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The Subcellular Distribution of β-Carotene in Bovine Corpus Luteum (41964)

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Abstract. The distribution of β-carotene was determined in various subcellular fractions of bovine corpus luteum. It was found in significant amounts in all subcellular fractions examined including nuclear, mitochondrial, microsomal, cytosolic, and floating lipid. Much of the β-carotene found in the crude nuclear and mitochondrial fractions was loosely bound and could be removed with repeated washings. In contrast, the microsomal β-carotene could only be removed by detergent extraction suggesting that it is an integral component of this membrane preparation. In the cytosol fraction β-carotene was bound to high-molecular-weight protein(s), quite possibly a plasma-derived lipoprotein. The subcellular distribution of β-carotene in corpus luteum is quite similar to the distribution of its metabolite, retinol, in liver. This finding coupled with other recently published data suggests that β-carotene could play a distinct role in corpora lutea function.

β-Carotene is the major dietary precursor of vitamin A in man and other mammals and is the source of the vitamin for free living herbivores. This polyprene is cleaved in the intestine by β-carotene 15,15′-dioxygenase (EC 1.13.11.21) to retinal and subsequently reduced to retinol by retinal reductase (EC 1.1.1.71) (1). The retinol produced is esterified and transported to the liver where it is stored. Retinol is released as needed and carried to specific receptors on target tissues via retinol binding protein (RBP) (2).

Aside from this precursor function β-carotene possesses interesting chemical properties of its own. It is a free radical trap and perhaps the most efficient natural quencher of singlet oxygen known (3). This is of importance since singlet oxygen is mutagenic and particularly effective in causing lipid peroxidation (4). β-Carotene and similar polyprenes which are present in carrots and all foods that contain chlorophyll appear to be the plant's major defense against the singlet oxygen which is generated as a by-product from the interaction of light and chlorophyll (5). Carotenoids have been shown to be effective anticarcinogens (6), to defend smokers presumably by scavenging radicals existing in cigarette smoke and tar (7), and to protect porphyrias patients whose photosensitivity is believed to be due to singlet oxygen formation (8). β-Carotene may also slow the aging process where the source of damage is due to oxygen radicals and lipid peroxidation (9).

Additionally, β-carotene has been purported to have a specific role in fertility distinct from that of serving as precursor to vitamin A. In nutritional studies, cows fed β-carotene-deficient diets exhibited delayed ovulation and an increase in the number of follicular and luteal cysts (10–12). However these studies have resulted in controversy for two major reasons. First, on theoretical grounds, since much of the ingested β-carotene would be converted to vitamin A these studies provide only circumstantial evidence of a distinct role for β-carotene. Second, other research groups have failed to substantiate these findings (13–16) although Folman *et al.* (17) have subsequently found an effect of β-carotene on fertility.

Recently, Rhodes (18) demonstrated antagonistic functions between retinol and β-carotene on interferon action in cultured cell systems. If an antagonism between retinol and β-carotene exists in other situations, e.g., fertility, then this could explain how controversy over β-carotene's role could arise. Cows with different β-carotene/retinol ratios may exhibit different effects.

The corpus luteum maintains pregnancy in several mammalian species through its synthesis of progesterone (19). In addition it is the tissue that can contain the highest levels of β-carotene (20). Knowledge of the distribution of β-carotene in this tissue could provide insight as to the possible influence of β-carotene on reproduction.

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Materials and Methods. *Chemicals.* Analytical grade cyclohexane, ethyl acetate, and toluene were products of J. T. Baker Chemical Company (Phillipsburg, N.J.). β-Carotene was a gift from Hoffmann–LaRoche, Inc. (Nutley, N.J.). All other biochemicals were purchased from Sigma Chemical Company (St. Louis, Mo.).

