Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 9124–9128, December 1989
Biochemistry

Enzymatic conversion of all-*trans*-β-carotene to retinal by a cytosolic enzyme from rabbit and rat intestinal mucosa

M. R. LAKSHMAN*, I. MYCHKOVSKY, AND MARK ATTLESEY

Lipid Research Laboratory, Veterans Administration Medical Center, Washington, DC 20422

Communicated by Nevin S. Scrimshaw, August 14, 1989

ABSTRACT **Enzymatic conversion of all-*trans*-β-carotene to retinal by a partially purified enzyme from rabbit and rat intestinal mucosa was demonstrated. The enzymatic product was characterized based on the following evidence: (*i*) The product gave rise to its *O*-ethyloxime by treatment with *O*-ethylhydroxylamine with an absorption maximum at 363 nm in ethanol characteristic of authentic retinal *O*-ethyloxime. High-pressure liquid chromatography (HPLC) of this derivative yielded a sharp peak with a retention time of 7.99 min corresponding to the authentic compound. The enzyme blank and boiled enzyme blank failed to show any significant HPLC peaks corresponding to retinal *O*-ethyloxime, retinal, or retinol. (*ii*) The mass spectrum of the *O*-ethyloxime of the enzymatic product was identical to that of authentic retinal *O*-ethyloxime (m/z 327: 45%, $M^{\ddagger}$ and m/z 282: 100%, M − ethoxy). (*iii*) The specific activity of the enzymatically formed [^{14}C]retinal *O*-ethyloxime remained constant even after repeated crystallization. (*iv*) The enzymatic product exhibited an absorption maximum at 370 nm in light petroleum characteristic of authentic retinal. Furthermore, it was reduced by horse liver alcohol dehydrogenase to retinol with an absorption maximum at 326 nm in light petroleum. This retinol was enzymatically esterified to retinyl palmitate by rat pancreatic esterase with a retention time of 10 min on HPLC corresponding to authentic retinyl palmitate. Thus, the enzymatic product of β-carotene cleavage by the partially purified intestinal enzyme was unequivocally confirmed to be retinal.**

It is well established that β-carotene is the precursor of vitamin A and its conversion into vitamin A *in vivo* has been unequivocally demonstrated in rats, pigs, and humans (1–7).† Subsequently, the *in vitro* enzymatic conversion of β-carotene to retinal by an enzyme preparation from rat intestine and liver was independently shown by Goodman *et al.* (8) and Olson and Hayaishi (9). This was extended by a number of workers (10–20) using different species as the source of the enzyme. However, a more recent report (21) claimed that it could not duplicate the original work (8, 9, 15) and hence raised the possibility that retinal may not be the true product of β-carotene conversion to vitamin A *in vitro*, although it did not question the validity of the formation of vitamin A from β-carotene *in vivo*.

In view of this controversy, we decided to systematically repeat the work using the intestinal mucosa from rabbit and rat as the source of the β-carotene cleavage (BCC) enzyme activity. It will be demonstrated conclusively in the present communication that retinal is, in fact, the product of BCC enzyme using (*i*) a method of synthesizing the *O*-ethyloxime of the enzymatic product as its derivative and subsequent separation, identification, and quantitation by high-pressure liquid chromatography (HPLC); (*ii*) unequivocal identification of the retinal *O*-ethyloxime derivative by its characteristic absorption spectrum, its mass spectrum, and its repeated crystallization to constant specific activity after it was enzymatically formed from [^{14}C]β-carotene; and finally (*iii*) enzymatic reduction of retinal to retinol and its subsequent esterification to retinyl palmitate with their characteristic absorption spectra and their distinctive elution profiles on HPLC. The structural formulae of the substrate, β-carotene, the product, retinal, and its *O*-ethyloxime derivative are depicted in Fig. 1.

EXPERIMENTAL

Animals. New Zealand White rabbits (2–3 kg) were procured from the National Institutes of Health; male Wistar-Furth albino rats were from Charles River Breeding Laboratories. The animals were maintained on their normal chow diets (Wayne Lablox; Allied Mills, Chicago) for at least 2 weeks before the experimentation.

Chemicals. All chemicals and reagents were of analytical or ultrapure grade. All organic solvents were of HPLC grade and were routinely filtered through a 0.45-μm filter before use. All solvents used for extraction had 50 mg of butylated hydroxytoluene per liter.

