Biosynthesis of retinal in bovine corpus luteum

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Abstract Bovine corpus luteum tissue was sliced and incubated with β-[15,15′-^{3}H]carotene. The conversion of radioactive β-carotene into radioactive retinal was substantiated utilizing column chromatography, thin-layer chromatography, high-speed liquid chromatography, and a derivative formation. Lowering of the incubation temperature to 20°C or boiling the tissue eliminated the conversion of β-carotene to retinal. In addition, other carotenoids and possible oxidation products of β-carotene in the corpus luteum were investigated. Our results indicate that the bovine corpus luteum possesses the ability to synthesize retinal in situ, which may play a role in reproductive functions.

Supplementary key words vitamin A · β-carotene · oxidation of carotenoids · dioxygenase · high-speed liquid chromatography

Bovine corpus luteum contains relatively high concentrations of β-carotene (1, 2), amounting to 60 μg/g of tissue weight.[1] Recently (3), retinal has been isolated from this tissue. Although the primary site of conversion of β-carotene to retinal is intestinal mucosa (4–6), the reaction occurs also in liver and kidney (7–9). The presence of provitamin A and retinal in the corpus luteum raises the question of a possible relationship between those compounds. It has been suggested that vitamin A may be required for efficient steroid hormone production (10–12) and that it plays a role in reproductive processes, e.g., maintenance of pregnancy (13, 14). It is also of interest that β-carotene was synthesized in vitro in the bovine corpus luteum from sodium [1-^{14}C]acetate (15).

MATERIALS AND METHODS

The β-[15,15′-^{3}H]carotene (sp act 147 μCi/mg) was a gift from Hoffmann-La Roche Inc. Unlabeled β-carotene and retinal were purchased from Sigma Chemical Co. All other reagents were analytical grade or glass distilled.

Corpora lutea were obtained from the ovaries of freshly slaughtered cows at a local slaughterhouse.

Incubation

Corpus luteum tissue was sliced, and 1-g samples were incubated in 20 ml of buffer medium with radioactive β-carotene in the dark at 37°C for 1 hr, with continuous shaking. The buffer solution contained 2 mmoles of KH_2PO_4, 0.3 mmole of nicotinamide, 0.1 mmole of glutathione, 0.12 mmole of sodium glycocholate, 0.12 mmole of $MgCl_2$, 0.02 mmole of $FeSO_4$, 4 mg of lecithin, 2 mg of α-tocopherol, and 1.25 μg of β-[^{3}H]carotene in 50 μl of acetone, pH 7.7. A total of 30 incubations were performed.

Extraction

After incubation, the tissue was homogenized in a mixture of chloroform–methanol 2:1 with a Polytron homogenizer, and the volume was brought to 150 ml. Then 25 ml of 0.1 N H_2SO_4 was added and the phases were separated in a separatory funnel. The chloroform layer was collected and evaporated in vacuo at 40°C. The residue was dissolved in hexane (1 ml) containing α-tocopherol (1 mg), β-carotene (100 μg), and retinal (100 μg).

Alumina column chromatography

The hexane solution was added to a column (6 × 1 cm) containing 5 g of alumina (Woelm neutral Al_2O_3, activity grade III) as in a hexane slurry. The elution, performed in the dark with solvents of increasing polarity (16), was as follows: β-carotene (20 ml of *n*-hexane); retinyl esters (20 ml of benzene–hexane 1:17[v/v]); retinal (20 ml of benzene–hexane 1:1), retinol (50 ml of benzene); more polar compounds (20 ml of methanol); acidic compounds, including retinoic acid (20 ml of methanol–25% acetic acid 3:1). The eluate was collected in 2-ml fractions with an ISCO model V volumeter and an ISCO model A fraction collector. Every fraction was sampled for radioactivity. An aliquot of 0.4 ml was evaporated and counted in 10 ml of Omnifluor (New England Nuclear, 4 g of a mixture of 98% 2,5-diphenyloxazole and 2% *p*-bis-(*o*-methylstyryl)benzene added to 1 l of toluene) in a Packard Tri-Carb model 3310 liquid scintillation spectrometer. If quenching was significant, an internal standard was added ([^{3}H]toluene, sp act 3.12 × 10^6 dpm/

[1] The bovine corpus luteum contains variable amounts of β-carotene, from traces in the winter to above 110 μg/g in the summer. Endogenous retinal level is about 1.2 μg/g of tissue. (B. Austin, Ph.D. Thesis, University of Massachusetts, 1969.)