Cell fractionation. Bovine corpus luteum was dissected from the ovaries of cows obtained at a local abattoir. The tissue was minced in 15 vol of ice-cold 0.25 *M* sucrose, 1 m*M* EDTA, 2 m*M* Tris–HCl (pH 7.4) and homogenized with seven passes of a Potter–Elvehjem homogenizer. The homogenate was fractionated as previously described (21) into crude nuclear (6×10^3 g-min sediment), crude mitochondrial (3.6×10^5 g-min sediment), microsomal (6×10^6 g-min sediment), cytosolic (6×10^6 g-min supernatant), and lipid (6×10^6 g-min floating layer) fractions. All pellets were resuspended in 15 vol of the sucrose buffer. This and all other procedures were performed under dim yellow light or in the dark if possible.

β-Carotene analysis. One-milliliter aliquots of the subcellular fractions were mixed with 1 ml of freshly prepared ethanolic potassium hydroxide (1 ml of 9 *N* KOH to 9 ml of 95% ethanol) in 16 × 125-mm screw-cap extraction tubes and incubated at 37°C for 1 hr. Five milliliters of hexane was then added and the tubes were vortexed for 5 min on a multitube vortexer (Scientific Manufacturing Industries, Inc., Emeryville, Calif.). The tubes were centrifuged for 5 min at 400*g*. The hexane layer was transferred to a 12 × 75-mm culture tube and evaporated to dryness under a stream of nitrogen in a 37°C water bath. For identification of β-carotene the contents of the tubes were redissolved in 50 μl hexane and 5-μl aliquots were applied to a thin-layer silica gel chromatography plate (No. 13179 without fluorescent indicator, Eastman Kodak, Rochester, N.Y.). The plates were developed in cyclohexane/toluene/ethyl acetate (50/30/20, v/v) (22). For quantification of β-carotene, the dried tubes were redissolved in 1.2 ml hexane and the absorbance of β-carotene was measured at 450 nm (23) where $E^{1\%}_{1cm}$ is 2620. Ninety percent of exogenously added β-carotene was recovered in the extraction procedure.

Pronase digestion. Five milligrams of Pronase was added to 1-ml aliquots of corpus luteum cytosol and incubated at 37°C for 2 hr. The mixtures were then applied to Sephadex G-75 columns.

Sephadex G-75 chromatography. One-milliliter corpus luteum cytosol fractions were applied to a 0.9 × 80-cm Sephadex G-75 column equilibrated at 4°C with 50 m*M* Tris–HCl (pH 7.4), 300 m*M* KCl, and 1 m*M* dithiothreitol. Flow rate was 3 ml/hr and 1.0-ml fractions were taken. The exclusion and total volumes of the column were measured with Dextran Blue 2000 and [^{14}C]-glucose, respectively.

Protein. The protein content of each subcellular fraction was determined by the method of Lowry *et al.* (24) with bovine serum albumin as standard.

Results. *Cellular localization of β-carotene.* The distribution of β-carotene in the subcellular fractions of bovine corpus luteum is presented in Table I. All five fractions contained significant amounts of β-carotene but most (67.7%) of the β-carotene is found in the crude nuclei and lipid. When the localization of β-carotene is expressed relative to the amount of protein (relative specific distribution), only the lipid fraction appeared to be enriched in β-carotene. This enrichment is not unexpected for such a lipophilic molecule as β-carotene. The lipid fraction, however, is not solely lipid as repeated centrifugation of this fraction reveals that it still contains approximately 2.5% of the total corpus luteum protein.

The crude nuclear and mitochondrial fractions are contaminated with material trapped from other cell fractions. In an attempt to characterize the association of β-carotene with these membrane fractions, the crude nuclei and mitochondria were repeatedly (five times) resuspended in homogenization buffer and centrifuged at 27,000*g* for 10 min. Of the β-carotene originally associated with these two fractions, 50–70% could be removed by washing. It appears unlikely that this loose association is due strictly to contamination by other subcellular fractions because it is much greater than expected (21, 25). Nevertheless it may be caused by nonspecific lipid–membrane interactions. Alternatively, the β-carotene may be associated with a

