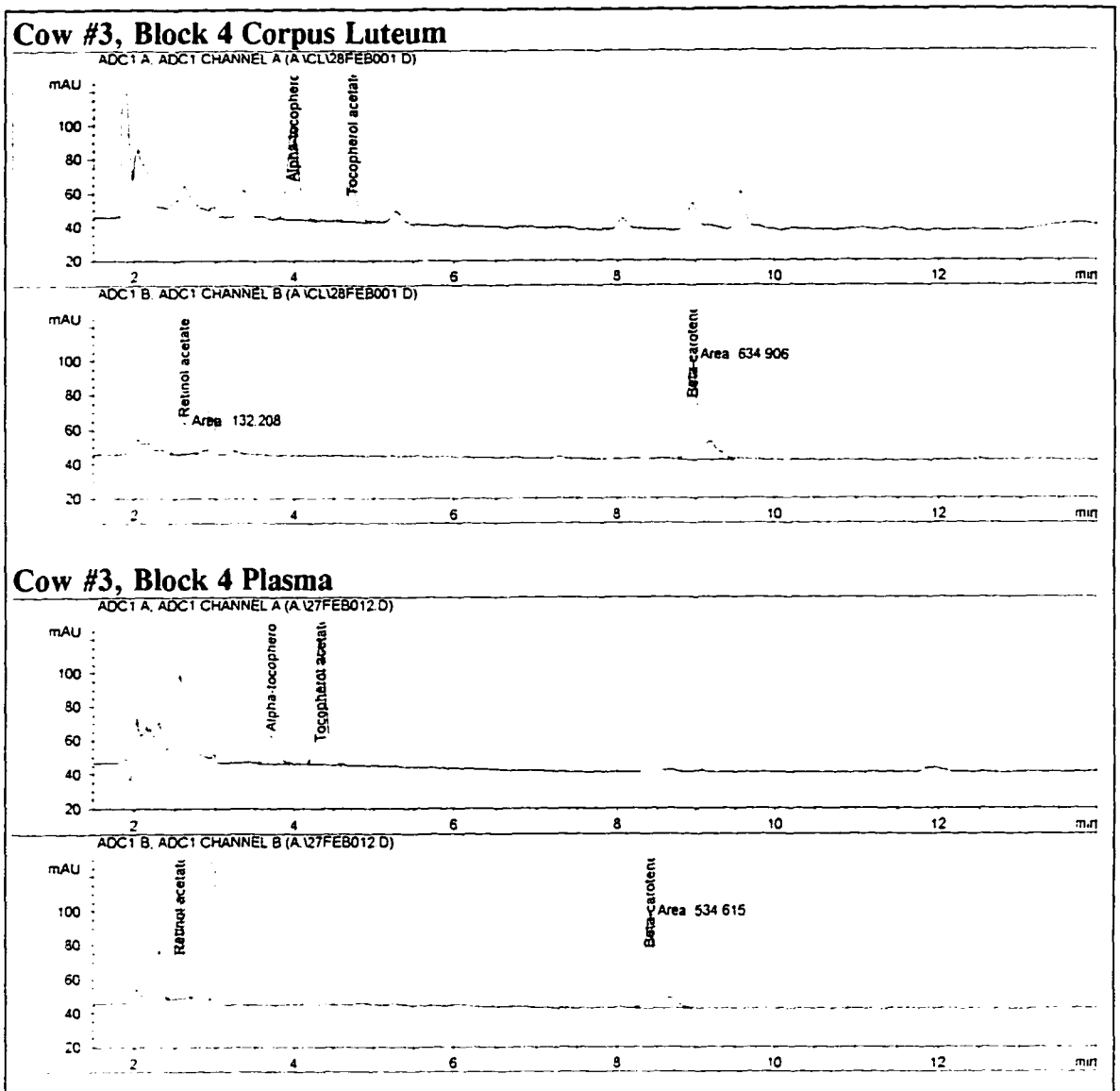

Figure 2 **Representative chromatograms.** Representative chromatograms of corpus luteum (top) and plasma (bottom) are shown. The top panel in each chromatogram is channel A, which represents the sample absorbance at 292 nm. The bottom panel in each chromatogram is channel B, which represents the sample absorbance at 325 nm from 0-3 min and 452 nm after 3 min. Identified peaks are labeled with their compound name: retinol acetate, beta-carotene, alpha-tocopherol, or tocopherol acetate.

	CL Beta-carotene (nmol/g)		
Replicate	**CL #1**	**CL #2**	**CL #3**
1	87.125	110.325	96.378
2	84.321	115.236	100.598
3	86.467	120.586	92.786
4	94.904	105.647	95.888
5	95.453	110.594	103.674
6	91.676	115.389	91.357
7	93.546	117.845	97.685
8	88.734	108.397	94.265
9	91.424	102.348	98.674
10	96.060	106.380	101.356
Coefficient of Variation	**4.3%**	**5.0%**	**3.8%**

Table 2 **Analytical variability**. The analytical variability in quantitation of the beta-carotene concentration within the bovine corpus luteum was determined by analyzing frozen tissue samples from three corpora lutea. Each analysis for any one CL was performed on a different day. The coefficient of variation is equal to the standard deviation divided by the mean BC concentration determined for that CL expressed as a percent.

Inter- and Intra- cow Variability

There is considerable variability in plasma BC concentrations in humans and in animals, which is difficult to account for by dietary intake alone. Accounting for this variability is important in assessing the number of animals needed for an experimental protocol. Because of this, and the fact that little is known about the variability of BC concentration within tissues, preliminary experiments with five non-lactating Jersey cows were conducted to determine the variability in plasma and CL beta-carotene between cows and between sampling periods in the same cow (***Table 3***). The intra-cow variability was 12.9% and 18.3% and the inter-cow variability was 26.5% and 36.3% for plasma BC and CL BC, respectively.

	Plasma Beta-carotene		Corpus Luteum Beta-carotene	
Cow-Sample	**(μmol/l)**	**C.V. (%)**	**(nmol/g)**	**C.V. (%)**
1-1	10.912		125.126	
1-2	10.154		115.625	
1-3	9.539	5.5	107.222	6.3
2-1	17.090		217.854	
2-2	12.381		248.399	
2-3	12.693	15.3	162.112	17.1
3-1	9.980		75.626	
3-2	10.208		172.050	
3-3	6.413	19.6	130.157	31.3
4-1	7.728		86.581	
4-2	6.611		105.239	
4-3	9.520	15.1	70.541	16.2
5-1	7.423		143.557	
5-2	8.498		200.345	
5-3	9.222	8.8	125.368	20.4
Average C.V. (%)	**26.5**	**12.9**	**36.3**	**18.3**

Table 3 Beta-carotene concentration variability. The variability in plasma and corpus luteum beta-carotene concentrations was determined for a group of five cows on a typical dry cow ration. The coefficient of variation is equal to the standard deviation divided by the mean BC concentration expressed as a percent. The intra-cow coefficient of variation (C.V.) is the mean of each cow's individual C.V., while the inter-cow C.V. is the variation between all measures.

Plasma Beta-carotene Depletion

Because of the accumulation of BC within the tissues, the adjustment of plasma BC concentrations to dietary modification may take some time. To determine how long it would take for the plasma BC concentration to level out in cows being depleted of BC, four cows were fed the basal ration, weekly blood samples were taken, and separated plasma was analyzed for BC concentration. Plasma BC concentrations decreased rapidly for the first three weeks, and then continued to decline more slowly.

Plasma BC concentration leveled out at about seven weeks (**Figure 3**). In one cow (Cow#5) which was fed the basal ration for fourteen weeks, plasma BC concentration declined from 4.077 μmol/l to 2.278 μmol/l at the end of seven weeks, and to 1.617 μ mol/l at the end of fourteen weeks (**Figure 4e**). Seven weeks was selected as the time for each block in the supplementation experiment because it allowed sufficient time for recovery and healing between CL harvests, and the rate of change in plasma BC concentration after this time point is small.

These data were also used to confirm that the basal ration, as formulated, would be sufficiently low in BC to cause a predicted serum response. During the depletion period, plasma alpha-tocopherol (AT) concentration was also measured. Plasma AT concentrations also declined during this period (36% reduction, data not shown). The decline in plasma AT was not anticipated and could confound the interpretation of any results due to changes in BC concentration, so the cows were fed Sunshine-Plus Pellets, a commercially-available AT supplemented grain, during the supplementation experiment to ensure that any effects seen were not due to AT depletion.

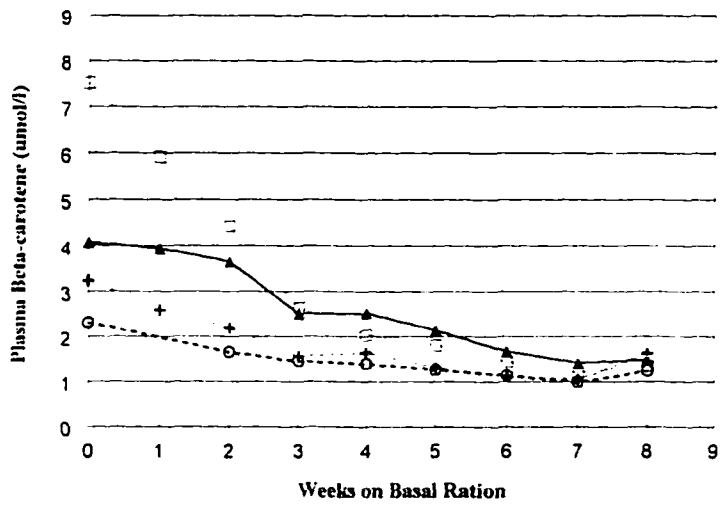

Figure 3 **Plasma beta-carotene depletion.** The change in plasma beta-carotene concentration (μmol/l) in four cows fed the basal ration containing low dietary beta-carotene. The rate of decline in plasma beta-carotene decreases, such that plasma beta-carotene concentrations change little after about seven weeks.

Supplementation Experiment

For the supplementation experiment, a total of eighteen corpora lutea were collected, four each from three cows and three each from two cows. One cow appeared to have a cystic ovary at the time of CL collection at the end of the third block and the CL of another cow, at the end of the fourth block, was not retrieved due to a build up of scar tissue. Some cows failed to show behavioral estrus, so corpora lutea were obtained thirteen or fourteen days after the second Lutalyse® injection. Since Lutalyse ® is a prostaglandin which induces ovulation within 80 hours, the corpora lutea obtained were mid-cycle (9-11 days post-ovulation). When cows did show behavioral estrus, corpora lutea were obtained ten days later.

Plasma Beta-carotene

The change of plasma BC with dietary modification over time is shown in **Figures 4a-4e**. These data show the plasma response to four different levels of supplemental BC. For all treatments, there was an unexpected general increase in the BC concentration after week eight, and a general downward trend after week twenty. These changes coincided with a change in the lot of straw which was being fed in the basal ration, suggesting that these variations were due to changes in the BC content of the basal ration. Because of this, the average plasma BC concentration observed for blocks two and three was greater than that for blocks one and four. As a consequence, during blocks two and three the CL beta-carotene concentration of cows receiving the lowest levels of supplementary BC were higher than those of cows receiving the same levels during blocks one and four (***Table 4***).

Plasma BC concentrations measured at the time of CL harvest reflected the amount of supplemental BC fed to the cow. Cows receiving the highest level of dietary BC had significantly higher plasma BC concentrations than any other treatment level ($p<0.01$), but there was not a statistically significant difference between the four lowest treatment levels. However, orthogonal contrast analysis showed that there was a significant linear response to the level of supplemental dietary BC ($p<0.001$) (**Figure 5**).

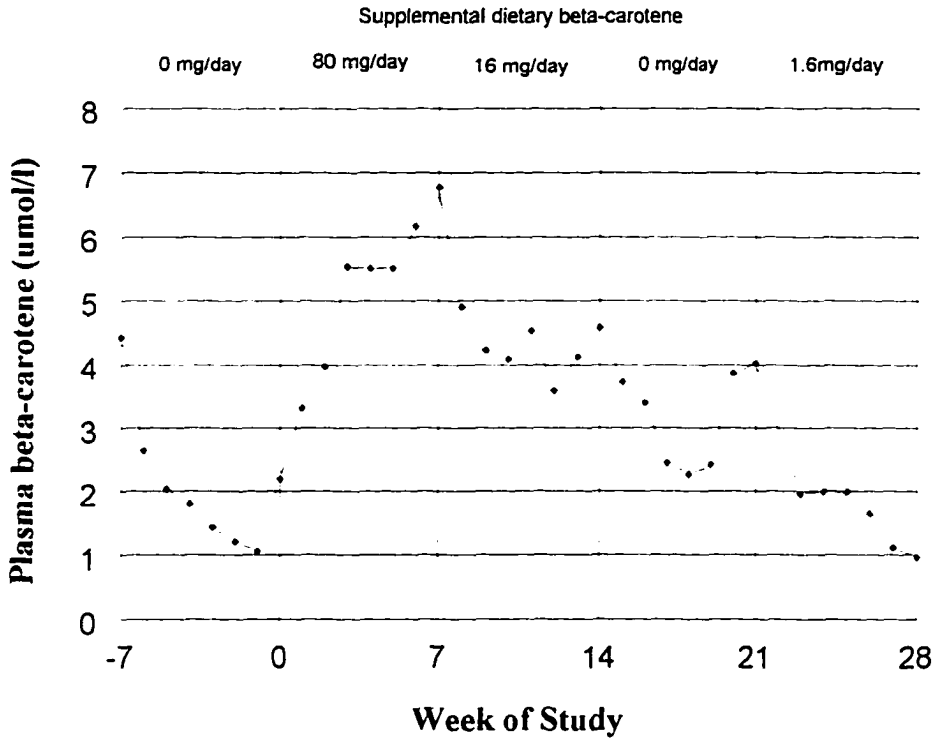

Figure 4a **Plasma beta-carotene timecourse (Cow#1).** The change in plasma beta-carotene concentration (μmol/l) in **Cow#1** over the seven weeks preceding, and the twenty-eight weeks of the experiment. Supplemental beta-carotene refers to the amount of beta-carotene fed per day in top-dressed grain during each seven week treatment period.