Isolation of BCC Enzyme. The procedure was essentially according to our earlier publication (16). Briefly, each animal was killed by aortic exsanguination under pentobarbital (50 mg/kg) anesthesia, and the proximal third of the intestine was isolated and washed with 0.154 M ice-cold saline. All subsequent procedures were carried out at 4°C unless otherwise stated. The intestine was slit open longitudinally and the mucosa was scraped into a beaker containing the homogenizing buffer (0.1 M potassium phosphate buffer, pH 7.8, containing 1 mM dithiothreitol). The mucosa was homogenized with 5 volumes of the homogenizing buffer and the homogenate was centrifuged at 100,000 × g for 1 hr. The supernatant solution was subjected to 0–60% ammonium sulfate saturation and centrifuged at 16,000 × g for 15 min. The pellet was dissolved in the homogenizing buffer to give a final protein concentration of ≈ 10 mg/ml and was further fractionated with cold acetone (−30°C) into 0–45% and 45–60% fractions. Both of the pellets were redissolved in original buffer and stabilized by addition of 1 mM reduced glutathione (GSH) and ammonium sulfate to 50% saturation. Under these conditions, the enzyme preparation could be stored for a month at −80°C without appreciable loss of activity. These enzyme fractions were assayed for BCC activity as described below. The protein in various fractions was determined according to Lowry *et al.* (22).

Enzyme Assay. The standard assay mixture was made up as follows: 100 nmol of β-carotene in 0.1 ml of benzene was mixed with 180 μl of 1:10 diluted Tween 20 in water, and the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: BCC, β-carotene cleavage.

*To whom reprint requests should be addressed at Lipid Research Laboratory (151T), Veterans Administration Medical Center, 50 Irving Street, N.W., Washington, DC 20422.

†The term "vitamin A" is used generically for all derivatives of β-ionone (other than the carotenoids) that possess the biological activity of all-*trans*-retinol or its structural relatives.

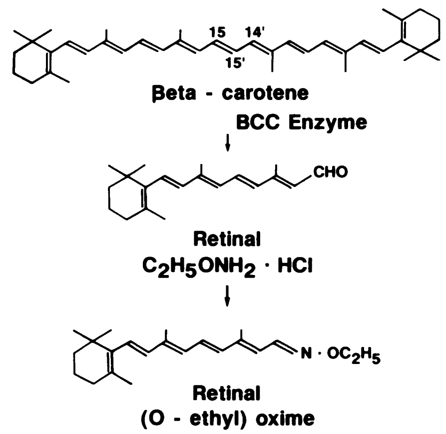

FIG. 1. Structural formulae of all-*trans* isomers of β-carotene, retinal, and retinal *O*-ethyloxime.

benzene was removed by a gentle stream of nitrogen. To this substrate the following components were added at the indicated final concentrations; potassium phosphate buffer (pH 7.8), 100 mM; GSH, 1 mM; ferrous sulfate, 1 mM; nicotinamide, 15 mM; and the intestinal enzyme fraction, ≈7 mg; the final volume was always made up to 2 ml with water. Blank tubes had either no enzyme preparation or an equivalent amount of boiled enzyme fraction (boiled at 100°C for 5 min). After incubation at 37°C for 60 min in a shaking water bath (50 excursions per min) under F40 gold fluorescent light, the reaction was stopped by addition of 2 ml of methanol. The *O*-ethyloxime derivative was prepared essentially according to Van Kuijk *et al.* (23). Briefly, 100 μl of 0.1 M *O*-ethylhydroxylamine hydrochloride in 0.1 M potassium phosphate buffer (pH 6.5) and 100 μl of methanol containing cholesterol (50 μg/ml) were added to the incubation mixture. After 10 min at 25°C, 6 ml of water was added and the whole reaction mixture was thoroughly extracted with three 10-ml portions of light petroleum. The lipid extracts were combined and evaporated to near dryness under a gentle stream of nitrogen, and the residue was finally redissolved in 1 ml of methanol. A 50-μl sample of the final methanol extract was subjected to HPLC on a 4.6 × 25 cm Vydac TP column (reverse-phase C_{18} column; 9% carbon load; particle size, 5 μm) with a linear gradient solvent system of methanol/water containing 0.5% ammonium acetate as the mobile phase at a flow rate of 1 ml/min. The linear gradient was increased over a 10-min period from 92%:8% to 98%:2% methanol/water system. Under these conditions authentic retinal *O*-ethyloxime had a retention time of 8.1 min, whereas retinol, retinal, and β-carotene had retention times of 4.8, 5.3, and 26.0 min, respectively. The recovery of added retinal as its *O*-ethyloxime was 95% ± 3% (n = 4) under these conditions. All HPLC analyses were carried out using a Gilson HPLC automated system equipped with a Kratos model 783 variable-wavelength detector.