TABLE I. CELLULAR LOCALIZATION OF β-CAROTENE IN BOVINE CORPUS LUTEUM

Fraction	Protein		β-Carotene		
	mg/g tissue	% of total	μg/g tissue	% of total	Relative specific distribution
Crude nuclei	80.6	35.7	10.3	32.6	0.9
Crude mitochondria	39.6	17.5	4.8	15.2	0.9
Microsomes	23.1	10.2	2.5	7.9	0.8
Cytosol	76.8	34.0	2.9	9.2	0.3
Lipid	5.6	2.5	11.1	35.1	14.0

Note. Cell fractions were prepared and protein and β-carotene concentrations were determined as described under Materials and Methods. Relative specific distribution per fraction is defined as percentage β-carotene ÷ percentage protein. The results in this table represent a typical experiment. In general the standard deviations of the percentage distributions were within 15%. However, the amount of β-carotene in corpus luteum depended on the diet of the animal and ranged between 10 and 50 μg/g.

lipoprotein that exerts a more specific, physiological binding with the membranes. Such a binding would also be washed away.

Physical relationship of β-carotene to microsomal membranes. In order to characterize the association of β-carotene with the microsomal membrane, the pellet was subjected to various treatments (Table II). These studies showed that neither repeated washing, polytronification, nor freeze–thawing released β-carotene from this membrane fraction. However, incubation of the membrane with Triton X-100 completely released it. These results suggest that β-carotene is an integral part of the bovine corpus luteum microsomal membrane, since it can be released only by an agent that causes extraction of intrinsic membrane components.

TABLE II. EXTRACTION OF β-CAROTENE FROM CORPUS LUTEUM MICROSOMAL MEMBRANE UNDER VARIOUS CONDITIONS

Treatment	β-Carotene (% of total)	
	Residue	Supernatant
(1) Untreated	100	n.d.[a]
(2) Washed	100	n.d.
(3) Polytronification	100	n.d.
(4) Freeze-thawed	98	2
(5) 1% Triton X-100	4	96

Note. The microsomal pellet was resuspended in a homogenization buffer. Aliquots were treated in the following ways: (1) untreated control; (2) repeated washing (five times); (3) polytronification (Brinkmann, setting 7) for 1 min, three times, 20 sec each time, at 0°C; (4) alternate freezing and thawing (five times); (5) addition of 1% Triton X-100 and incubation at 37°C for 30 min. Following these treatments the samples were centrifuged at 105,000*g* for 1 hr. The respective residues and supernatants were each extracted and analyzed for β-carotene.

[a] Not detectable.

Gel filtration of corpus luteum cytosol. Since β-carotene is a lipophilic molecule it is likely to be protein-bound in order to facilitate intracellular transport and prevent nonspecific membrane partitioning. In order to obtain such evidence cytosol was chromatographed on a Sephadex G-75 column. In the cytosol, as shown in Fig. 1, endogenous β-carotene is bound to high-molecular-weight protein(s) that elutes in the exclusion volume of the column. Pronase digestion destroys this elution pattern (Fig. 1) and β-carotene becomes part of a lipid aggregate that does not enter the column.

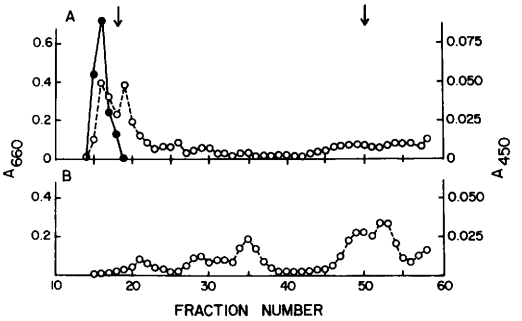

FIG. 1. Sephadex G-75 gel filtration patterns showing optical density of β-carotene at 450 nm (●) and protein at 660 nm (○) in bovine corpus luteum cytosol. (A) Untreated cytosol and (B) cytosol after incubation with Pronase. The arrows indicate the void volume (V_0) and total volume (V_t) of the columns.

