Characterization of β-Apo-13-carotenone and β-Apo-14′-carotenal as Enzymatic Products of the Excentric Cleavage of β-Carotene[†,‡]

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ABSTRACT: Two new products from the incubation of β-carotene with intestinal mucosa homogenates of human, monkey, ferret, and rat were isolated using high-performance liquid chromatography (HPLC). Identification by comparing retention times in HPLC, by monitoring ultraviolet/visible spectra, by reduction to corresponding alcohol, by oxime formation, and by mass spectrometry demonstrated that they are β-apo-13-carotenone and β-apo-14′-carotenal. These compounds were not found in incubations done without intestinal homogenates or with disulfiram as an inhibitor. Under standard incubation conditions, these products increased linearly for 60 min and up to a protein concentration of 1.5 mg/mL and increased along with increasing concentrations of β-carotene. Therefore, they are enzymatic cleavage products from β-carotene. The formation of the β-apo-13-carotenone and β-apo-14′-carotenal provides direct evidence for an enzymatic excentric cleavage mechanism.

The hypothesis that β-carotene might serve as a dietary anticarcinogen, first proposed by Peto et al. (Peto et al., 1981), and subsequently supported by a variety of epidemiological (Ziegler, 1991) and biological studies (Krinsky, 1991), has stimulated renewed interest in β-carotene metabolism, with respect to both its mechanism and regulation (Ganguly, 1989; Olson, 1965). Two pathways have been proposed for the conversion of β-carotene to vitamin A in mammals. The central cleavage pathway (Olson, 1965; Goodman & Huang, 1965) postulated the formation of 2 molecules of retinal from a single β-carotene molecule by cleavage at the 15,15′ double bond, whereas the excentric pathway postulated a single molecule of retinal formed by a stepwise oxidation beginning at any of the other double bonds of the polyene chain (Glover, 1960; Ganguly & Sastry, 1985). We have shown in a previous paper the in vitro enzymatic conversion of β-carotene into retinoic acid, retinal, and β-apo-12′-, 10′-, and 8′-carotenals by mammalian tissues (Wang et al., 1991), which supported an excentric cleavage mechanism of β-carotene metabolism. In addition, we reported the appearance of unidentified enzymatic metabolites which were formed in significant amounts during the incubation of mammalian tissues with β-carotene. In the present paper we have identified these enzymatic metabolites of β-carotene as β-apo-13-carotenone and β-apo-14′-carotenal that would arise from the enzymatic excentric cleavage of β-carotene.

Experimental Procedures

Chemical Products. All chemicals and reagents were of analytical or ultrapure grade. All organic solvents were of HPLC grade (from J. T. Baker Chemical Co., Philipsburg, NJ) and were routinely filtered through a 0.45-μm filter before use. β-Carotene (type IV), *all-trans*-retinal, PIPES, β-NAD,[1] DTT, and propylene glycol were obtained from Sigma (St. Louis, MO). Standards of β-apo-8′-, 10′-, and 12′-carotenal were gifts from Hoffmann-LaRoche (Nutley, NJ), as was the methyl β-apo-14′-carotenoate. *O*-Ethylhydroxylamine was from Fluka (Ronkonkoma, NY).

Preparation and Incubation of Tissues. The preparation and incubation procedures for tissues from rats, ferrets, monkeys, and humans were described in a previous paper (Wang et al., 1991). In order to collect a sufficient amount of the incubation products for mass spectrometry analysis and for the derivatization experiments, the incubation assay for rat intestinal mucosa homogenate was extended to a scale of 100 times greater than those of the standard incubation (vide supra).