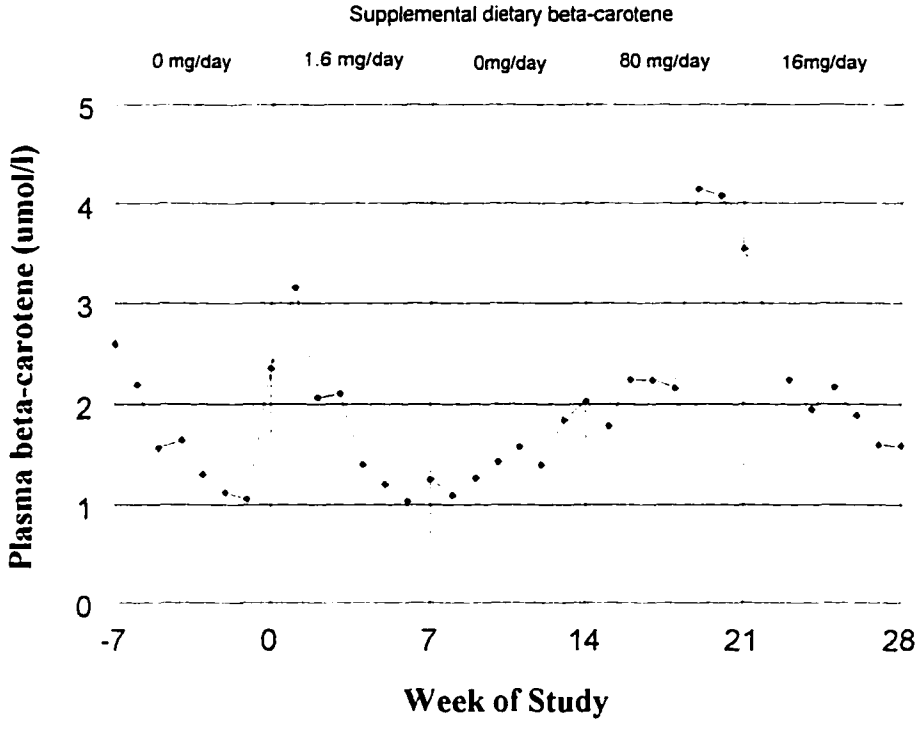

Figure 4b **Plasma beta-carotene timecourse (Cow#2).** The change in plasma beta-carotene concentration (μmol/l) in **Cow#2** over the seven weeks preceding, and the twenty-eight weeks of the experiment. Supplemental beta-carotene refers to the amount of beta-carotene fed per day in top-dressed grain during each seven week treatment period.

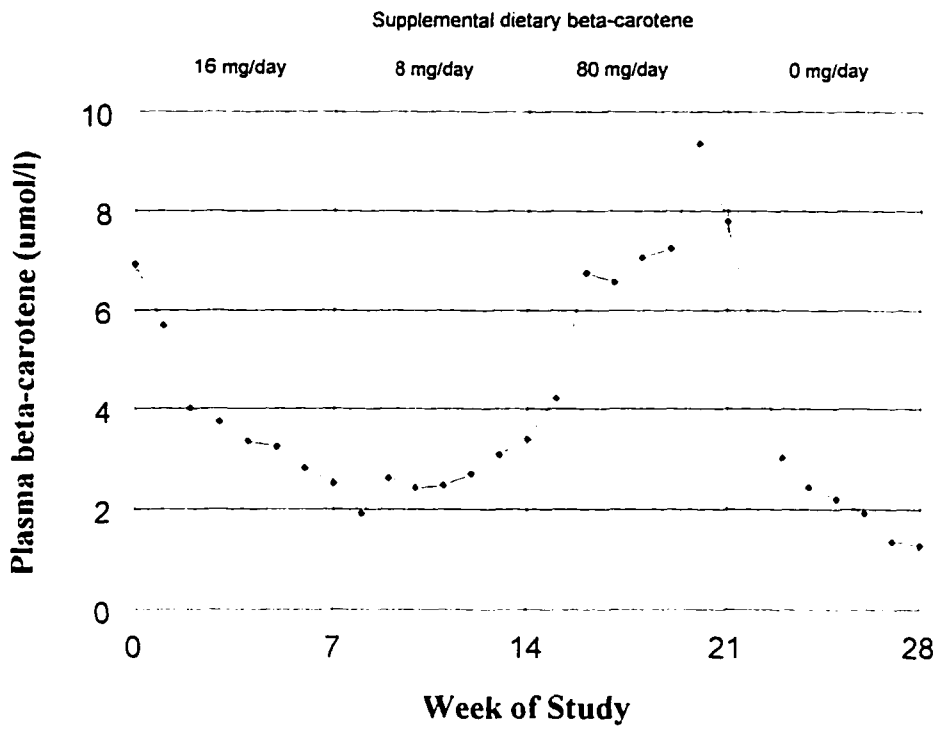

Figure 4c **Plasma beta-carotene timecourse (Cow#3).** The change in plasma beta-carotene concentration (μmol/l) in **Cow#3** over the twenty-eight weeks of the supplementation experiment. Supplemental beta-carotene refers to the amount of beta-carotene fed per day in top-dressed grain during each seven week treatment period.

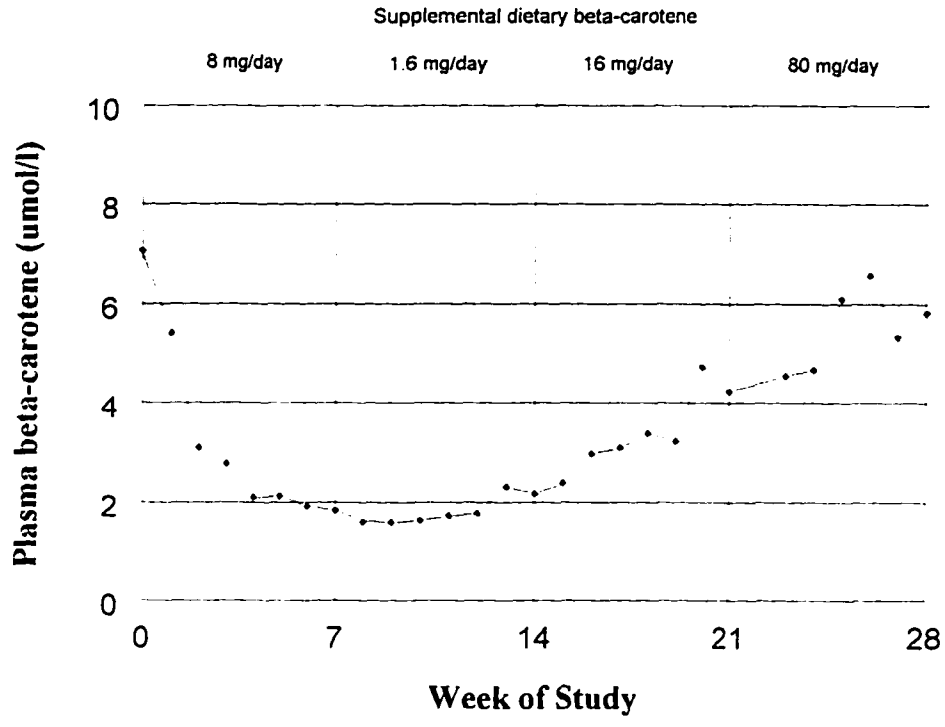

Figure 4d **Plasma beta-carotene timecourse (Cow#4).** The change in plasma beta-carotene concentration (μmol/l) in **Cow#4** over the twenty-eight weeks of the supplementation experiment. Supplemental beta-carotene refers to the amount of beta-carotene fed per day in top-dressed grain during each seven week treatment period.

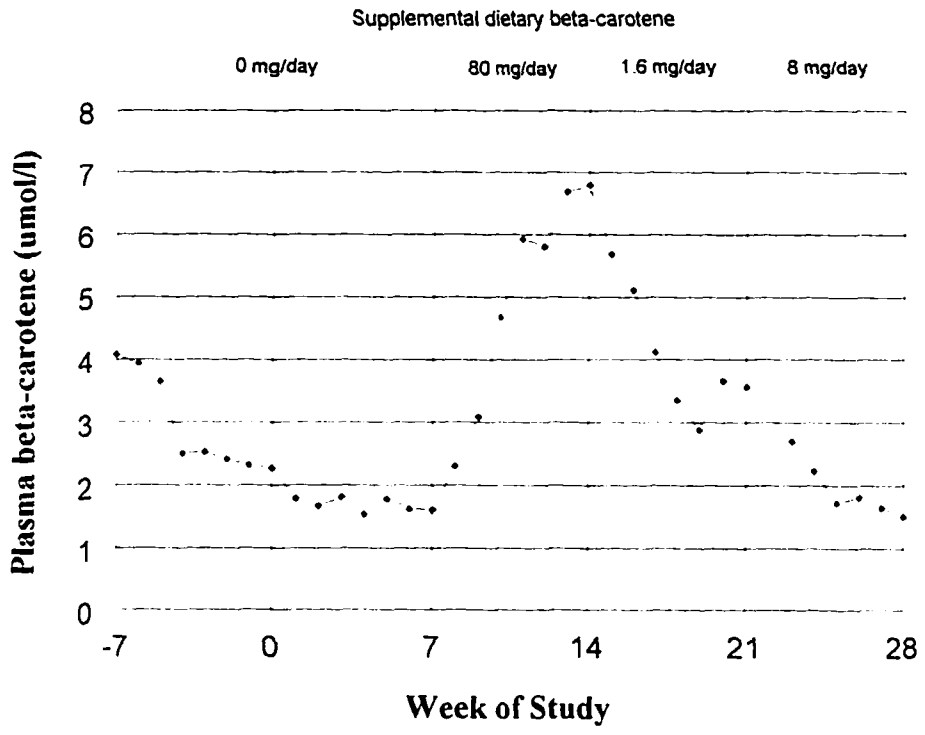

Figure 4e **Plasma beta-carotene timecourse (Cow#5).** The change in plasma beta-carotene concentration (μmol/l) in **Cow#5** over the seven weeks preceding, and the twenty-eight weeks of the experiment. Supplemental beta-carotene refers to the amount of beta-carotene fed per day in top-dressed grain during each seven week treatment period.

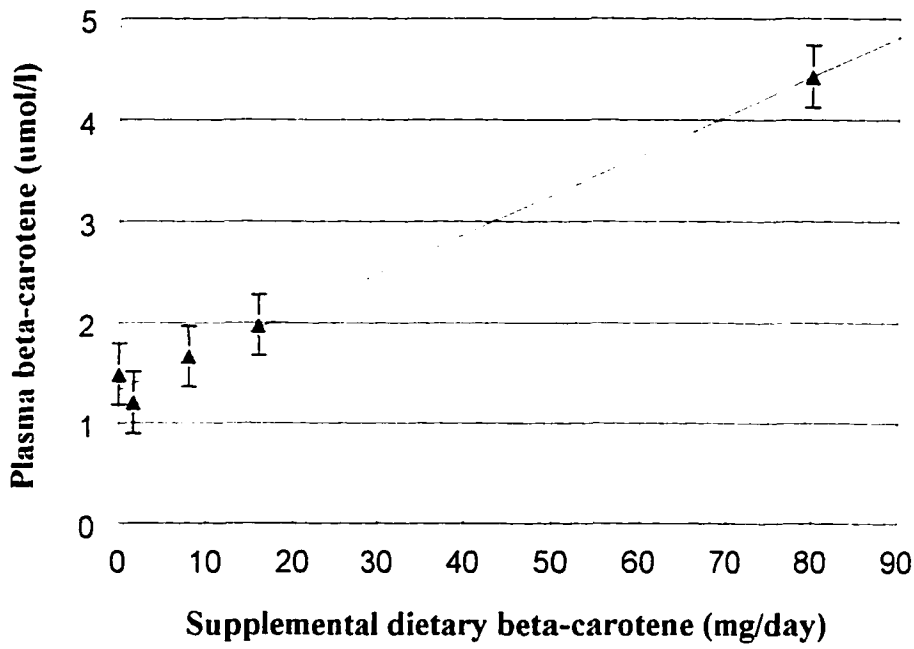

Figure 5 **Plasma beta-carotene.** Plasma beta-carotene concentration (μmol/l) on day of corpus luteum harvest grouped by supplemental dietary beta -carotene (mg/day) received in the preceding seven weeks. Values are least squares means ± SEM. n = 4 for each treatment. There was a significant linear response to the level of supplemental dietary beta-carotene ($p < 0.001$).