In retrospect, the fact that β-carotene is bound to protein(s) of high molecular weight in the cytosol might be expected. In serum β-carotene is associated with both high-density (HDL) and low-density (LDL) lipoproteins (mol wt 2×10^5 and 3×10^6, respectively) (26, 27). These cholesterol-containing lipoproteins bind to specific cell surface receptors on target tissues. Presumably, β-carotene would enter corpus luteal tissue bound to such lipoproteins in a manner analogous to cholesterol entry. As these proteins are internalized by endocytosis they would be expected to be found temporarily intact in the cytosol. The fact that protein-bound β-carotene elutes from Sephadex G-75 in the same fractions as LDL and HDL (data not shown) is consistent with this information. Whether HDL or LDL is found within a cell depends on the tissue and species (28).

Discussion. The distribution of β-carotene in corpus luteum is similar in many respects to the distribution of retinol in liver. In liver retinol is found in crude nuclear and mitochondrial fractions (29–31), and in microsomes (29, 30). Its extraction from microsomes likewise requires detergent treatment supporting the view that both vitamin A and β-carotene are integral components of the endoplasmic reticulum.

Retinol is also found in both the cytosol (29–33), where it is bound to a specific protein called cellular-retinol-binding protein (CRBP) (34), and in a floating lipid fraction (29, 32). The latter fraction probably consists of lipid globules which exist for the storage of retinyl ester and carotenoids (35).

As mentioned previously, it has been suggested that β-carotene may play a unique role in reproduction (10–12). It was also noted that separation of this role from that of retinol precursor is difficult since β-carotene would be converted to retinol in the animal's intestinal mucosa. Furthermore β-carotene can be converted to retinol by bovine corpus luteum tissue slices (36, 37). Here the carotene cleavage activity changes with the ovulatory cycle and the highest level is present at midovulation (37). Ostensibly, the observations of Lotthammer *et al.* might be accounted for in terms of a retinol, rather than a β-carotene, deficiency.

What might actually be relevant, however, is not that both β-carotene and retinol are present in corpus luteum per se but rather what their ratios are. Rhodes (18) has recently demonstrated antagonistic functions between retinol and β-carotene on human interferon action in cultured cell systems. The observation that β-carotene can affect the outcome of a retinol-dependent event (and vice versa) and even induce a new event depending on the β-carotene:retinol ratio has important consequences and sheds new light on the possibility of a separate function for β-carotene. Ratios are characteristic of many diverse systems, e.g., cAMP:cGMP; estrogen:progesterone. It may be reasonable to envision a similar concept for β-carotene and retinol, the experimental possibility of which has already been obtained (18).

The need to have the correct β-carotene:retinol ratio might explain why others (13–16) have been unable to confirm Lotthammer's work. It could also explain the necessity to have a β-carotene cleavage system in the corpus luteum. Such a system could maintain a constant β-carotene:retinol ratio in the face of a rising β-carotene concentration. Other variations are also possible.

The ratio concept allows retinol to be present for retinol-dependent functions but yet ensures an overall β-carotene-directed cell. It follows that as the corpus luteum would be a β-carotene-directed tissue, the liver would be a retinol-directed one. Both may be model areas of corresponding functions since each represents extreme and opposite β-carotene:retinol ratios.

Since mammalian species do not synthesize β-carotene it is difficult to envision a reproductive role for β-carotene in those animals that do not absorb it. One very interesting report however, which awaits conformation by other investigators, presents evidence that β-carotene can be synthesized *de novo* from acetate by corpora luteal tissue (in this instance, bovine) (38). If this is true of corpora luteal tissue in all species, β-carotene function could be universal.

In this paper we have made an attempt to determine where β-carotene may exert its prospective function(s) by characterizing its subcellular location in bovine corpus luteum.

Since it is present in fractions of the cell where retinol is found, β-carotene may influence retinol's role in corpora lutea function.

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