HPLC Procedures. The products from incubations were analyzed using reverse-phase HPLC. The incubated mixtures were extracted without saponification using $CHCl_3/CH_3OH$ (2:1 v/v). Retinyl acetate and γ-carotene were added as internal standards during extraction but were not used in the large-scale incubations. After centrifugation, the chloroform layer was removed and evaporated to dryness under nitrogen at 40 °C and the residue was redissolved in 100 μL of ethanol. A-50 μL aliquot of the final extract was injected onto the HPLC. The HPLC system consisted of two Waters 510 pumps (Waters, Milford, MA), an ISS-100 autosampler, a Pecosphere-3 C18 0.46 × 8.3 cm cartridge column (Perkin-Elmer, Inc., Norwalk, CT), and a Waters 860–Digital 380 data station. The HPLC mobile phase was $CH_3CN/THF/H_2O$ (solvent A, 50:20:30 v/v/v, or solvent B, 50:44:6 v/v/v, with 1% ammonium acetate in H_2O). The gradient procedure at a flow rate of 1 mL/min was as follows: 100% solvent A is used for 7 min followed by a 6-min linear gradient to 100% solvent B, a 13-min hold at 100% solvent B, then a 2-min gradient back to 100% solvent A. The Waters 490E multiwavelength spectrophotometer detector was set at 340 nm for retinoids and at 450 nm for carotenoids. β-Apo-13-carotenone and β-apo-14′-carotenal were quantitated by determining peak

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[1] Abbreviations: DTT, dithiothreitol; β-NAD, β-nicotinamide adenine dinucleotide; HPLC, high-performance liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid.

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areas calibrated against known amounts of standards. β-Apo-13-carotenone was prepared by radical-initiated cleavage of β-carotene (Handelman et al., 1991), and β-apo-14′-carotenal was prepared by reduction of methyl β-apo-14′-carotenoate with lithium aluminum hydride and oxidation of β-apo-14′-carotenol with manganese dioxide (Das & Becker, 1978; Bergen et al., 1988). The extinction coefficients (ε) used were 25 490 for β-apo-13-carotenone at 340 nm and 51 590 for β-apo-14′-carotenal at 399 nm (Das & Becker, 1978). A Hewlett-Packard 1090 diode array liquid chromatograph (Hewlett-Packard, Avondale, PA) was used to record the absorption spectra for these compounds.

Chemical Identification. Reductions were carried out by evaporating the appropriate HPLC fraction under N_2 and redissolving the residue in 150 μL of methanol. A few crystals of KBH_4 and 20 μL of H_2O were added, and the mixture was incubated in the dark at room temperature for 2 h (Krinsky & Goldsmith, 1960). After the addition of 150 μL of H_2O, the mixture was extracted three times with 600 μL of hexane, and the combined extract was evaporated under N_2, the residue was redissolved in 80 μL of methanol, and a 25-μL aliquot was injected onto the HPLC column.

The *O*-ethyl oxime derivatives were made as described earlier (Handelman et al., 1988). The desired fraction from the HPLC eluate was collected and evaporated to dryness under N_2. The residue was redissolved in 200 μL of methanol, and 10 μL of 0.1 M *O*-ethylhydroxylamine (in 0.1 M PIPES, adjusted to pH 4.7) was added. Following incubation at ambient temperature for 4–23 h, the incubation mixture was diluted with an equal volume of water and the oximes were extracted with hexane and were subjected to HPLC analysis, using a diode array detector to record their absorption spectra.

Mass Spectrometry. Fraction containing the peaks to be analyzed by GC–MS were purified immediately before analysis through a new Nova-C18 4-μm column (Waters, Milford, MA) using 10–20% H_2O in methanol as the eluting solvent.

For GC–MS analysis, the purified compounds were dissolved in methanol and a 1-μL aliquot was injected onto a Varian 3400 gas chromatograph installed with a 30 m (length) × 0.32 mm (i.d.) 0.25-mm film thickness DB-1 capillary column. On-column injection was used with the injector temperature set at 250 °C. The initial column temperature was 100 °C. The temperature program was 10 deg/min until 290 °C was reached. The carrier gas was helium with a flow rate set at 1.0 mL/min. The mass spectrometer used was a Finnigan MAT 8230 with source temperature at 250 °C and electron ionization at 70 eV (Ouyang et al., 1980).

Chemical ionization was carried out to confirm the existence of the molecular ion. Isobutane was used as reagent gas and source pressure was about 0.7 torr.

Statistical Analysis. Results were expressed as mean ± SEM, and the significances of differences were calculated by Student's *t* test.