Mass Spectrum of the *O*-Ethyloxime Derivative of the Enzymatic Product and Crystallization of [¹⁴C]Retinal *O*-Ethyloxime Formed From [¹⁴C]β-Carotene on Incubation with BCC Enzyme. Twenty microcuries of [15,15′-^{14}C]β-carotene (specific activity, 21 μCi/μmol; 1 Ci = 37 GBq) was mixed with 1.5 μmol of unlabeled β-carotene (both from Hoffmann–La Roche) and chromatographed on a 1 × 30 cm column of 10% (vol/wt) water-deactivated neutral alumina (30 g; Brockman grade I; Sigma). The fraction that was eluted with 1% acetone in petroleum ether was collected and showed the characteristic absorption spectrum of β-carotene. The recovery of β-carotene based on radioactivity and extinction coefficient amounted to 70% of the starting material. The purified β-carotene was used in an experiment identical to the standard assay described above except that 100 nmol of [15,15′-^{14}C]β-carotene (specific activity, 17,600 dpm/nmol) was added as the substrate. At the end of 60 min of incubation, 10 such assay mixtures were combined, treated with *O*-ethylhydroxylamine, and extracted for total lipids as described above. A 50-μl sample of the lipid extract (2.5 ml) was subjected to HPLC as described above coupled to Flo-One β-radioactive flow detector (model IC; Radiomatic Instruments and Chemical, Tampa, FL). The rest of the extract was subjected to thin-layer chromatography on silica gel G (250 μm thick) using 1.5% (vol/vol) acetone in isooctane as the developing solvent. The derivative of the product, retinal *O*-ethyloxime (R_f, 0.43), was completely separated from the substrate, β-carotene (R_f, 0.83). The product band was scraped off the thin-layer plate and extracted thoroughly with acetone. One-tenth of the total amount of this extract was evaporated under a gentle stream of nitrogen, redissolved in 10 μl of ethanol, and analyzed for its mass spectrum in a LKB model 9000 mass spectrometer. The conditions were 70 eV (1 eV = 1.602 × 10^{-19} J) by direct insertion probe; source temperature, 270°C; ionization current, 20 μA.

The other nine-tenths of the extract was combined with 87 mg of authentic nonradioactive retinal *O*-ethyloxime in 2 ml of ethanol and allowed to crystallize at −80°C. The orange-yellow needle crystals were redissolved in ethanol and recrystallized from ethanol two more times. The ^{14}C specific activity (dpm/mg) of the crystalline retinal *O*-ethyloxime was determined at each crystallization step.

Enzymatic Reduction and Esterification of Retinal Formed from β-Carotene Reduction. The lipid extract of the standard incubation mixture of β-carotene with the BCC enzyme of the rabbit intestine was incubated for 1 hr at 37°C with 250 mM $NADH_2$, 1 mM GSH, and 1 mg of horse liver alcohol dehydrogenase (Sigma) in 2 ml (final volume) of 0.1 M potassium phosphate buffer (pH 6.0). The reaction was stopped by addition of 2 ml of ethanol followed by extraction with light petroleum. The lipid extract was tested for its absorption spectrum and for its HPLC profile.