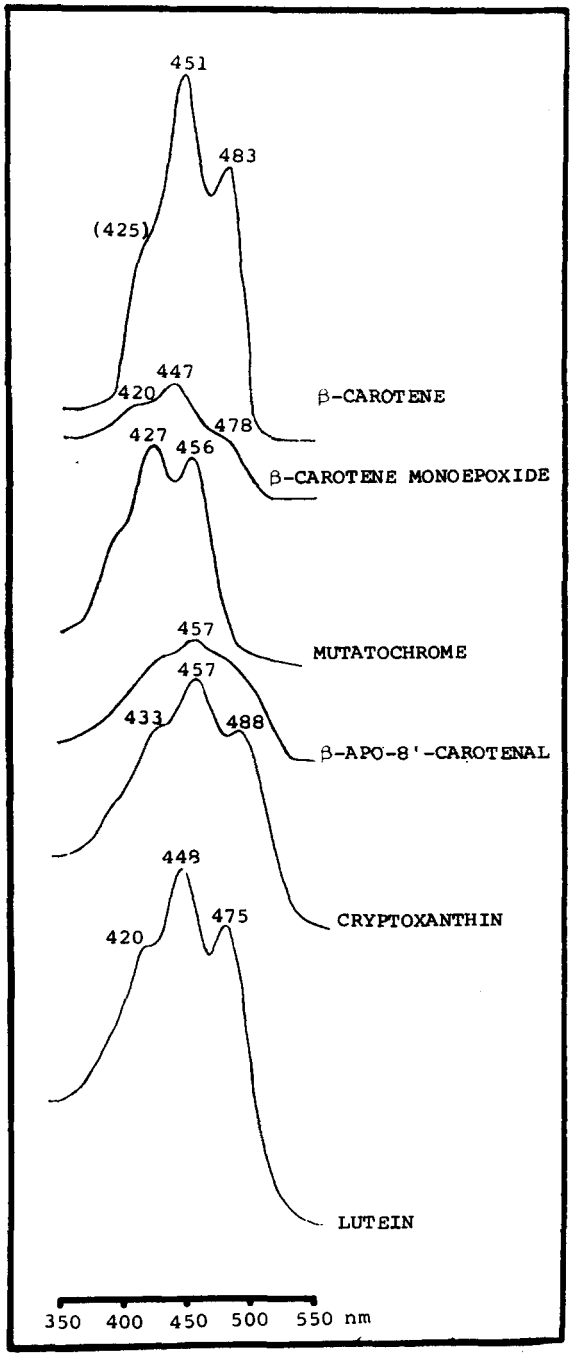

Fig. 1. Visible spectra of carotenoids isolated from corpus luteum (in hexane).

ml, New England Nuclear) and the sample was counted again.

High-speed liquid chromatography

Radioactive fractions containing carrier retinal were pooled, evaporated to dryness, and redissolved in minimal amounts of hexane. Aliquots of this solution were injected onto a Corasil II column (2 × 1050 mm) and eluted with 1% ethyl acetate in Skellysolve B (bp 61–70°C) at a flow rate of 0.65 ml/min under 150–200 psi pressure delivered by a Milton Roy model 196-31 pump. The UV absorbing peaks were detected in the eluate by a Laboratory Data Control model 1280 UV detector (280 nm) and a recorder. The peaks were collected in separate vials and counted or subjected to further analysis.

Thin-layer chromatography

The samples were spotted on silica gel G plates, 250 μm thick, with authentic retinal and β-carotene, and the plates were developed in a closed tank in the dark. The following solvent systems were used: I, acetone–petroleum ether 1:9 (3); II, hexane–diethyl ether 1:1 (17); and III, methanol–benzene 15:85. The spots were scraped into scintillation vials and counted in Omnifluor or eluted with hexane–ethanol 9:1 and used for further tests.

Derivative formation

Retinal was reduced to retinol with $NaBH_4$ (16). The retinal solution was evaporated under nitrogen and dissolved in 0.3 ml of ethanol; 1 mg of solid $NaBH_4$ was added, and the mixture was left in the dark for 2 hr. Retinol was separated on a thin-layer chromatography plate with solvent system II. Retinol was detected by fluorescence under UV light, eluted, and counted. Spectra of retinal, retinol, and β-carotene were obtained with a Cary 14 (Varian) spectrophotometer.