Results

HPLC Profiles for in Vitro Metabolites of β-Carotene. The incubation of β-carotene with rat intestinal mucosa homogenate produced peaks 1, 2, and 3 when monitored at 340 nm (Figure 1A) and peaks 4–8 when monitored at 450 nm (Figure 1B). Peaks 1, 3, 5, 6, and 7 had retention times of 3.8, 9.8, 14.0, 14.4, and 15.2 min, respectively, and matched the retention times of authentic standards of retinoic acid, retinal, and β-apo-12′-, 10′-, and 8′-carotenal, respectively. The formation of these products has been discussed in our previous paper (Wang et al., 1991). Peak 8 has a retention time at 17.2 min and absorption maxima at 425, 450, and 476 nm and was identical to an authentic standard of β-carotene 5,6-epoxide (Handelman et al., 1991). Two major unidentified peaks were identified as peak 2 eluting at 6.7 min (Figure 1A) and peak 4 eluting at 12.0 min (Figure 1B). These two unknowns had maximal absorption at 349 and 405 nm, respectively, when the spectra were recorded during chromatography using a diode array detector (Figure 2). Addition of antioxidants such as butylated hydroxytoluene (0.1%) or α-tocopherol (1–10 μM) had no effect on the formation of the above metabolites (data not shown). When β-carotene was incubated in the absence of homogenate, extremely small amounts of peak 8 could be detected. Incubation of β-carotene in the absence of the homogenate did not yield any of the peaks described above (data not shown).

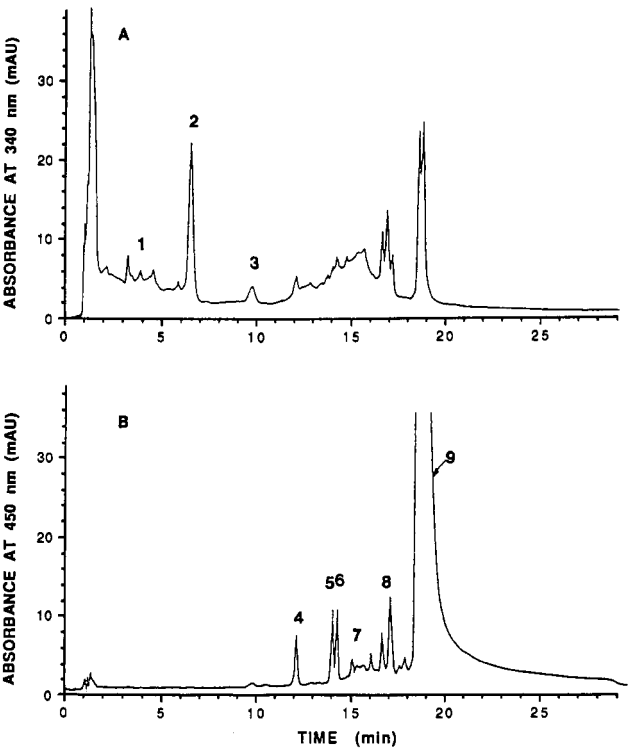

FIGURE 1: HPLC chromatography from the incubation of β-carotene with rat intestinal mucosa homogenate. The detector was set at 340 nm (chromatograph A) and 450 nm (chromatograph B) to monitor cleavage products. Peaks are as follows: (1) retinoic acid, (2) unknown compound, (3) retinal, (4) unknown compound, (5) β-apo-12′-carotenal, (6) β-apo-10′-carotenal, (7) β-apo-8′-carotenal, (8) β-carotene 5,6-epoxide, and (9) β-carotene. Column: Pecosphere-3 C18 0.46 × 8.3 cm cartridge column. Solvents: $CH_3CN/THF/H_2O$ (50:20:30 v/v/v, 1% ammonium acetate in H_2O, solvent A) and $CH_3CN/THF/H_2O$ (50:44:6 v/v/v, solvent B). Flow rate: 1 mL/min.