Cow	Block	Supplemental BC (mg/day)	Plasma BC (μmol/l)	Corpus luteum BC (nmol/g)
3	4	0.0	1.267	4.762
5	4	8	1.521	7.073
1	4	1.6	0.968	8.922
2	1	1.6	0.709	11.035
3	1	16	1.421	15.939
4	1	8	1.040	16.388
5	1	0	0.916	18.077
1	3	0	2.279	19.568
4	2	1.6	1.230	21.101
5	3	1.6	2.019	25.121
3	2	8	1.923	26.212
2	2	0	1.150	32.901
1	2	16	2.599	39.745
4	3	16	2.399	41.135
2	3	8	2.009	53.017
1	1	80	3.835	53.283
5	2	80	3.849	57.770
4	4	80	5.828	58.445
2	4	16	1.588	No CL
3	3	80	4.416	No CL

Table 4 Beta-carotene concentrations by cow and block. Plasma and corpus luteum beta-carotene concentrations of cows on day of corpus luteum harvest during the supplementation experiment. Supplemental beta-carotene refers to the amount of beta-carotene in top-dressed grain. Values are sorted by corpus luteum beta-carotene concentration (corpora lutea were not obtained from two cows, indicated by No CL).

Relationship between plasma and HDL beta-carotene

Because of the narrow range of BC concentrations (6.41-17.09 μmol/l plasma) in previous experiments (**Table 3**), preliminary experiments failed to show the significant relationship between plasma and HDL beta-carotene concentrations (Pearson's R = 0.456, p=.217). However, when this data was combined with the data from the dietary BC manipulation experiment, which resulted in a broader range

of BC concentrations (0.96-17.09 μmol/l plasma) and increased the sample size, the relationship was clear (Pearson's R= 0.868, $p<0.01$). There was a direct relationship between plasma BC concentration and the BC associated with high density lipoprotein (**Figure 6**). On average, 33% of the plasma BC was associated with the HDL fraction.

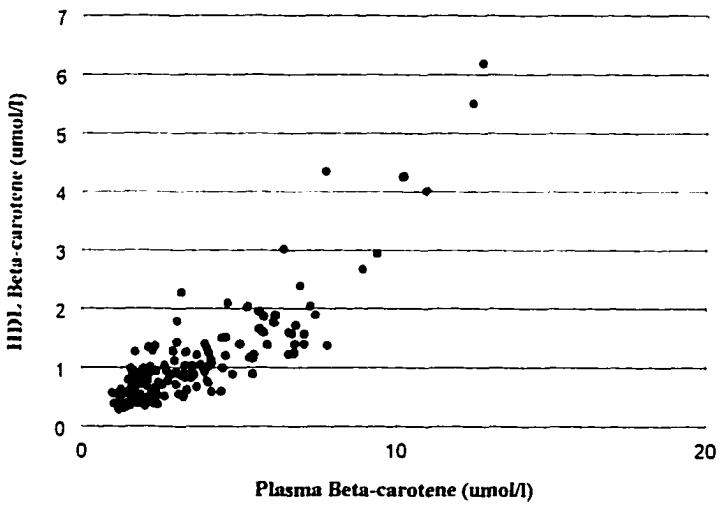

Figure 6 **Plasma vs. HDL beta-carotene.** Relationship between plasma beta-carotene concentration and the amount of beta-carotene carried in the high density lipoprotein fraction of plasma (n=148). Pearson's R= 0.868, $p<0.01$.

Corpus luteum beta-carotene

To determine the effect of dietary BC on the tissue concentration of BC, the concentration of BC was determined for each CL collected (***Table 4***). As shown by linear orthogonal contrast, the concentration of BC in the CL was directly related to the amount of dietary BC received by the cow in the preceding seven week block ($p<0.001$) (**Figure 7**). Further, this effect was mediated by the changes in plasma BC concentrations, as evidenced by the direct linear relationship between the concentration of BC in the plasma and the concentration of BC in the CL of cows from both the preliminary and supplementation experiments (**Figure 8**) (Pearson's R = 0.903,$p<0.001$, n=27).

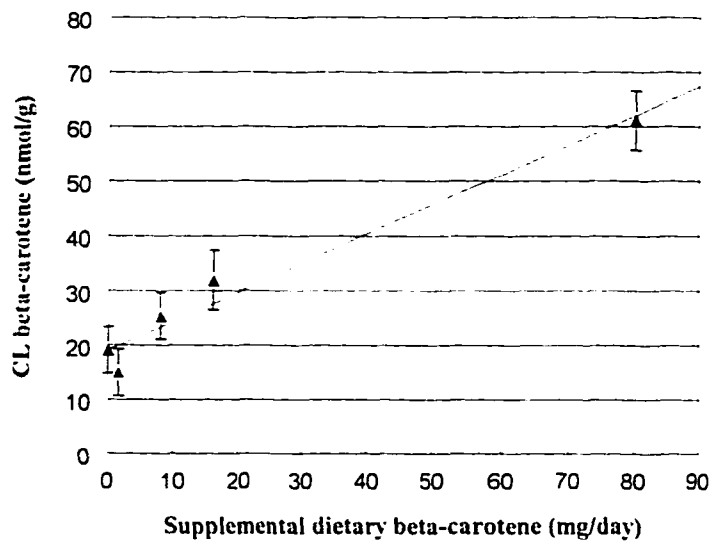

Figure 7 **Corpus luteum beta-carotene.** Corpus luteum beta-carotene concentration (nmol/g) grouped by supplemental dietary beta-carotene (mg/day) received in the preceding seven weeks. Values are least squares means ± SEM, n = 4 for each treatment, except 16 and 80 mg/day where n = 3. There was a significant linear response to the level of supplemental dietary beta-carotene ($p<0.001$).

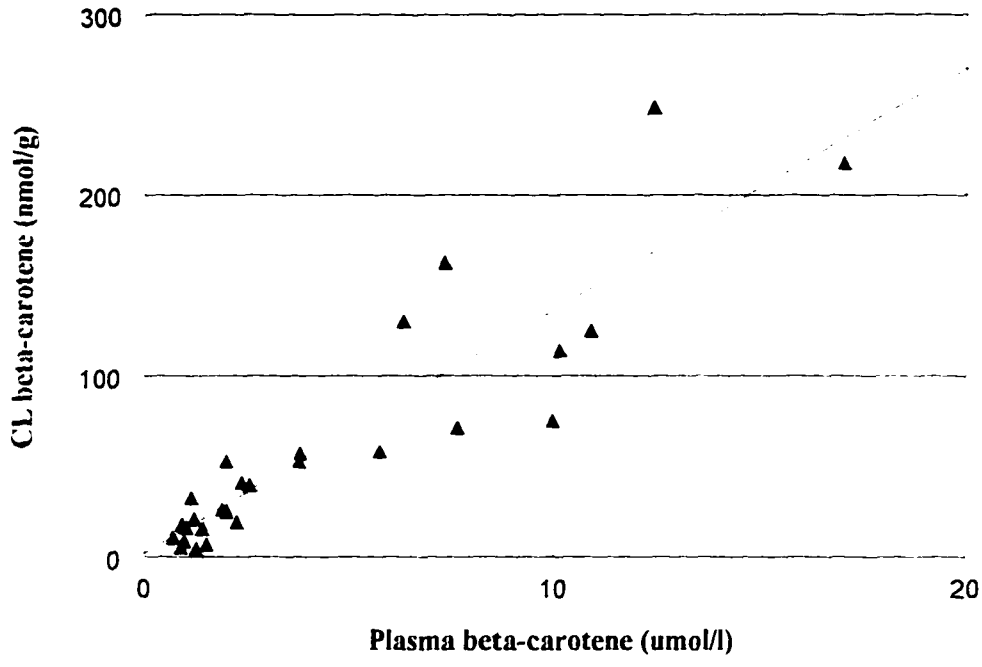

Figure 8 **Plasma vs. corpus luteum beta-carotene.** Relationship between plasma beta-carotene concentration (μmol/l) on day of CL harvest and corpus luteum beta-carotene concentration (nmol/g). The relationship (Pearson's R = 0.903) was significant ($p < 0.001$, $n = 27$).

Subcellular Fractionation

To determine if the change in CL beta-carotene concentration was due to changes in BC within lipid droplets or within organelles, subcellular fractionation was performed. A portion of each of the eighteen corpora lutea collected during the supplementation experiment were fractionated, as described, to obtain crude mitochondrial, nuclear, microsomal, and lipid fractions. The amount of BC in each fraction was determined and expressed relative to the amount of protein in that fraction.

Graphical depictions of the results are below (**Figures 9a-9d**). Changes in total CL beta-carotene concentration were paralleled by each subcellular fraction.

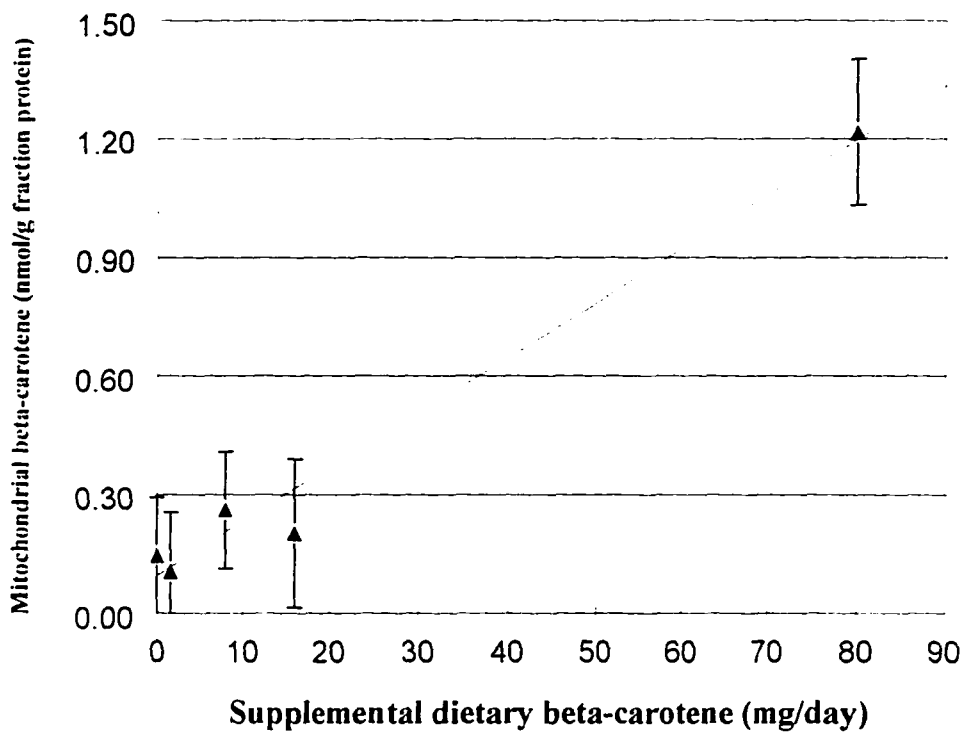

Figure 9a **Mitochondrial fraction beta-carotene.** Corpus luteum mitochondrial beta-carotene concentration (nmol/g fraction protein) grouped by supplemental dietary beta-carotene (mg/day) received in the preceding seven weeks. Values are least squares means ± SEM, n = 4 for each treatment, except 16 and 80 mg/day where n = 3. There was a significant linear response to the level of supplemental dietary beta-carotene ($p < 0.01$).

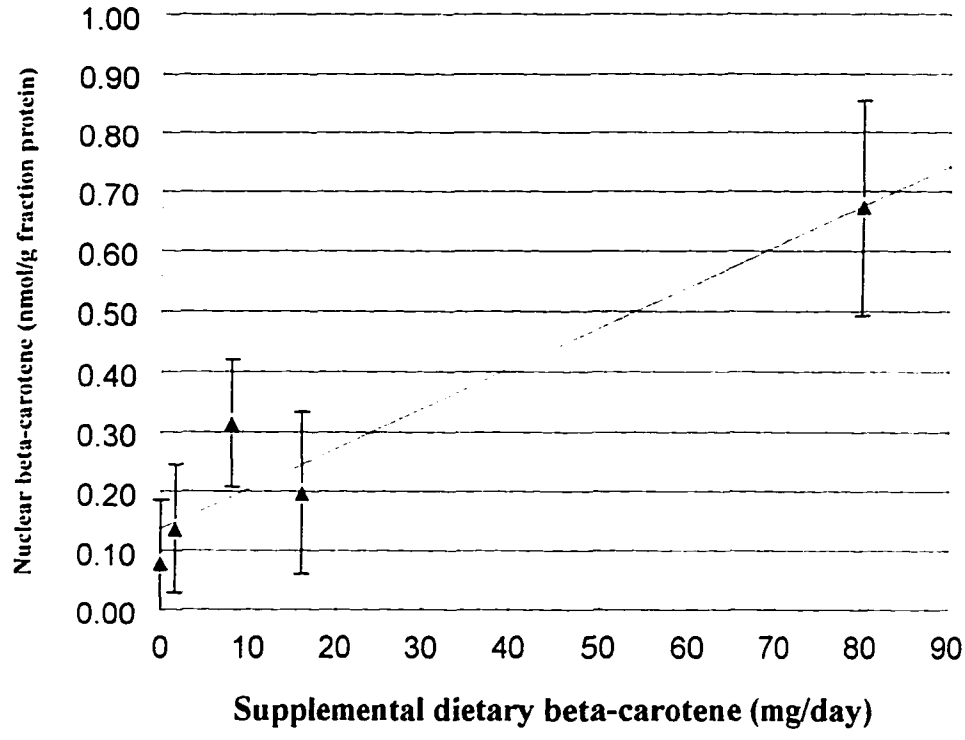

Figure 9b **Nuclear fraction beta-carotene.** Corpus luteum nuclear beta-carotene concentration (nmol/g fraction protein) grouped by supplemental dietary beta-carotene (mg/day) received in the preceding seven weeks. Values are least squares means ± SEM, n = 4 for each treatment, except 16 and 80 mg/day where n = 3. There was a significant linear response to the level of supplemental dietary beta-carotene ($p < 0.01$).