Recovery

All the operations were performed in dim light, red light, or, if possible, complete darkness. The addition of 0.05% BHT (butylated hydroxytoluene, Aldrich Chemical Co.) as an antioxidant helped in obtaining excellent recovery in all analytical procedures for β-carotene and retinal as confirmed by the radioactivity and absorbance measurements. Recovery of β-carotene and retinal in various procedures was over 95%.

RESULTS AND DISCUSSION

Oxidation of β-carotene

Goodman and coworkers (6, 16) advise purification of radioactive β-carotene on an alumina column before using it in the incubation. In this investigation it was found that oxidation products of β-carotene contaminated every fraction in alumina column chromatography, with the greatest amount of radioactivity in the methanol eluate. When 0.05% BHT was added to all solvents, the oxidation products became insignificant. Subsequently, the solution of labeled β-carotene was used without a daily alumina column purification. Apparently, most contaminants are formed on the alumina column itself if β-carotene is not protected by antioxidants. In further experiments we used

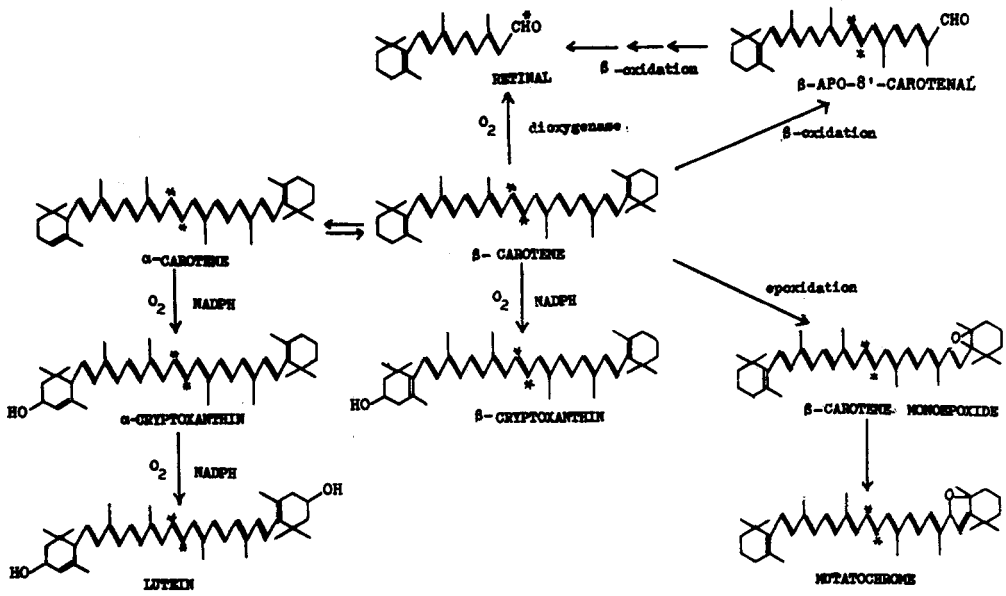

Fig. 2. Scheme of β-carotene oxidation in corpus luteum.

0.05% BHT in all solvents for extraction and alumina or thin-layer chromatography.

Besides β-carotene, corpora lutea of examined cows contained other carotenoids. It was possible to isolate them by alumina chromatography and to identify tentatively visible spectra of β-carotene monoepoxide (447, 478 nm) in fraction 2, mutatochrome (427, 456 nm) and β-apo-8′-carotenal (457 nm) in fraction 3, cryptoxanthin (450, 480 nm) in fraction 4, and lutein (420, 448, 478 nm) in fraction 5 (**Fig. 1**). The last fraction, 6, always contained a pink-brown pigment with a sharp absorption band at 398 nm, identical with hemin from cattle blood subjected to identical extraction and chromatography.

Lutein and cryptoxanthin have been found before in cow's blood and milk (17, 18). Mutatochrome was mentioned as a possible β-carotene autoxidation product in fats and organic solvents (19, 20).

After incubation of β-[^{3}H]carotene with active corpus luteum tissue, the isolated carotenoids contained some radioactivity. They should be considered as possible stages in the β-carotene changes in corpus luteum tissue, at least in vitro (**Fig. 2**). Fraction 3 obviously required further purification to separate retinal from mutatochrome and β-apo-8′-carotenal.