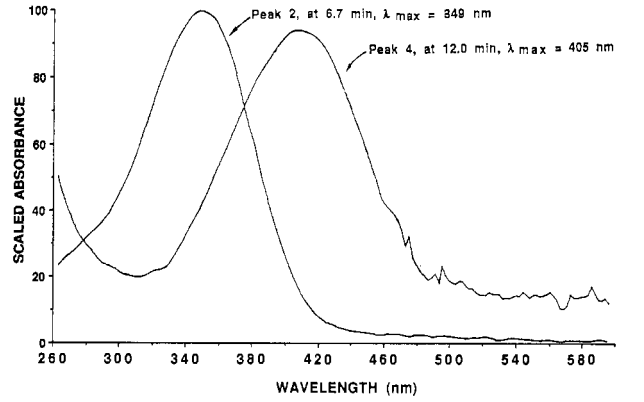

FIGURE 2: The spectra of unknown compounds, peak 2 and peak 4 (from Figure 1) recorded by Hewlett-Packard 1090.

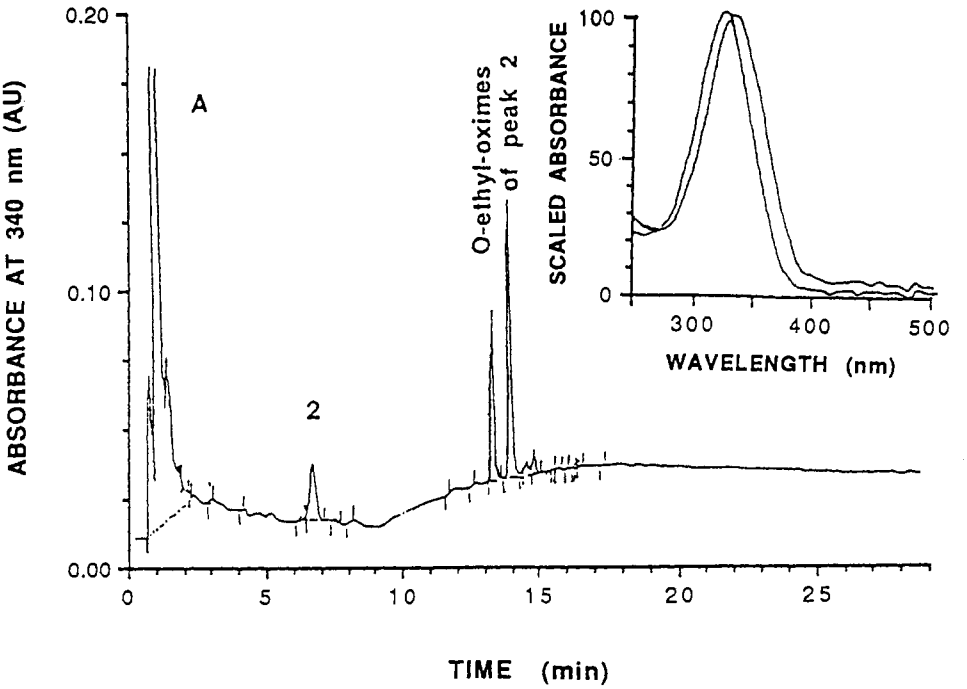

FIGURE 3: HPLC chromatography for *O*-ethyl oximes of peak 2 (from Figure 1). The inset is the absorbance spectra for the *syn*- and *anti*-*O*-ethyl oximes of peak 2.