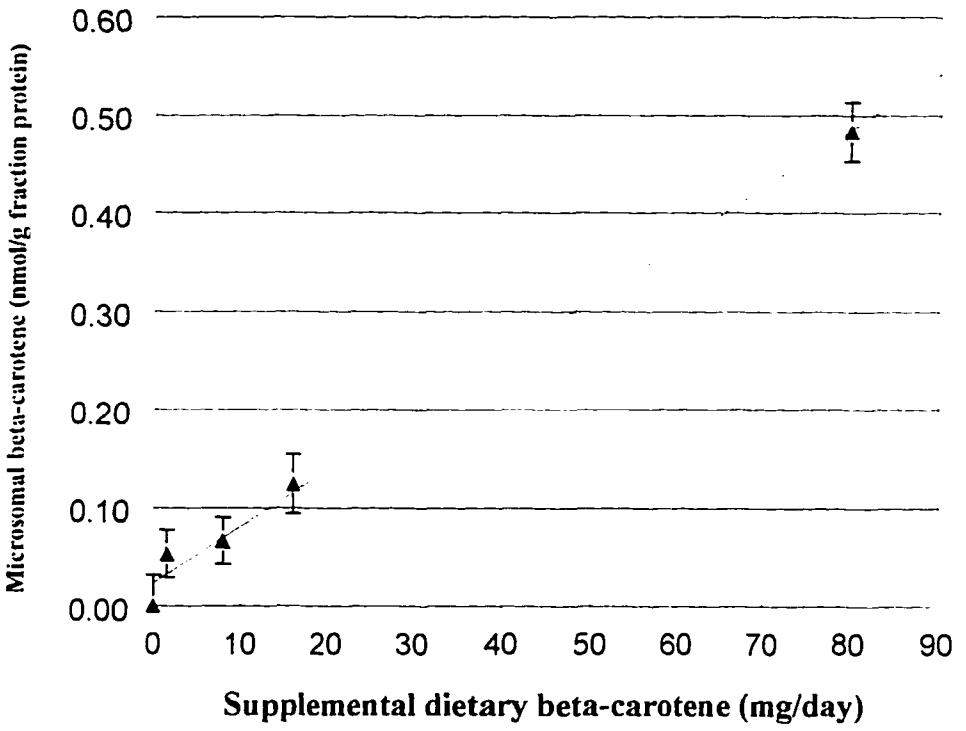

Figure 9c **Microsomal fraction beta-carotene.** Corpus luteum microsomal beta-carotene concentration (nmol/g fraction protein) grouped by supplemental dietary beta-carotene (mg/day) received in the preceding seven weeks. Values are least squares means ± SEM, n = 4 for each treatment, except 16 and 80 mg/day where n = 3. There was a significant linear response to the level of supplemental dietary beta-carotene ($p < 0.001$).

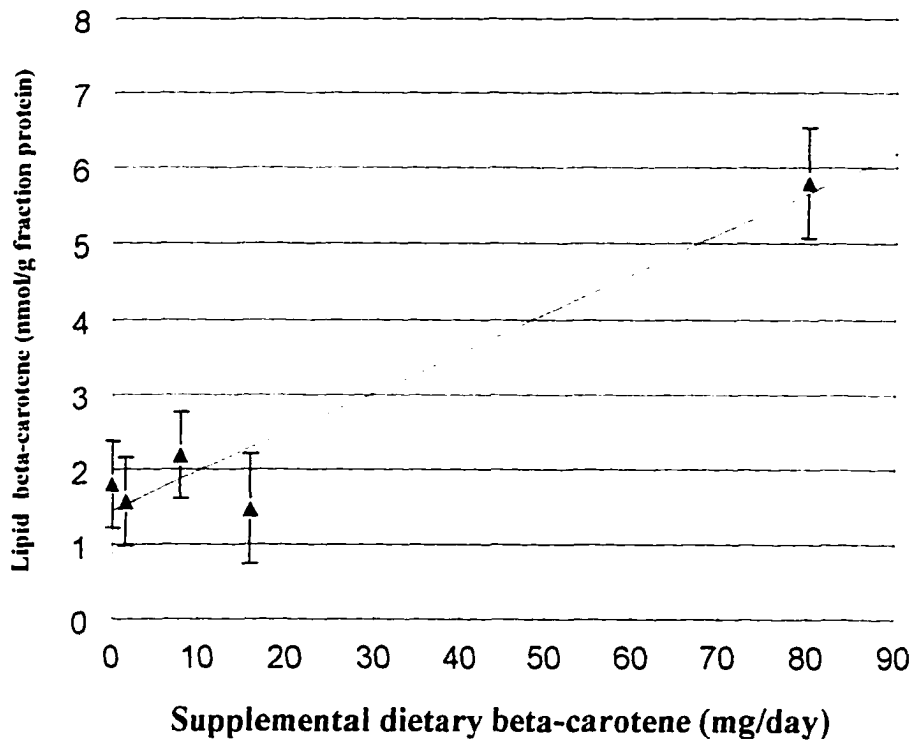

Figure 9d **Lipid fraction beta-carotene.** Corpus luteum lipid fraction beta-carotene concentration (nmol/g fraction protein) grouped by supplemental dietary beta-carotene (mg/day) received in the preceding seven weeks. Values are least squares means ± SEM, n = 4 for each treatment, except 16 and 80 mg/day where n = 3. There was a significant linear response to the level of supplemental dietary beta-carotene ($p < 0.01$).

Western Immunoblots

While it has been reported that BC reduces the cross-linking of Adx and P450scc in luteal cells in culture, the effect of BC on cross-linking in vivo has not been studied. To assess the effect of CL beta-carotene concentration on the cross-linking of Adx and P450scc, immunoblotting was performed on the separated proteins from all corpora lutea collected during the supplementation experiment. Polyclonal antibodies were made to bovine adrenodoxin isolated from frozen cow adrenals. The isolated protein was analyzed by automated amino acid sequence analysis (**Figure 10**), and the first 19 amino acids in the protein isolate (S-S-S-E-D-K-I-T-V-H-F-I-N-R-D-G-E-T-L) are the same as for mature adrenodoxin, as determined by Tanaka et al. (98). Based on the relative amount of other amino acids, the purity of the isolate was estimated to be ~80%. The antisera was purified by affinity column chromatography, and the various eluents were compared for their reactivity with adrenodoxin standard in Western immunoblots.

Antisera to adrenodoxin and to cytochrome P450scc, apart from recognizing their respective proteins, also recognized a protein of higher relative molecular mass (M_r) (~63kDa) in their respective immunoblots (**Figures 11a and 11b**). The M_r of this protein was equal to the combined M_r of Adx (~14 kDa) and P450scc (49 kDa) and corresponds to the cross-linked Adx-P450scc protein seen by other researchers (84). The figures show the corresponding CL beta-carotene concentrations for each lane of the Western blots. Based on visual observations, the cross-linked protein appears consistently in the lanes where CL beta-carotene concentrations were below ~11 nmol/g. On the other hand, the cross-linked protein is not apparent in lanes

where the CL beta-carotene concentrations were above ~50 nmol/g. In lanes where the CL beta-carotene concentrations are between 11 and 50 nmol/g, the cross-linked protein is sometimes apparent, but not consistently.

Control immunoblots were performed to verify the specificity of the antibodies used (**Figure 11c**). The adrenodoxin antibody reacts with the purified Adx standard, but not with lysozyme, a protein of similar molecular weight. Additionally, CL homogenates failed to exhibit any reactive protein bands when incubated with serum from the rabbits obtained prior to immunization as the primary antibody, showing that the antibody to the reactive protein is produced only after immunization with Adx. Also, no reactive proteins were identified when blots were not incubated with a primary antibody, but only the secondary antibody, showing that there was negligible non-specific binding of the enzyme-labeled secondary antibody.

Other researchers have observed the cross-linking of Adx and P450scc when luteal cells are grown in culture without supplementation of BC (84). Therefore, as a positive control, homogenates of luteal cells cultured for 48 hours without supplementary BC were analyzed by Western immunoblotting for the presence of the cross-linked protein. All cell homogenates (n=14) contained the cross-linked protein (immunoblots not shown).

Amino Acid	Ser	Ser	Ser	Glu	Asp	Lys	Ile	Thr	Val	His	Phe	Ile	Asn	Arg	Asp	Gly	Glu	Thr	Leu
Abbrev	S	S	S	E	D	K	I	T	V	H	F	I	N	R	D	G	E	T	L
Cycle 1	**231.2**			30.8	26.5	18.7	16.5	11.3	18.7	0.0	29.6		22.6	0.0		21.5			31.3
Cycle 2		**139.0**		47.6	48.8	17.3	11.0	9.0	36.9	0.0	6.8		17.8	0.0		21.9			19.1
Cycle 3			**118.4**	58.4	53.1	14.1	14.6	11.5	18.8	0.0	4.5		6.2	31.8		17.8			19.7
Cycle 4			34.4	**133.0**	53.5	69.3	13.9	14.9	12.2	0.0	39.4		5.5	8.7		16.7			17.2
Cycle 5			21.6	48.3	**162.8**	50.2	28.8	15.0	9.9	3.3	32.1		5.4	5.0		24.2			17.9
Cycle 6			13.2	31.4	65.1	**159.7**	20.3	37.8	9.2	0.0	16.6		5.5	4.1		25.3			22.1
Cycle 7			14.7	35.4	40.0	66.5	**102.6**	34.7	21.1	0.0	28.8		6.1	3.2		24.0			20.5
Cycle 8			14.2	31.7	33.1	58.0	36.0	**94.1**	20.3	10.5	17.1		6.2	4.2		22.6			26.8
Cycle 9			13.6	31.2	38.6	45.4	24.4	36.3	**93.7**	25.2	19.3		6.0	4.5		27.0			20.4
Cycle 10			17.3	32.5	37.3	32.9	23.4	27.6	38.6	**76.1**	16.1		6.3	4.9		27.7			18.7
Cycle 11			16.9	31.7	31.6	32.0	28.6	19.9	26.6	29.8	**74.6**		12.5	6.3		23.9			24.2
Cycle 12			15.0	30.2	27.0	35.4		18.5	26.9	16.2	32.0	**74.8**	13.8	10.2		22.0			23.8
Cycle 13			6.7	23.8	39.3	29.6		23.2	20.0	7.9	16.9	35.8	**51.7**	14.5		22.7			24.9
Cycle 14			12.1	19.5	37.4	26.0		23.0	21.0	6.1	11.9	23.2	24.2	**58.1**		22.8			23.6
Cycle 15			14.5	21.6		24.7		17.0	23.2	6.5	9.3	18.0	14.1	30.3	**64.6**	21.3			24.2
Cycle 16			15.5	21.1		23.4		17.2	20.5	4.6	8.3	17.1	13.2	21.1	45.1	**47.4**			26.4
Cycle 17			13.6			22.0		18.7	18.6	5.4	9.1	14.7	9.6	8.7	36.0	31.9	**33.2**		26.3
Cycle 18			12.9			20.6			22.1	4.8	7.4	13.8	8.3	6.7	32.6	23.7	21.4	**39.7**	25.3
Cycle 19			6.5			21.3			19.7	6.8	7.6	14.3	10.1	6.8	29.3	21.4	16.1	27.7	**46.0**

Figure 10 **Amino acid sequence of isolated protein.** The amino acid sequence (first 19 amino acids) of the isolated protein was determined. This table shows the amount of each amino acid detected during sequencing in picomoles. The bold and shaded numbers are the predominant amino acid detected for that cycle. The amino acids (columns) are arranged to show the sequence of the protein (from left to right).