Conversion of β-carotene to retinal

In order to investigate the possibility of enzymatic splitting of β-carotene to retinal in corpus luteum, the incubations were performed at 20°C, with boiled tissue, and at the optimal temperature of 37°C. Goodman and Olson (16) reported that the intestinal enzyme is completely inactivated by heating at 64°C for 55 sec. **Table 1** shows that the uptake of β-carotene by tissue was reduced to one-tenth due to boiling. Lowering of incubation temperature also significantly reduced β-carotene uptake (35%). In fraction 3, where retinal is eluted, no radioactivity was found in the case of boiled tissue, and twice as much was found in the 37°C incubation as in the 20°C incubation. High-speed liquid chromatography revealed that most of the radioactivity in the 20°C incubation resided in products other than retinal. In the extract from the 37°C incubation, most of the radioactivity was found in the retinal peak (**Fig. 3**). Thin-layer chromatography of fraction 3 confirmed these results. No radioactivity was found in the retinal spot from the 20°C incubation, whereas the 37°C incubation did produce radioactive retinal.

Another proof of authentic retinal formation from radioactive β-carotene in corpus luteum tissue was obtained from the conversion of purified retinal to radioactive retinol by quantitative reduction with $NaBH_4$. Retinal with nonlabeled carrier was extracted and purified by alumina chromatography and thin-layer chromatographic separation in system II (R_F = 0.50). It was eluted and reduced with $NaBH_4$. The retinol thus obtained was purified on

TABLE 1. Radioactivity distribution in different fractions from alumina column chromatography

Fraction	Boiled Tissue, 37°C	20°C	37°C
	cpm/g	*cpm/g*	*cpm/g*
1 (β-Carotene)	1,524	9,795	15,193
2 (Retinyl esters)	32	40	261
3 (Retinal)	0	227	478
4 (Retinol)	34	464	473
5	75	298	416
6 (Retinoic acid)	20	238	211
Total[a]	1,685	11,062	17,032

[a] Total radioactivity taken up by the tissue.

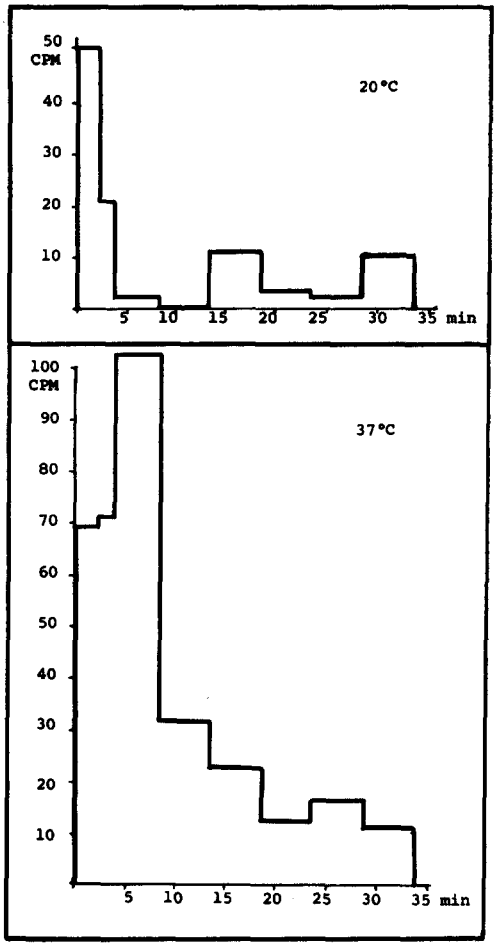

Fig. 3. High-speed liquid chromatography elution pattern of radioactive compounds in the retinal fraction (fraction 3) from incubations at different temperatures.

another thin-layer chromatography plate and visualized under UV light (R_F = 0.25). After elution, retinol contained 88% of the retinal radioactivity (360 and 408 cpm, respectively). There was no radioactivity at the retinal R_F in the second chromatography system.

The bovine corpus luteum possesses an enzymatic mechanism capable of converting β-carotene to retinal, although it may be not as effective as that of rat intestinal mucosa. Goodman and coworkers reported a 50% conversion yield (6, 16), but others usually obtained lower values (9, 21, 22). Recently, it was reported (23) that purified carotene 15,15′-dioxygenase of rabbit intestine could produce retinal from β-apo-10′-carotenol with a 10–15% yield. The enzyme is known to be 12.5 times more effective with β-apo-10′-carotenol than with β-carotene as a substrate. When the high concentration of β-carotene in the corpus luteum is considered, the conversion of this substrate to retinal in situ may have physiological significance in reproductive functions.