Esterification. The enzymatically formed retinol fraction from the above experiment was evaporated under nitrogen, redissolved in 0.05 ml of ethanol, and incubated with 0.14 mM palmitic acid bound to 0.14 mM fatty acid-free bovine serum albumin, 0.14 mM coenzyme A, and 10 mg of the rat pancreatic acetone powder (source of esterase) in 0.6 ml (final volume) of potassium phosphate buffer (pH 7.3). The reaction was stopped by addition of 1.6 ml of ethanol. After addition of 1 ml of water, the reaction mixture was thoroughly extracted twice with 10-ml portions of light petroleum. After a 5-min centrifugation at 600 × g the light petroleum extracts were combined, evaporated under nitrogen, and finally redissolved in 0.5 ml of methylene chloride. An aliquot of this fraction was subjected to HPLC on an ODS column (4.6 mm × 10 cm, 3-μm particle size) using a solvent system of 100% methanol containing 0.5% (wt/vol) ammonium acetate. The initial flow rate of the solvent, 1 ml/min for the first 3 min, was increased linearly to 2 ml/min over the next minute and thereafter kept constant at 2 ml/min for the subsequent 10 min. Under these conditions authentic retinol and retinyl palmitate completely separated from each other, exhibiting sharp peaks with retention times of 1.97 min and 10.01 min, respectively.

RESULTS AND DISCUSSION

In spite of the fact that a number of workers (8–20) have demonstrated the enzymatic conversion of β-carotene to retinal by a cytosolic enzyme preparation from intestine and liver of different species, this has been recently questioned by Hansen and Maret (21). It is therefore important to unequivocally prove that β-carotene is cleaved by an enzyme from

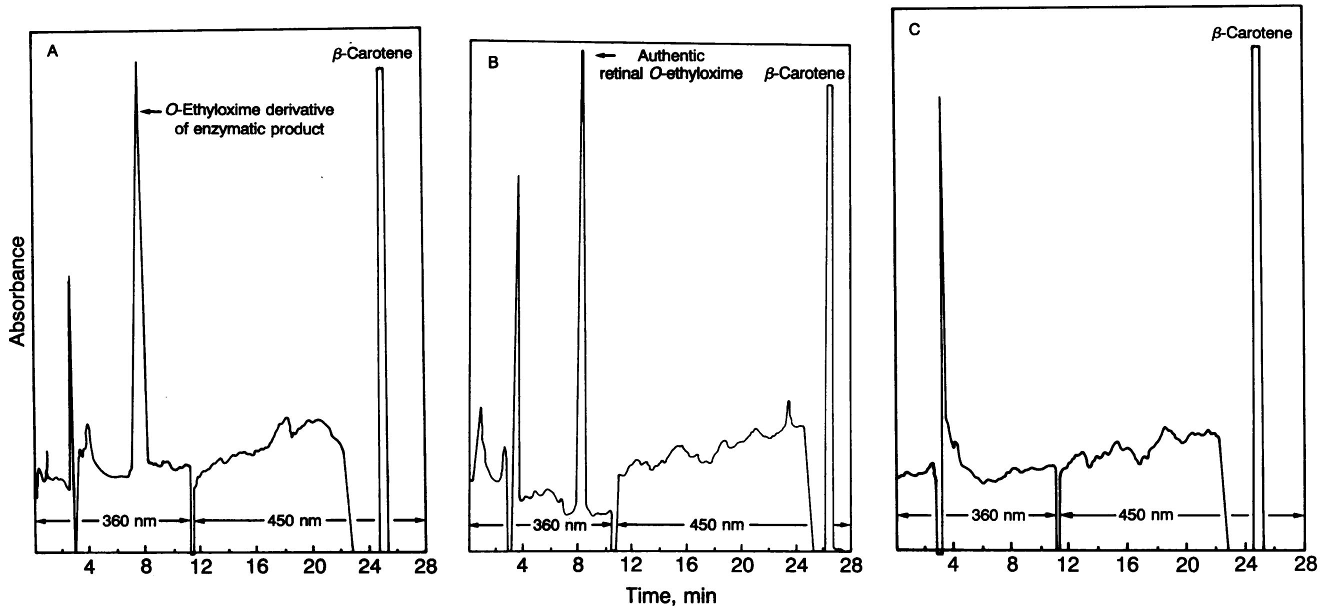

FIG. 2. HPLC profiles of the lipid extracts from the standard assay mixtures from rabbit BCC enzyme (*A*), authentic retinal *O*-ethyloxime (*B*), and boiled rabbit BCC enzyme (*C*).