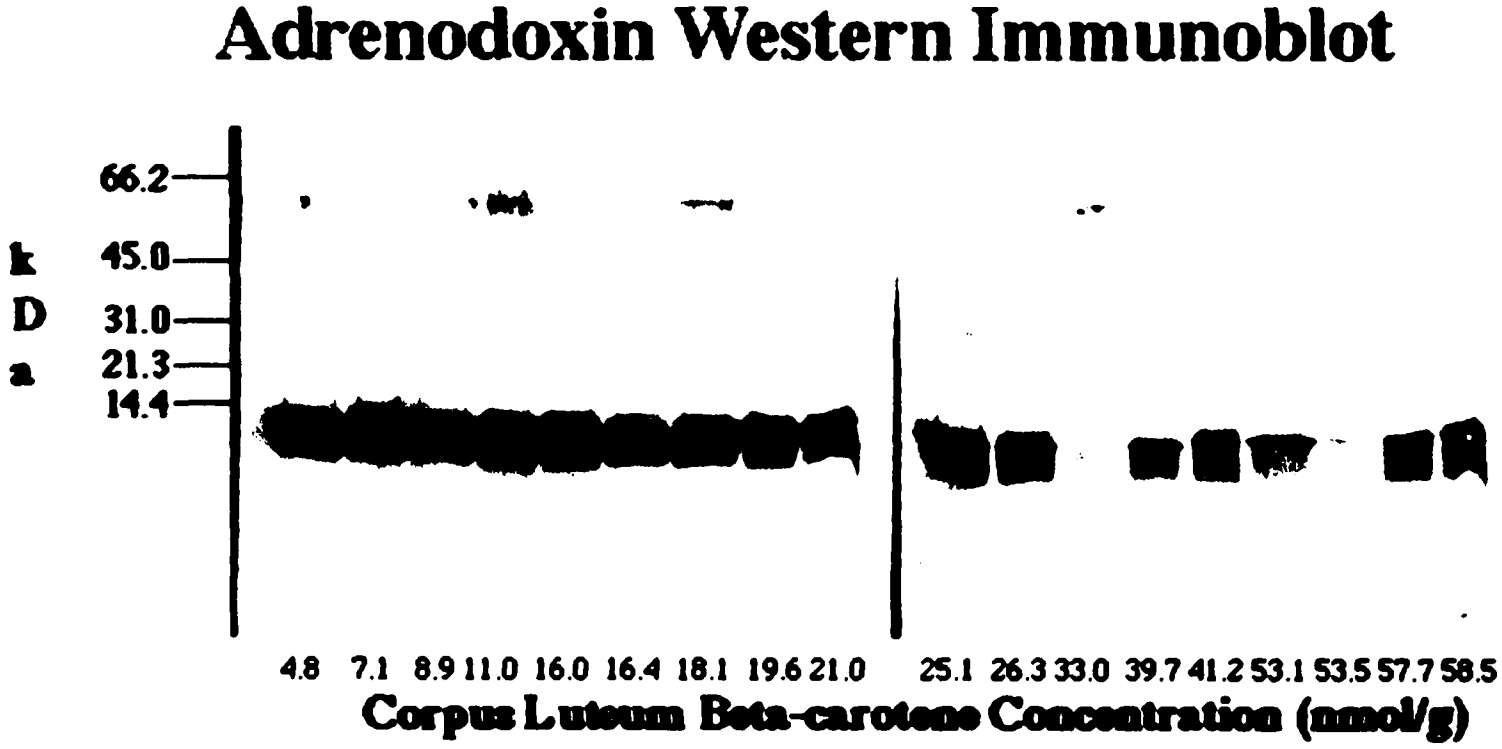

Figure 11a **Western immunoblots of corpus luteum tissue from cows receiving various levels of dietary beta-carotene, developed with antibody to adrenodoxin. In addition to the adrenodoxin band, at ~14 kiloDaltons (kDa), a second protein, at ~63 kDa, was detected by the antibody in some lanes. Corpus luteum beta-carotene concentration for each respective lane is shown.**

Cytochrome P-450scc Western Immunoblot

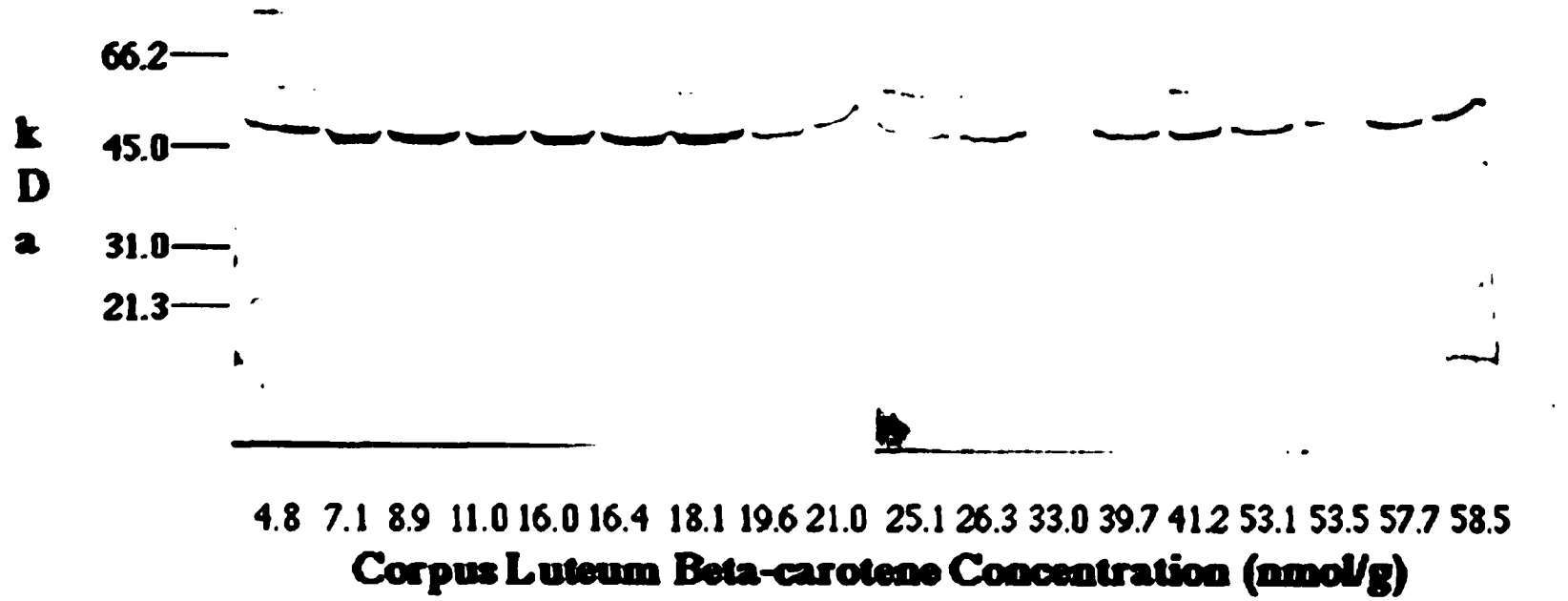

Figure 11b **Western immunoblots of corpus luteum tissue from cows receiving various levels of dietary beta-carotene, developed with antibody to cytochrome P-450scc. In addition to the P-450scc band, at ~49 kiloDaltons (kDa), a second protein, at ~63 kDa, was detected by the antibody in some lanes. Corpus luteum beta-carotene concentration for each respective lane is shown.**

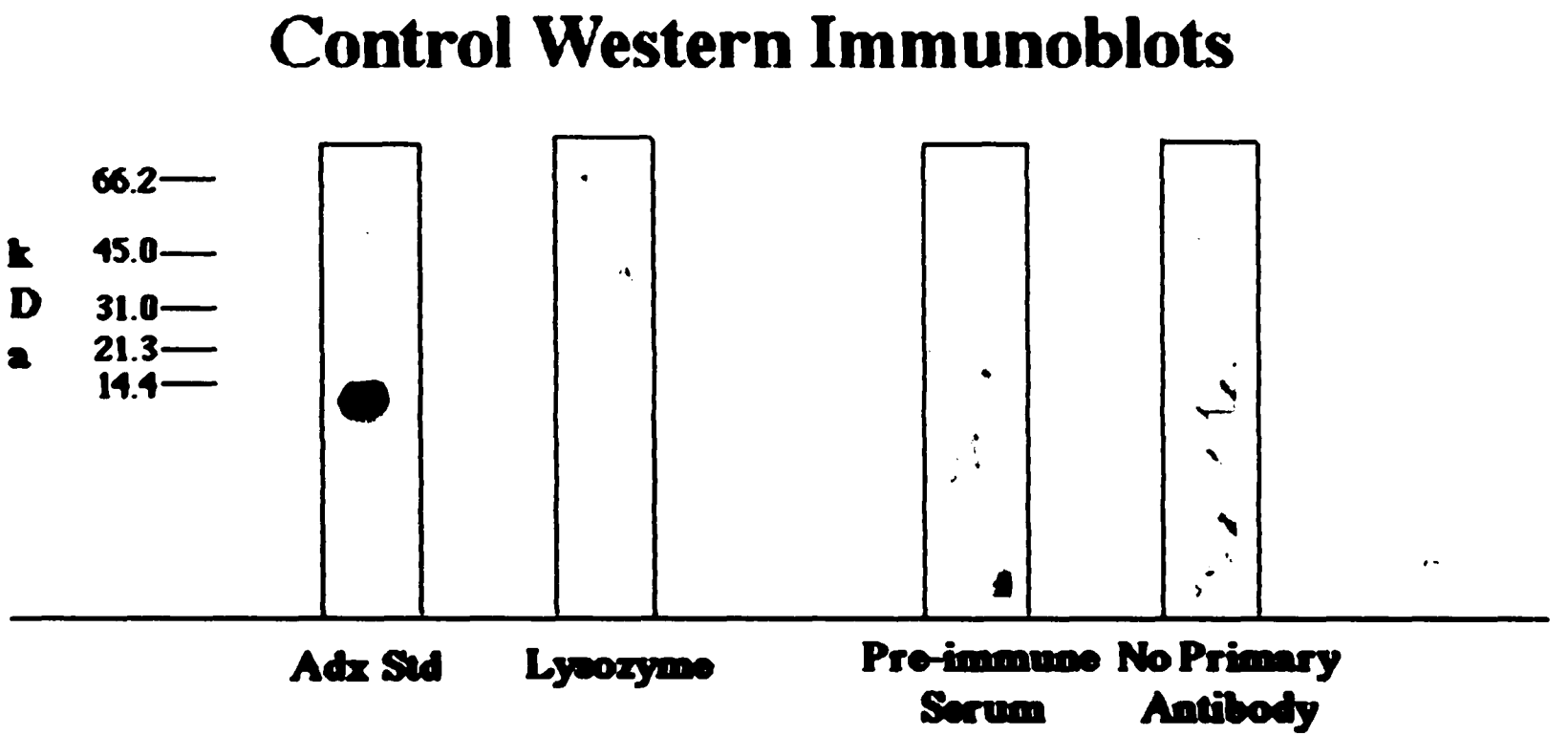

Figure IIc Representative lanes from control western immunoblots. Lane 1 is the purified Adx standard developed in the same manner as the corpus luteum homogenates. Lane 2 is the purified protein lysozyme, developed in the same manner as the corpus luteum homogenates. Lane 3 is corpus luteum homogenate developed with pre-immune serum as the primary antibody. Lane 4 is corpus luteum homogenate developed with no primary antibody. No protein bands are observed when specific antibody is not used as the primary antibody, and the Adx antibody does not recognize lysozyme, a protein of similar molecular weight (~ 14.3 kDa).

Plasma Progesterone

Cross-linking of Adx and P450scc, two enzymes involved in progesterone synthesis within the CL, may be expected to affect plasma progesterone (P4) concentrations. Therefore, plasma and corpus luteum P4 concentrations were determined for each cow on the day of CL harvest in an attempt to determine if the changes in dietary BC had any effect on CL function. There were no significant differences in plasma or CL progesterone concentration between treatment groups (**Figure 12a and 12b**). Nor was there any difference in total CL progesterone content between treatment groups (**Figure 12c**).

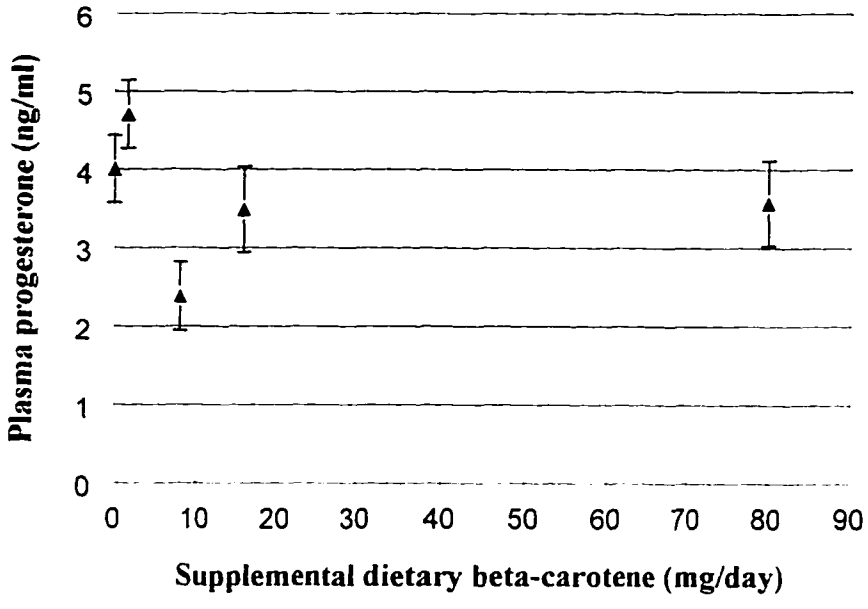

Figure 12a **Plasma progesterone.** Plasma progesterone concentration (ng/ml) on day of corpus luteum harvest grouped by supplemental dietary beta-carotene (mg/day) received in the preceding seven weeks. Values are least squares means ± SEM, n = 4 for each treatment, except 16 and 80 mg/day where n = 3. There was no statistically significant difference between treatment groups.