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REFERENCES

1. Escher, H. 1913. Über den Farbstoff des Corpus Luteum. *Hoppe-Seyler's Z. Physiol. Chem.* **83:** 198–211.
2. Kuhn, R., and E. Lederer. 1932. Bestimmung von Carotinoiden. *Hoppe-Seyler's Z. Physiol. Chem.* **206:** 41–64.
3. Austern, B. M., and A. M. Gawienowski. 1969. The isolation of retinal from the bovine corpus luteum. *J. Reprod. Fert.* **19:** 203–205.
4. Thompson, S. Y., R. Braude, M. E. Coates, A. T. Cowie, J. Ganguly, and S. K. Kon. 1950. Further studies of the conversion of β-carotene to vitamin A in the intestine. *Brit. J. Nutr.* **4:** 398–420.
5. Olson, J. A. 1964. The effect of bile and bile salts on the uptake and cleavage of β-carotene into retinol ester (vitamin A ester) by intestinal slices. *J. Lipid Res.* **5:** 402–408.
6. Goodman, DeW. S., H. S. Huang, M. Kanai, and T. Shiratori. 1967. The enzymatic conversion of all-*trans* β-carotene into retinal. *J. Biol. Chem.* **242:** 3543–3554.
7. Bieri, J. G., and C. J. Pollard. 1954. Studies of the site of conversion of β-carotene injected intravenously into rats. *Brit. J. Nutr.* **8:** 32–44.
8. Worker, N. A. 1957. Site of conversion of carotene into vitamin A in the rat: further studies on aqueous dispersions administered intravenously. *Brit. J. Nutr.* **11:** 44–47.
9. Olson, J. A., and O. Hayaishi. 1965. The enzymatic cleavage of β-carotene into vitamin A by soluble enzymes of rat liver and intestine. *Proc. Nat. Acad. Sci. USA.* **54:** 1364–1370.
10. Grangaud, R., M. Nicol, and D. Desplanques. 1969. Effect of vitamin A on the enzymic conversion of the Δ^5-3-β-hydroxy into Δ^4-3-oxosteroids by adrenals of the rat. *Amer. J. Clin. Nutr.* **22:** 991–1002.
11. Juneja, H. S., S. K. Murthy, and J. Ganguly. 1966. The effect of vitamin A deficiency on the biosynthesis of steroid hormones in rats. *Biochem. J.* **99:** 138–145.
12. Piziak, V. K., and A. M. Gawienowski. 1972. The effect of added vitamin A alcohol on the metabolism of progesterone in the placenta of the guinea pig, *Cavia porcellus*. *Comp. Biochem. Physiol.* **42B:** 201–203.
13. Moore, T. 1957. Vitamin A. Elsevier, Amsterdam. 329.
14. Thompson, J. N. J., McC. Howell, and G. A. J. Pitt. 1965. Nutritional factors affecting fertility: vitamin A. *In* Agents Affecting Fertility. C. R. Austin and J. S. Perry, editors. J. & A. Churchill Ltd., London. 34–46.
15. Austern, B. M., and A. M. Gawienowski. 1969. In vitro biosynthesis of β-carotene by bovine corpus luteum tissue. *Lipids.* **4:** 227–229.
16. Goodman, DeW. S., and J. A. Olson. 1969. The conversion of all-*trans* β-carotene into retinal. *Methods Enzymol.* **15:** 462–475.
17. Gillam, A. E., and I. M. Heilbron. 1935. The carotenoids of butter. *Biochem. J.* **29:** 834–836.
18. Gillam, A. E., and M. S. El Ridi. 1935. Carotenoids and vitamin A in cow's blood serum. *Biochem. J.* **29:** 2465–2468.

19. Hunter, R. F., and R. M. Krakenberger. 1947. The oxidation of β-carotene in solution by oxygen. *J. Chem. Soc. London.* 1–4.
20. McWeeny, D. J. 1968. Deterioration of β-carotene in certain hydrogenated fats. II. Products of β-carotene deterioration and nature of green pigment. *J. Sci. Food Agr.* **19:** 254–258.
21. Olson, J. A. 1961. The conversion of radioactive β-carotene into vitamin A by the rat intestine in vivo. *J. Biol. Chem.* **236:** 349–356.
22. Gronowska-Senger, A., and G. Wolf. 1970. Effect of dietary protein on the enzyme from rat and human intestine which converts β-carotene to retinal. *J. Nutr.* **100:** 300–308.
23. Lakshmanan, M. R., H. Chansang, and J. A. Olson. 1972. Purification and properties of carotene 15,15′-dioxygenase of rabbit intestine. *J. Lipid Res.* **13:** 477–482.