the intestinal mucosa and that the product of this reaction is, in fact, retinal. It has been previously observed (18) that there may be accompanying activities in the cytosol fraction of the intestine that could catalyze the further metabolism of retinal to retinol or retinoic acid. Furthermore, the crude cytosol fraction from the rat intestine, kidney, testes, and liver has also been shown to convert β-carotene to retinoic acid (24). In view of these observations, we decided to fractionate the cytosol fraction of the rabbit intestinal mucosa as described in *Experimental*. The BCC activity was localized in the 45–60% acetone pellet fraction of the intitial 0–60% ammonium sulfate pellet fraction of the cytosol. More importantly, this fraction was devoid of either retinal reductase or retinal oxidase activity (data not shown). Since our aim was to test whether or not retinal was the product of BCC, we used the 45–60% acetone pellet fraction of the intestinal mucosa throughout this study.

As shown in Fig. 2, the HPLC analysis of the lipid extract from the incubation of β-carotene with the rabbit intestinal enzyme preparation (Fig. 2*A*) showed a peak with a retention time of 7.99 min corresponding to authentic retinal *O*-ethyloxime with a retention time of 8.1 min (Fig. 2*B*). In contrast, as shown in Fig. 2*C*, the extract from the boiled enzyme incubation mixture failed to show any HPLC peaks corresponding to retinal *O*-ethyloxime, retinal, or retinol. The absorption spectrum in ethanol of the *O*-ethyloxime derivative of the enzymatic product corresponded very well with that of authentic retinal *O*-ethyloxime with an absorption maximum at 363 nm. The enzyme preparation from rat intestinal mucosa also showed similar results. Furthermore, the mass spectrum of the *O*-ethyloxime derivative of the enzymatic product was identical to that of authentic retinal *O*-ethyloxime (m/z 327: 45%, $M^{+\cdot}$ and m/z 282: 100%, M − ethoxy). These results conclusively prove the identity of the enzymatic product as retinal.

It is significant, however, to point out that the HPLC profiles of the lipid extracts from the enzyme and the boiled enzyme incubation mixtures (Fig. 2 *A* and *C*) showed small peaks with retention times of 18–21 min. Authentic β-apo-10′-carotenal *O*-ethyloxime had a retention time of 17.7 min under these conditions. Thus, the nonenzymatic formation of minute quantities of β-apocarotenals from β-carotene may take place as reported by Hansen and Maret (21). Whether or not other enzymes capable of converting β-carotene to β-apocarotenals exist in the intestinal mucosa or other tissues remains to be clarified.

Table 1. Cocrystallization of enzymatically formed [^{14}C]retinal as its *O*-ethyloxime with authentic nonradioactive retinal *O*-ethyloxime

Stage of crystallization	Amount of the oxime, mg	Total radioactivity, dpm	Specific radioactivity, dpm/mg
Before	87	117,000	1345
First	75	102,000	1360
Second	46	61,040	1327
Third	43	57,600	1340

The enzymatic product formed from [^{14}C]β-carotene was converted into its *O*-ethyloxime derivative, mixed with 87 mg of authentic nonradioactive retinal *O*-ethyloxime in 2 ml of ethanol, and allowed to crystallize at −80°C. The crystals were redissolved in the indicated volumes of ethanol and the crystallization process was repeated through the third crystallization. Based on the total radioactivity and the amount of retinal *O*-ethyloxime (assuming an $\varepsilon^{1\%}_{1cm}$ value of 1800 at 363 nm in ethanol) recovered at each stage of crystallization, the specific radioactivity (dpm/mg) at each crystallization step was calculated.

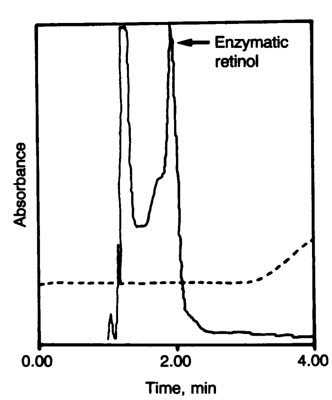

FIG. 3. HPLC profile of the reduction product of horse liver alcohol dehydrogenase on the enzymatic product of the rabbit BCC enzyme. Detection wavelength, 325 nm.