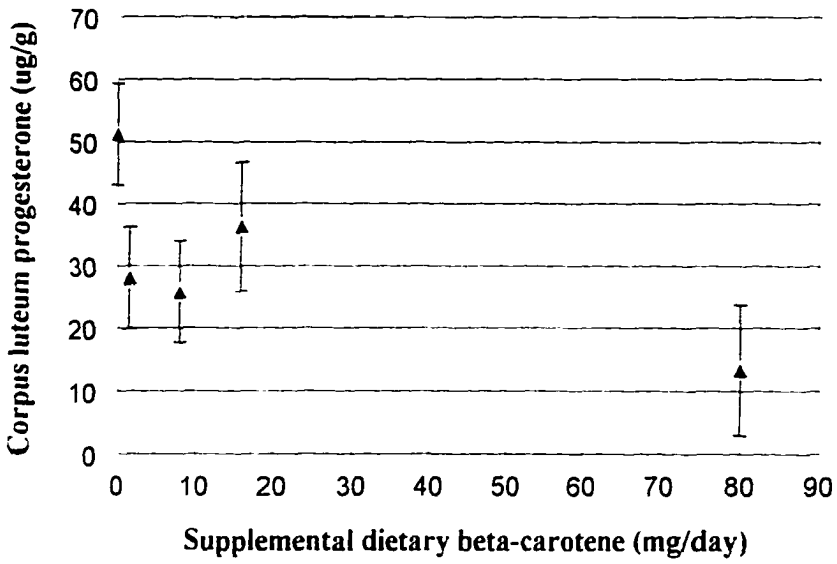

Figure 12b **Corpus luteum progesterone concentration**. Corpus luteum progesterone concentration (μg/g) on day of corpus luteum harvest grouped by supplemental dietary beta-carotene (mg/day) received in the preceding seven weeks. Values are least squares means ± SEM, n = 4 for each treatment, except 16 and 80 mg/day where n = 3. There was no statistically significant difference between treatment groups.

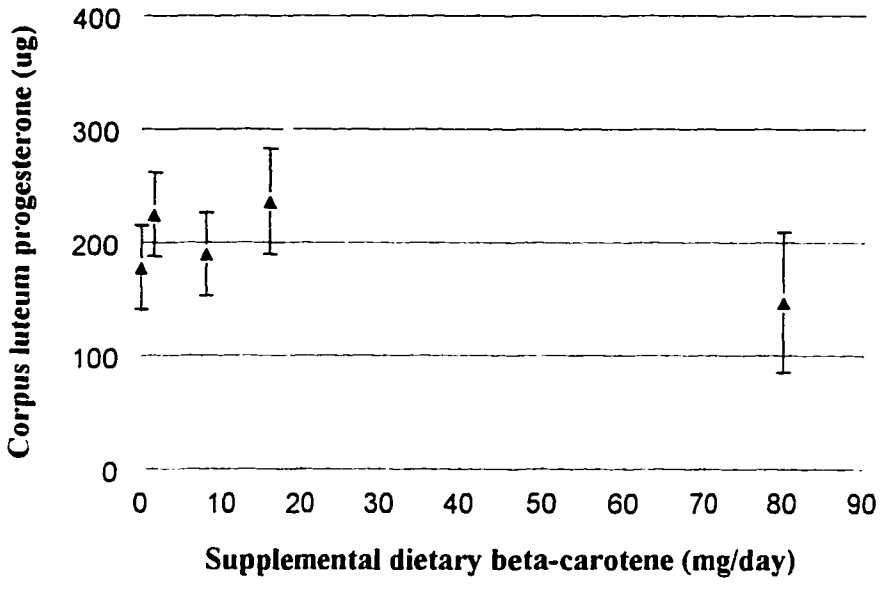

Figure 12c **Corpus luteum progesterone content.** Corpus luteum progesterone content (μg) on day of corpus luteum harvest grouped by supplemental dietary beta-carotene (mg/day) received in the preceding seven weeks. Values are least squares means ± SEM, n = 4 for each treatment, except 16 and 80 mg/day where n = 3. There was no statistically significant difference between treatment groups.

Alpha-tocopherol

Plasma and CL alpha-tocopherol (AT) concentrations were determined. There was not a significant difference in either plasma AT concentration (**Figure 13a**) or in CL alpha-tocopherol concentration (**Figure 13b**) between cows receiving different levels of dietary BC. Additionally, the AT to BC ratio in the CL was directly related to the ratio in plasma (**Figure 13c**), suggesting that there was not preferential uptake of either.

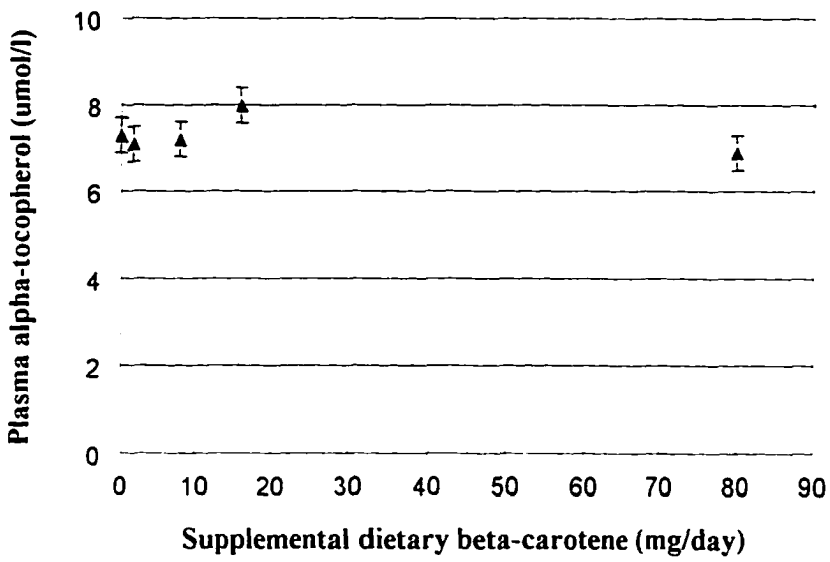

Figure 13a **Plasma alpha-tocopherol.** Plasma alpha-tocopherol concentration (μmol/l) on day of corpus luteum harvest grouped by supplemental dietary beta-carotene (mg/day) received in the preceding seven weeks. Values are least squares means ± SEM, n = 4 for each treatment. There was no statistically significant difference between treatment groups.

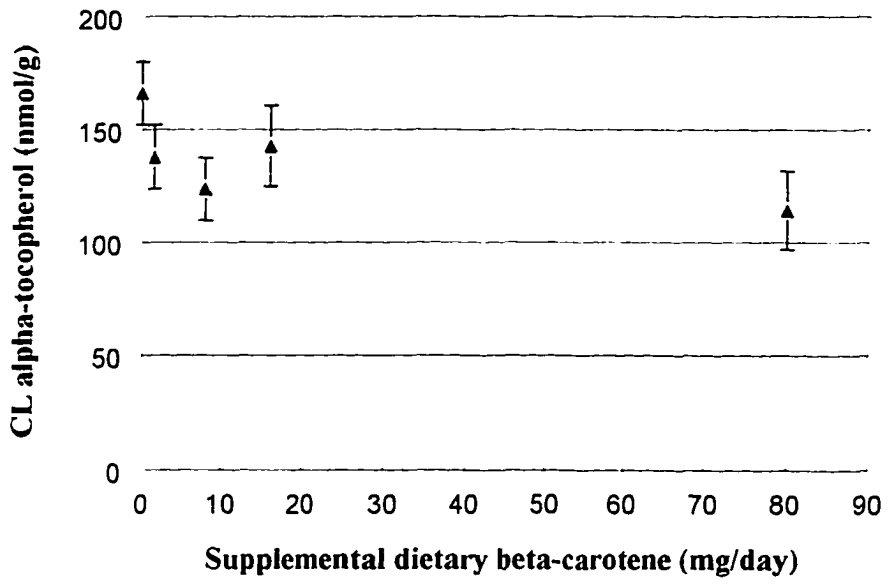

Figure 13b **Corpus luteum alpha-tocopherol.** Corpus luteum alpha-tocopherol concentration (μmol/l) on day of corpus luteum harvest grouped by supplemental dietary beta-carotene (mg/day) received in the preceding seven weeks. Values are least squares means ± SEM, n = 4 for each treatment, except 16 and 80 mg/day where n = 3. There was no statistically significant difference between treatment groups.

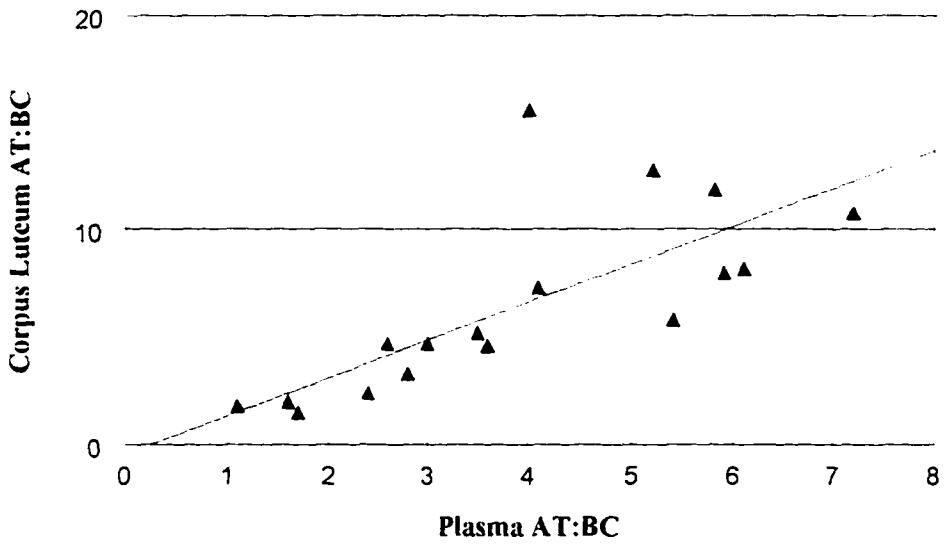

Figure 13c **Plasma vs. corpus luteum alpha-tocopherol to beta-carotene ratio.** Relationship between plasma alpha-tocopherol to beta-carotene ratio on day of CL harvest and corpus luteum alpha-tocopherol to beta-carotene ratio. The linear relationship (Pearson's R = 0.756) was significant ($p<0.001$, $n=18$).

Histology and Cell Ultrastructure

In order to assess the effects of BC on the organization of the CL and on the ultrastructure of its cells, tissue sections for light microscope and transmission electron microscope (TEM) examination were prepared. Light microscopy at 25 and 100x magnification revealed no obvious differences in tissue organization between treatments (**Figures 14 and 15**). The CL contained cells of varying sizes, representing small and large luteal cells and non-steroidogenic cells in an extensive extracellular matrix. The luteal cells possessed a uniform granular cytoplasm with many vacuoles which were shown to be lipid filled by TEM. Electron micrographs, taken at 5000 and 10000 x magnification, of tissue sections from corpora lutea from blocks 1 and 2 were examined to determine if there were differences in mitochondria appearance between treatments(**Figure 16**). No apparent differences were seen. Luteal cells contained numerous mitochondria and the mitochondria appeared morphologically normal (i.e., intact double membrane and tubular cristae were visible, no gigantism or swelling observed, and no abnormal inclusions). The luteal cells also exhibited a great deal of smooth endoplasmic reticulum and numerous membrane-bound lipid droplets, two additional hallmarks of steroidogenic cells.