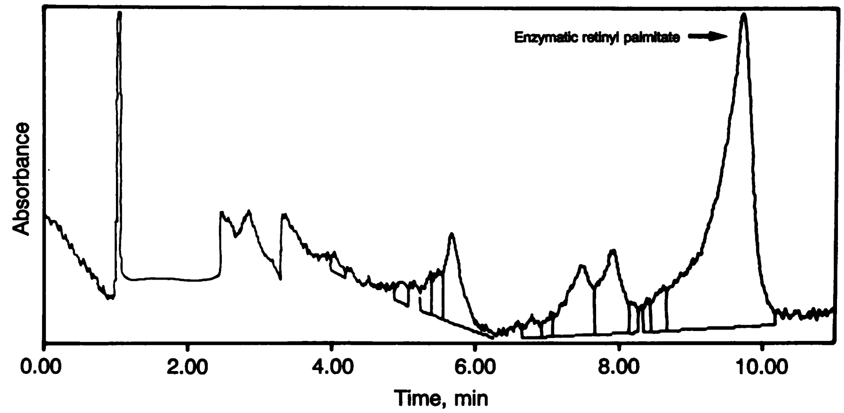

FIG. 4. HPLC profiles of the esterification product of rat pancreatic esterase on the enzymatically formed retinol. Detection wavelength, 325 nm.

The activity of the enzyme in standard (100 nmol of β-carotene per 2 ml) and large-scale (1000 nmol of β-carotene per 20 ml) incubations was calculated based on the HPLC measurement of retinal *O*-ethyloxime formed. The BCC enzyme activity of the rabbit intestinal mucosa was calculated to be on the average 1.0 nmol of retinal formed per mg of protein per hour. The BCC activities of the enzyme from rat (11), hog (14), and rabbit (16) reported earlier were 0.57, 0.8 and 0.72 nmol of retinal per mg per hr, respectively, and, thus, are reasonably comparable to the value in the present study.

^{14}C tracer analysis of the eluant fractions from the HPLC of an aliquot of the extract from the incubation of labeled β-carotene showed a sharp peak with a retention time of 8.0 min, corresponding to retinal *O*-ethyloxime. The radioactivity recovered under this peak was found to be 7.4% of the total β-carotene radioactivity injected. This amounted to an enzyme activity of 1.04 nmol of retinal per mg per hr, a value close to the value based on HPLC peak area of the oxime.

Table 1 summarizes the persistence of radioactivity in crystalline retinal *O*-ethyloxime formed after the lipid extract from the incubation of [15,15′-^{14}C]β-carotene with the partially purified (45–60% acetone pellet fraction) BCC enzyme fraction from rabbit intestine. Nearly 87% of the original radioactivity in the final ethanol solution containing the concentrate of retinal *O*-ethyloxime was recovered in its crystalline form with a concomitant recovery of the product (first crystallization). It is clear that even after three crystallizations the specific activity of the isolated retinal *O*-ethyloxime remained constant (1343 dpm/mg). This is further evidence for the identity of the enzymatic product to be retinal.

The enzymatic product had an absorption spectrum in light petroleum similar to that of authentic retinal with an absorption maximum at 370 nm. Furthermore, it gave rise to retinol with an absorption spectrum in light petroleum similar to that of authentic retinol with its absorption maximum at 326 nm when incubated with $NADH_2$ and horse liver alcohol dehydrogenase. This enzymatically formed retinol, on HPLC, had a retention time of 1.96 min (Fig. 3), identical to that of authentic retinol. Its identity was further confirmed by its ready conversion to retinyl palmitate on incubation with palmitic acid, coenzyme A, and rat pancreatic esterase as evidenced by the appearance of a HPLC peak with a retention time of 10.0 min (Fig. 4) corresponding to authentic retinyl palmitate.

In view of the above evidence, it is unequivocally proven that retinal is, indeed, the enzymatic product of BCC by the intestinal mucosal enzyme, and therefore, the original findings of Goodman and Huang (8) and Olson and Hayaishi (9) are confirmed. The inability of Hansen and Maret (21) to demonstrate this important metabolic conversion *in vitro* is puzzling. Some of the possible causes may be as follows. (*i*) The isolated enzyme might have been inactive for any number of reasons. For example, BCC enzyme is a sulfhydryl enzyme, and these workers presumably did not use any -SH protecting agents, such as reduced GSH or dithiothreitol, in all of their enzyme isolation steps. (*ii*) The recovery of the product, retinal, might have been poor; these authors failed to report the recovery of added retinal in their system. (*iii*) Although it is not absolutely essential, it is important to fractionate the BCC enzyme activity from other interfering activities, such as the retinal reductase or the oxidase, to characterize the product and estimate its true activity. It is possible that these interfering activities far exceeded the BCC activity in the previous studies (21, 24), presumably because of very low BCC enzyme activity to start with due to reasons listed above. Thus, the present study *in vitro* fully confirms the *in vivo* observations (1–7) that β-carotene is the precursor of vitamin A in biological systems and that retinal is the true product of BCC even in *in vitro* systems. Once the mechanism of this cleavage reaction is fully understood, some of the provocative criticisms against such a reaction raised by Hansen and Maret (21) based on theoretical considerations can be addressed.

The authors are grateful to Drs. Henry M. Fales and William E. Comstock, Division of Chemistry, National Institutes of Health, for the mass spectrometric analyses and identification of retinal *O*-ethyloxime in this study. We also acknowledge Dr. P. R. Sundaresan, Division of Nutrition, Food and Drug Administration, Washington, DC, for his expert advice in HPLC analysis and for critically reading this manuscript, and we gratefully acknowledge Dr. H. S. Bhagawan, Hoffmann-LaRoche, for the generous gift of [^{14}C]β-carotene. This work was supported by a grant from the National Cancer Institute (CA 39999).

1. Goodman, D. S., Blomstrand, R., Werner, B., Huang, H. S. & Shiratori, T. (1966) *J. Clin. Invest.* **45**, 1615–1619.
2. Thompson, S. Y., Braude, R., Coates, M. E., Cowie, A. T., Ganguly, J. & Kon, S. K. (1950) *Br. J. Nutr.* **4**, 398–402.
3. Wagner, H., Wyler, F., Rindi, G. & Bernhard, K. (1960) *Helv. Physiol. Pharmacol. Acta* **18**, 438–443.
4. Huang, H. S. & Goodman, D. S. (1965) *J. Biol. Chem.* **240**, 2839–2842.
5. Olson, J. A. (1961) *J. Biol. Chem.* **236**, 349–352.
6. Olson, J. A. (1964) *J. Lipid Res.* **5**, 402–407.
7. Zachman, R. D. & Olson, J. A. (1963) *J. Biol. Chem.* **238**, 541–546.
8. Goodman, D. S. & Huang, H. S. (1965) *Science* **149**, 879–880.
9. Olson, J. A. & Hayaishi, O. (1965) *Proc. Natl. Acad. Sci. USA* **54**, 1364–1369.
10. Goodman, D. S., Huang, H. S. & Shiratori, T. (1966) *J. Biol. Chem.* **242**, 1929–1932.
11. Goodman, D. S., Huang, H. S., Kanai, M. & Shiratori, T. (1967) *J. Biol. Chem.* **242**, 3543–3554.

12. Goodman, D. S. (1969) *Am. J. Clin. Nutr.* **22,** 963–965.
13. Lakshman, M. R., Pope, J. L. & Olson, J. A. (1968) *Biochem. Biophys. Res. Commun.* **33,** 347–352.
14. Fidge, N. H., Smith, F. R. & Goodman, D. S. (1969) *Biochem. J.* **114,** 689–694.
15. Goodman, D. S. & Olson, J. A. (1969) *Methods Enzymol.* **15,** 462–475.
16. Lakshman, M. R., Chansang, H. & Olson, J. A. (1972) *J. Lipid Res.* **13,** 477–482.
17. Gawienowski, A. M., Stacewicz, M. & Longley, R. (1974) *J. Lipid Res.* **15,** 375–378.
18. Singh, H. & Cama, H. R. (1974) *Biochim. Biophys. Acta* **370,** 49–61.
19. Sklan, D. (1983) *Int. J. Nutr. Res.* **53,** 23–26.
20. Sklan, D. & Havely, O (1984) *Br. J. Nutr.* **52,** 107–114.
21. Hansen, S. & Maret, W. (1988) *Biochemistry* **27,** 200–206.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
23. Van Kuijk, F. J. G. M., Handelman, G. J. & Dratz, E. A. (1985) *J. Chromatogr.* **348,** 241–251.
24. Napoli, J. L. & Race, K. R. (1988) *J. Biol. Chem.* **263,** 17372–17377.