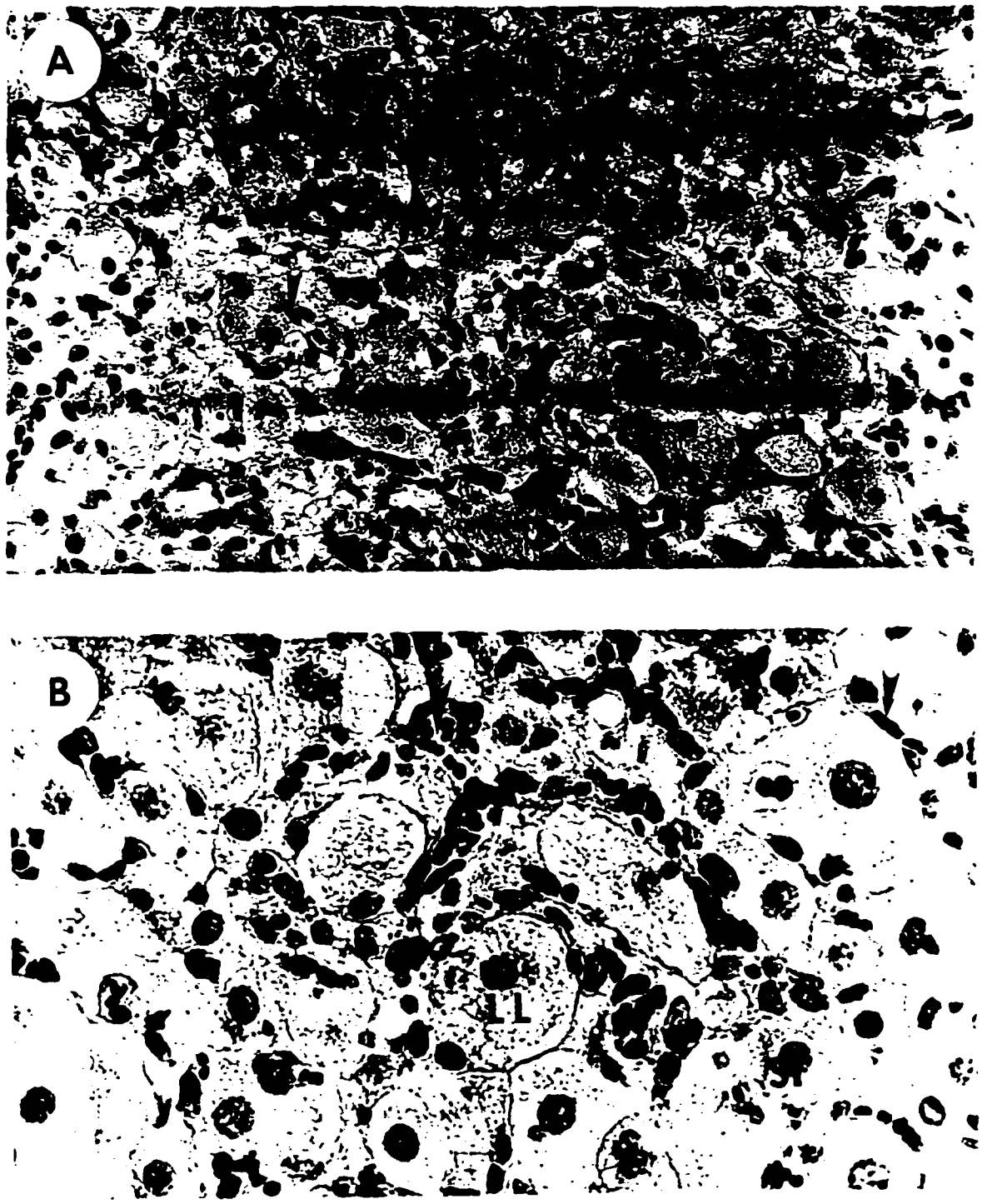

Figure 14 **Hematoxylin and eosin-stained sections.** Pictured, are representative photographs of corpus luteum sections which were stained with hematoxylin and eosin (photographed at 25 (A) and 100 (B) x magnification). Note the heterogeneous cell size, extensive vascularization, and uniform granular cytoplasm. Symbols: LL = large luteal cell, SL = small luteal cell, Small arrow = stromal cell, and large arrow = capillary with red blood cells.

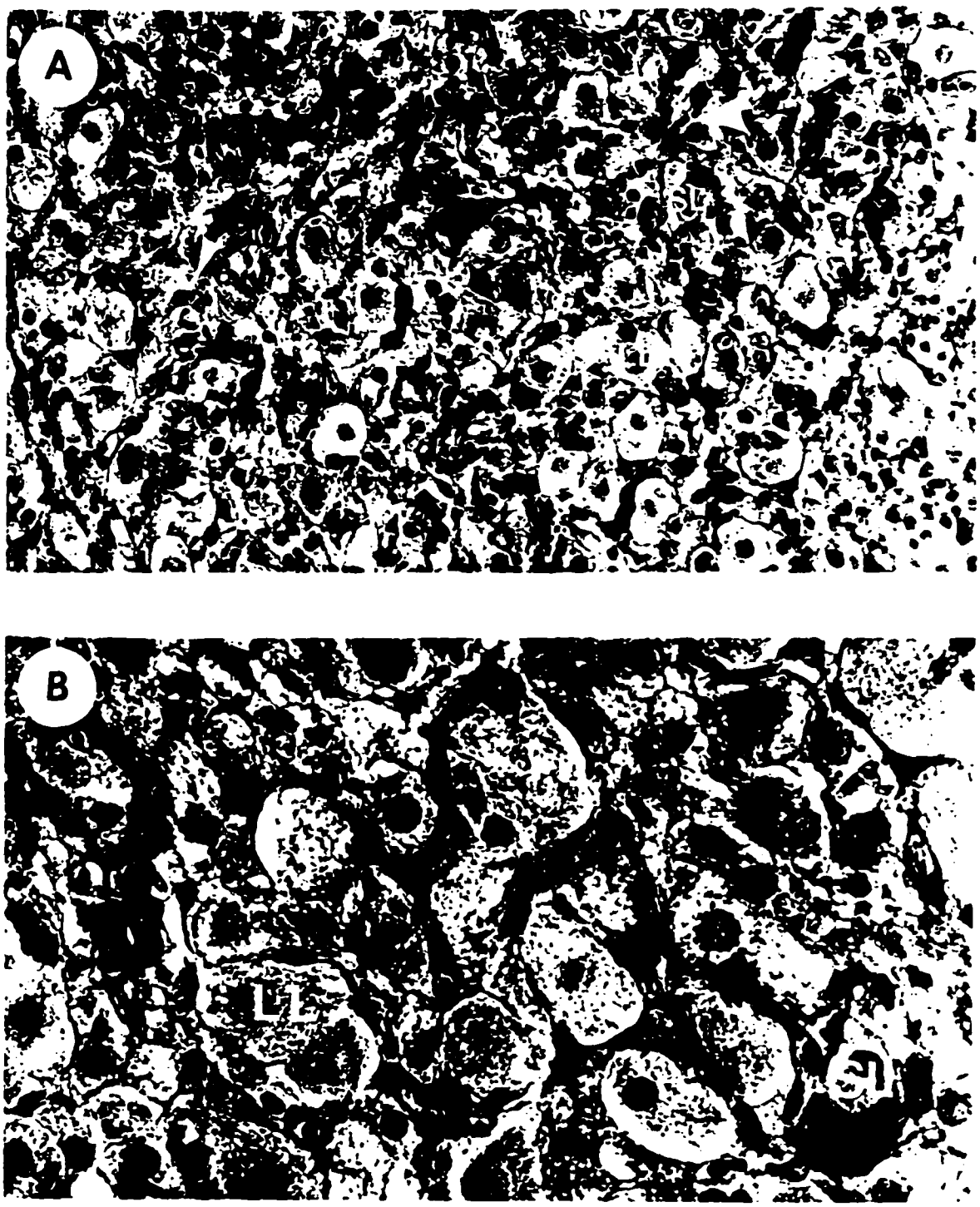

Figure 15 **Masson's trichrome-stained sections.** Pictured, are representative photographs of corpus luteum sections which were stained with Masson's trichrome (photographed at 25 (A) and 100 (B) x magnification). Note the heterogeneous cell size, extensive vascularization, and uniform granular cytoplasm. Symbols: LL = large luteal cell, SL = small luteal cell, Small arrow = stromal cell, and large arrow = capillary with red blood cells.

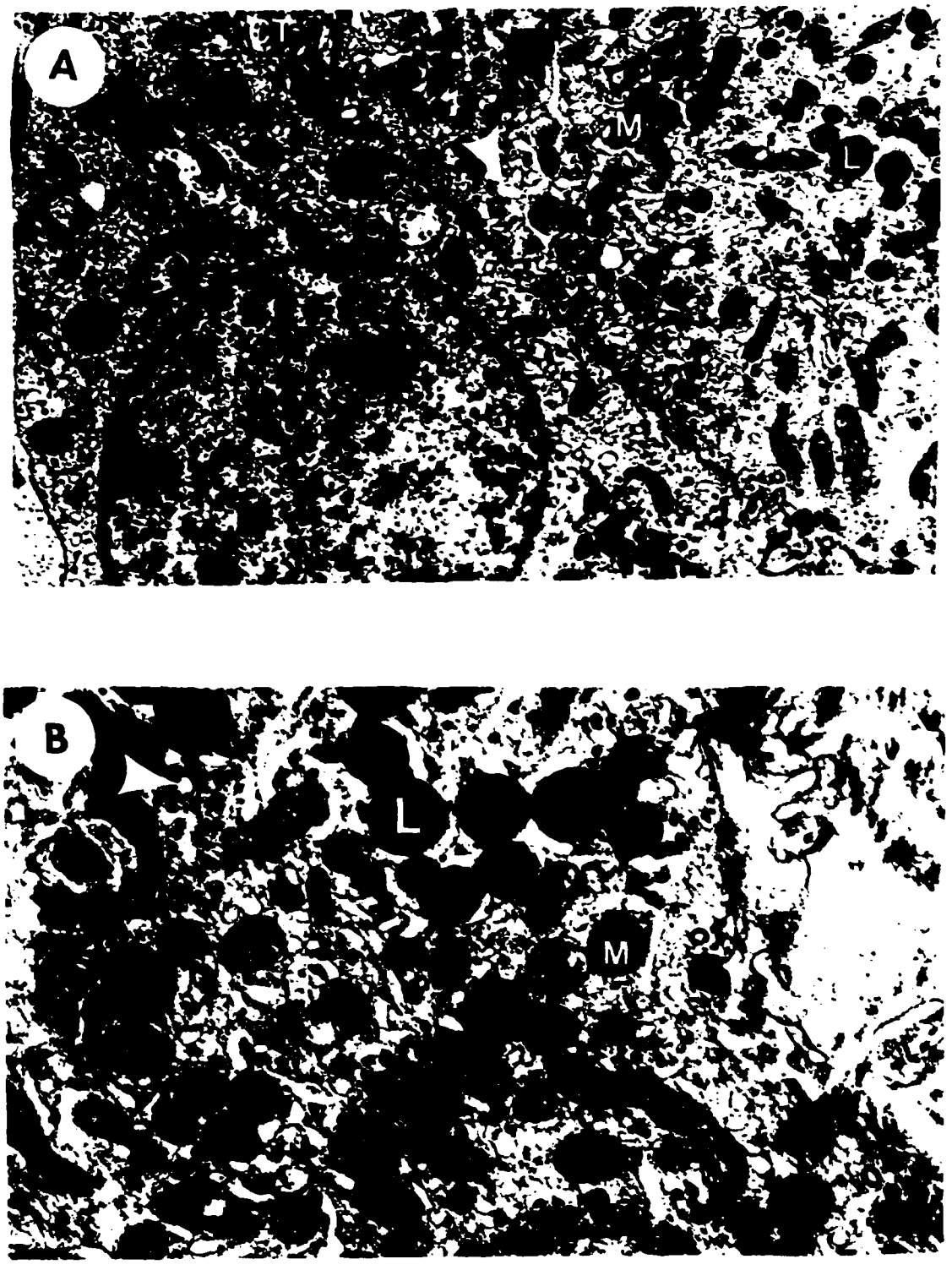

Figure 16 **Electron micrographs.** Pictured, are representative micrographs of corpus luteum sections (magnification of photograph: A = 9,000 x ; B = 18,000 x). Note the numerous lipid droplets (L), mitochondria (M), and smooth endoplasmic reticulum (arrowhead) characteristic of steroidogenic tissue.

CHAPTER IV

DISCUSSION

Numerous epidemiological studies have shown an inverse relationship between the consumption of fruit and vegetables rich in beta-carotene (BC) and the incidence of chronic diseases such as cardiovascular disease, some cancers, and age-related macular degeneration (27). However, double-blind intervention trials which expose subjects to BC supplements have failed to show consistent benefit. Indeed, two studies suggest a negative effect of supplemental BC on cancer incidence in high-risk populations (29,99). The hypothesis that BC is the protective component in fruits and vegetables, was formed based largely on in vitro experiments and observational data (6). To date, no definitive in vivo function of BC has been shown, other than its role as a vitamin A precursor.

Likewise, in the cow there have been conflicting reports regarding the positive impact of BC on fertility. These reports have been based on both observational data and dietary supplementation trials. Hemken and Bremel (79) reported on several studies in Germany which showed an increased length of estrous period and a decreased conception rate in cows fed a low BC ration compared to a supplemented group (0.3 mg / kg body weight). They also found that corpus luteum progesterone concentration was lower in unsupplemented cows. Graves et al. (80) found that plasma progesterone

was positively correlated with plasma BC concentrations in a 39 cow study and Jackson (82) saw an inverse correlation between plasma BC and number of services to conception. On the other hand, Ascarelli et al. (81) saw no effect of supplemental BC (>500 mg/day) on reproductive parameters.

One problem with interpreting the results of intervention trials in both humans and cows is the basal BC status of the population. If an individual already has adequate BC, then no effect of supplemental BC would be expected. Folman et al. (83) reviewed the case for beta-carotene's enhancement of fertility in cows and found that, in some experiments where the basal BC levels were below 0.931 μmol/l, BC supplementation increased fertility. However, there was no case in which fertility was affected when plasma BC exceeded 2.794 μmol/l. Therefore, using control groups which have sufficiently low BC status may be crucial to discerning an effect of supplemental BC.

In an attempt to resolve some of the conflicting results in the literature, it is necessary to investigate the function of BC in vivo to determine the mechanism which would explain the observed associations. While most measures of BC status are based on plasma concentrations, if BC is affecting fertility or the incidence of disease, it is most likely that these outcomes are being mediated in the tissues at the cellular level. Therefore, determining the relationship between plasma and tissue BC concentrations is an important step in the interpretation of plasma BC data collected from a large population.

Collecting tissue from humans is problematic. What little information is available on BC in human tissues is based on needle biopsies or biopsy samples at time of surgery for some other cause (89), non-invasive techniques such as cell scrapings

(100), or indirect techniques such as light remittance of skin (34). Therefore, an animal model which absorbs and accumulates intact BC is necessary. The cow may be one such model, because of its high plasma and tissue concentrations of BC. The possible associations between BC and fertility in dairy cows provides an added incentive to investigate BC metabolism in this animal.

The studies presented in this dissertation were designed to elucidate the relationship between plasma and tissue BC in the cow, determine the subcellular distribution of BC within the bovine corpus luteum, and assess the impact of dietary BC on the steroidogenic function of the CL. For the sake of clarity, when referring to the final study in which dietary BC intake was manipulated, the acronym DAISS (Dietary Alteration's Impact on Steroidogenesis Study) will be used. The purpose of these studies was two-fold: to determine the suitability of the bovine CL to serve as a model of tissue BC metabolism and to identify a function of BC which might explain its reported effect on fertility in dairy cows. Because of its high concentration of BC, the CL may be the site of beta-carotene's influence on fertility.

As in humans (34, 35), there is variability in the plasma concentration of BC among cows being fed the same diet. In preliminary experiments, the variability within and between cows was determined for various measures (***Table 3***). The intercow variability of plasma BC of 26.5% is in good agreement with that of Hoppe et al. (47). They found an intercalf variability of ~30% for plasma BC and an intracalf variation of ~7%. This compares to the preliminary studies' intracow variability of 12.9%. However, the intracalf variability determined by Hoppe was based on seven

measurements at four hour intervals, while this study compared 3-4 measurements over a six month period.

Because of the difficulty associated with obtaining successive tissue samples from one individual, little information is available regarding the intraindividual variability of BC concentration of tissues. The CL is easily accessible and corpora lutea can be obtained repeatedly from the same cow, thus making it available for study. Preliminary experiments demonstrated that the inter- and intracow variability in CL beta-carotene concentration were 36.3% and 18.3%, respectively (***Table 3***). This variability is slightly greater than the 26.5% and 12.9% seen in plasma values.

The variability in plasma and tissue BC, both within and between cows, needs to be considered when designing experiments. When only single measures are taken on individuals, any variation in response due to interindividual variation becomes part of the error sum of squares. As a result, it is more difficult to obtain statistically significant results, and the number of subjects may need to be increased. By contrast, studies which take repeated measures on the same individual allow the partitioning of the sums of squares into both within and between cow variability. Repeated measures are practical in the cow for both plasma and CL parameters, because of the ease of blood sampling and the ability to obtain successive corpora lutea, thus reducing the needed sample size, and increasing statistical power. Therefore, experimental designs which utilize repeated-measures and the crossover from one treatment to another may be particularly valuable in reducing the confounding effect of interindividual variability.

Dairy cows, in areas where forage is plentiful, are usually fed rations which contain feeds like corn silage, hay, and/or haylage. These forages contain high BC concentrations and contribute to the high BC concentration found in cows' plasma and tissue (73). When consuming a typical ration, cows in preliminary studies (***Table 3***) had mean plasma BC concentrations of 9.891 μmol/l (Standard deviation (SD)= 2.717; Range= 6.413-17.090) and mean corpus luteum concentrations of 139.053 nmol/g (SD= 52.236; Range= 70.541-248.399). According to Folman (83), there are no studies which reported a beneficial effect of supplemental BC when cows had plasma BC concentrations greater than 2.8 μmol/l. Plasma concentrations this low are only seen in cows which are receiving minimal amounts of forage, or poor quality forages such as straw. This occurs in regions of the world where arable land is scarce and dedicated mainly to the production of foods for human consumption, such as occurs in Israel (81). In these regions, cows are fed grains and feed by-products which provide the bulk of energy in the ration, and given forage in quantities sufficient to maintain rumen function. In the United States, in contrast, forages make up the bulk of the ration, with concentrates being fed to increase energy density of the ration and ensure adequate caloric intake in high-producing dairy cows.

To achieve low plasma and corpus luteum BC concentrations in the cows in the DAISS, a ration composed largely of straw was formulated to meet the nutritional requirements of the cows. Straw, the stalks and leaves of grain plants (in this case, wheat), is allowed to dry in the field and is then baled for transport to the farm. Because the grain from these plants is harvested after it ripens and dries, these plants have been dead for several weeks prior to harvest. This, combined with the field-

curing, result in a low BC content in straw. The remainder of the ration consisted of a small amount of corn silage (to enhance palatability), soy bean meal (to increase ration protein content), corn meal (to increase energy density), and Sunshine Plus Pellets (to provide energy, protein and vitamin E). The ration was top-dressed with a molasses-coated grain, which is very palatable and provided a vehicle to incorporate supplemental BC. When fed the basal ration with no supplemental BC for seven weeks, cows in the DAISS had mean plasma BC concentrations of 1.403 μmol/l (SD= 0.602, Range= 0.916-2.279) (***Table 4***). These concentrations fall within the range in which previous studies have shown a beneficial effect of supplemental BC on fertility (83). Feeding of this ration also resulted in a decrease in the mean corpus luteum BC concentrations in these cows to 18.827 nmol/g (SD= 11.504, Range= 4.672-32.901) (***Table 4***). This represents a substantial decrease from the tissue/blood concentrations seen when cows are fed a typical dairy ration.

The plasma BC concentrations achieved in cows receiving no supplemental BC or the lowest BC supplementation levels are approaching the range of the human population aged four years and older (0.09-0.91 μmol/l, 5^{th}-95^{th} percentiles) (Unpublished NHANES III data, NCHS, 1996. Presented at EB '96, Washington, DC 4/17/96). Nierenberg et al. (39) found that in humans supplemented with 30 mg/day BC for one year, mean plasma BC concentration was 3.163 μmol/l. In DAISS, all cows, except those receiving 80 mg/day BC, had plasma BC concentrations less than 2.6 μmol/l (***Table 4***). Additionally, the corpus luteum BC concentrations of unsupplemented cows ranged from 4.672 to 32.901 nmol/g. Yokoe and Takenaka (77) reported human CL concentrations of BC ranging from ~2.0 to 93.1 nmol/g. Thus,

by manipulating dietary BC intake in the cow, CL and plasma BC concentrations comparable to those seen in humans can be achieved. This is critical if the bovine CL is to be developed as a model for BC metabolism and inferences are to be drawn regarding the function of BC in humans.

There was a direct linear relationship between the amount of supplemental BC fed and the CL and plasma BC concentrations measured in DAISS (***Figure 8***). This relationship gives future researchers the ability to control and predict the BC content of the CL based on dietary intake. This will be useful in studies examining the effects of different cellular BC concentrations on function within the CL, because desired cellular BC concentrations can be predictably achieved.

The change in cellular BC concentration is also reflected in the subcellular organelles (***Figures 9a-9d***). The CL contains lipid droplets which provide the stores of cholesterol needed as a substrate for steroid synthesis. The DAISS shows that the change in corpus luteum BC concentration is not due solely to changes in the BC content of these lipid droplets. This is significant, because presumably BC can only function within the cell if it is located at critical sites. Beta-carotene associated with the lipid droplets might protect the lipid from peroxidation, but would be unable to scavenge free radicals at other sites within the cell. Nuclear, mitochondrial, and microsomal fractions had increased BC concentrations which paralleled the increase in CL beta-carotene in those cows receiving supplemental BC. Therefore, BC would be in a location to scavenge free-radicals formed in these organelles.

The reported association between BC and fertility in dairy cows, while inconsistent, suggests a function for BC. Since the CL is a site of BC accumulation

and the primary function of the CL is the production and secretion of progesterone, it seemed likely that BC would have some relationship with the steroidogenic capacity of the CL. Adrenodoxin and cytochrome P450scc are two enzymes which catalyze the first and rate-limiting reaction in the formation of steroids from cholesterol. Young et al. (84) observed that luteal cells grown in culture exhibited cross-linking between Adx and P450scc, except when the culture medium was supplemented with BC.

To determine if this effect occurs in vivo, the cross-linking of Adx and P450scc was assessed by Western immunoblotting in the corpora lutea obtained from cows during DAISS (***Figures 11a and 11b***). The cross-linked protein (~ 63 kDa) was found in all corpora lutea which had BC concentrations of 11.035 nmol/g or lower, and was seen in some which had BC concentrations below 41.135 nmol/g. No cross-linking was observed in corpora lutea which had BC concentrations of 53.017 nmol/g or higher. In terms of plasma BC concentration, no cross-linking was seen in cows when their plasma BC concentration was higher than 2.599 μmol/l. This finding is interesting when one considers Folman's (83) interpretation of the literature that no beneficial effect of supplemental BC is seen in those cows with plasma BC concentrations greater than 2.8 μmol/l. This suggests that there is some minimal level of BC needed within the CL to prevent the cross-linking, and that increasing the concentration beyond that point has no beneficial effect.

In spite of the cross-linking seen in some of the corpora lutea, there was no effect seen on the plasma progesterone concentration on the day of CL harvest (***Figure 12***), nor on the morphology of the mitochondria, nor on the structure of the tissue as observed in histological sections (***Figures 14-16***). Therefore, it is unclear whether

cross-linking signifies a detrimental condition within the CL or whether it is simply a curious observation. However, cross-linking may be a cumulative process, that would result in functional or morphological defects after a longer period of time. The corpora lutea obtained during this study were 9-11 days old. In normal cycles, if fertilization occurs, the CL continues to produce progesterone, and the CL's functioning beyond the 150^{th} day is necessary to sustain pregnancy. If cross-linking occurs in these corpora lutea, it is possible that inadequate quantities of progesterone would be produced and fertility would be affected. Investigation of this possibility will be more difficult, because harvesting the CL would result in the termination of the pregnancy, and corpora lutea from pregnant slaughterhouse animals would be from cows with unknown dietary BC history.

The CL is a metabolically active tissue and may, therefore, produce large quantities of free radicals. Steroidogenesis has been shown to produce free-radicals in vitro (67) and hydrogen peroxide is produced in the rat CL (68). Cells typically contain a host of enzymatic and non-enzymatic defenses to protect against the damaging effects of free-radicals (101). Beta-carotene is an antioxidant in vitro and functions in plants to protect chlorophyll from the damaging effects of free-radicals. Therefore, BC may be inhibiting cross-linking by scavenging free-radicals which cause the cross-linking.

The observed cross-linking may have implications for other tissues also. Because of its short life-span (18 days or 273 days in non-fertile or fertile cycles, respectively) the oxidative damage may be of limited consequence within the CL. However, in other tissues, the lifetime accumulation of oxidative damage may have profound consequences. Free-radical damage has been suggested to mediate the effects

of aging and the pathogenesis of many chronic diseases such as, cancer, arthritis, and cardiovascular disease (102). Beta-carotene may scavenge free-radicals in other tissues and affect these conditions.

During DAISS, a second antioxidant, vitamin E, was maintained at normal levels. Since vitamin E is also an antioxidant, it is possible that low plasma and CL concentrations of vitamin E might confound any results due to altered BC concentrations. The basal ration resulted in a decline in plasma vitamin E concentrations until Sunshine Plus Pellets were added, at which time plasma vitamin E concentrations returned to the normal range (data not presented). This suggests that in cows receiving low forage diets, declines in plasma BC may be paralleled by declines in plasma vitamin E. Since both nutrients are antioxidants, their simultaneous decline may result in far more oxidative damage. It would be interesting to perform a two-factor experiment in which BC and/or vitamin E were supplemented and the effect on cross-linking observed.

Clearly, the CL has potential to serve as a model for the study of BC function in tissue. DAISS shows that successive corpora lutea can be obtained from the same cow, and that the concentration of BC within the CL can be modified by dietary manipulation over a short period of time. Because the CL is formed anew each cycle, the BC concentrations found in the CL reflect the BC concentrations of the plasma at the time it develops. Other tissues that accumulate BC may retain high concentrations for an extended time after plasma concentrations decline. This lack of a "memory" of past BC exposure, allows rapid manipulation of the tissue BC content in the CL.

The concentration of BC associated with the nuclear fraction was also altered in DAISS. This may be valuable in research seeking to elucidate a functional mechanism to explain the relationship between BC and cancer. Since cancer is a disease associated with changes in DNA, the nucleus is the site at which one would expect to see an effect of BC, if an effect exists. Using the CL as a model, researchers could manipulate the nuclear content of BC and assess its impact on induced mutagenesis and transformation.

Another area of research which may benefit from the CL as a model is the area of intracellular trafficking of BC. Beta-carotene is highly hydrophobic, and is therefore insoluble in the aqueous environment of the cell. How BC is transported within the cell and incorporated into organelles, is a question which has not been addressed.

The DAISS study presented here demonstrates that, through the manipulation of dietary BC, plasma and corpus luteum concentrations within the cow can be altered and lowered to concentrations comparable to those seen in humans. Combined with the ability to obtain successive corpora lutea from the same cow, this provides researchers with a feasible model to study BC accumulation and metabolism. Applying these techniques, beta-carotene was shown to inhibit the cross-linking of the steroidogenic enzymes adrenodoxin and cytochrome P450scc in vivo. Whether this cross-linking may explain the reported association between BC and fertility in dairy cows remains to be determined.

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APPENDIX

Serum Carotenoid Reference Ranges 5th-95th percentiles, age 4 years and older Source: CDC/NCHS, NHANES III, 1988-94		
CAROTENOID	**μg/dL**	**μmol/L**
Lycopene	7-44	0.13-0.82
Lutein & Zeaxanthin	9-41	0.16-0.72
Beta-Carotene	5-49	0.09-0.91
Beta-Cryptoxanthin	3-21	0.05-0.38
Alpha-Carotene	1-12	0.02-0.22

Unpublished NHANES III data, NCHS, 1996.
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