Identification of Compounds Eluted as Peak 2 and Peak 4. There was no change in the hexane extractability of peak 2 even after it was neutralized with dilute alkali, thus, indicating that it was not an acid. However, peak 2 was readily reduced with KBH_4, as evidenced by a 57-nm blue shift in the spectrum to 292 nm and a 0.5-min decrease in the retention time (data not shown). Furthermore, treatment of peak 2 with *O*-ethylhydroxylamine resulted in the formation of products which separated as two peaks on HPLC (Figure 3), with absorption maxima at 326 and 336 nm (23- and 33-nm blue shift, see insert in Figure 3). The retention times of these two products were 6.7 and 7.2 min longer than the original peak 2 (Figure 1A). Under similar conditions, retinal was reduced to form retinol with a 53-nm blue shift in the absorption spectrum and a retention time that was 2 min faster than retinal. Treatment of retinal with *O*-ethylhydroxylamine yielded two isomers of retinal *O*-ethyl oxime with maximum absorption at 365 and 370 nm (13- and 8-nm blue shift, data not shown) and with retention times which were 5.2 and 5.4 min longer than retinal. These two peaks presumably represent the syn and anti isomers of retinal *O*-ethyl oxime, in analogy with the formation of these two isomers from retinal oxime (Groenendijk et al., 1979). Similarly, the *O*-ethyl oxime of peak 4 showed a retention time 2.1 min longer and a maximum absorption 13 nm shorter than those of peak 4 (data not shown). A similar *O*-ethyl oxime derivative of authentic β-apo-12′-carotenal resulted in an oxime peak with a retention time 2.0 min longer and with a maximum absorption at 426 nm (4-nm blue shift). These observations provided evidence that peaks 2 and 4 were conjugated carbonyl compounds.

Moreover, peak 2 had the retention time which was 3.2 min shorter than retinal and an absorption maximum 29 nm blue shifted from that of retinal. Peak 4 had a retention time which was 2.1 min longer than that of retinal and an absorption maximum 27 nm red shifted from that of retinal. Therefore, the length of the polyene chain of peak 2 was one double bond shorter than retinal and that of peak 4 was one double bond longer than retinal. Thus, β-carotene appeared to undergo excentric cleavage at the 13,14 double bond to produce β-apo-13-carotenone and β-apo-14′-carotenal (Figure 4).

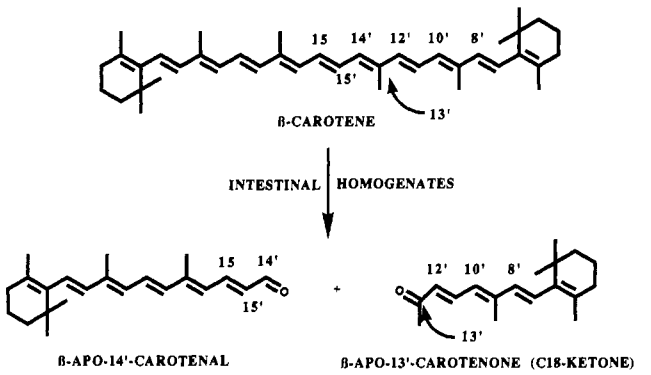

FIGURE 4: The excentric cleavage of β-carotene yielding β-apo-14′-carotenal and β-apo-13-carotenone.

This hypothesis was confirmed by GC–MS analysis. Peak 2 and peak 4 were isolated in large amounts by first collecting the appropriate fractions in HPLC eluate of large-scale incubations. The crude compounds were then redissolved in methanol and rechromatographed using 20% H_2O in methanol as the mobile phase for peak 2 and 10% H_2O in methanol as the mobile phase for peak 4. Purified fractions were passed through a previously unused Nova-C18 column immediately before GC–MS. As seen in Figure 5, the molecular ion of peak 2 had an *m/e* of 258. Principal fragments were at *m/e* 243 (M-CH_3), *m/e* 173, *m/e* 145, *m/e* 131, and *m/e* 91 (Figure 5A). The molecular ion for peak 4 was at an *m/e* of 310. The principal fragments were at *m/e* 281 (M-CHO), *m/e* 123, *m/e* 107, and *m/e* 55 (Figure 5B). These mass spectra were essentially identical to the mass spectra for β-apo-13-carotenone and β-apo-14′-carotenal, which have been reported by Ouyang et al. (1980) and for β-apo-13-carotenone reported by Handelman et al. (1991). In addition, the mass spectra obtained from chemical ionization of these two compounds showed an *m/e* of 259 (M + 1) for peak 2 and an *m/e* of 311 (M + 1) for peak 4 (data not shown), further confirming the structural assignment.

Effect of Incubation Time, Enzyme Concentration, and β-Carotene Concentration on the Formation of β-Apo-13-

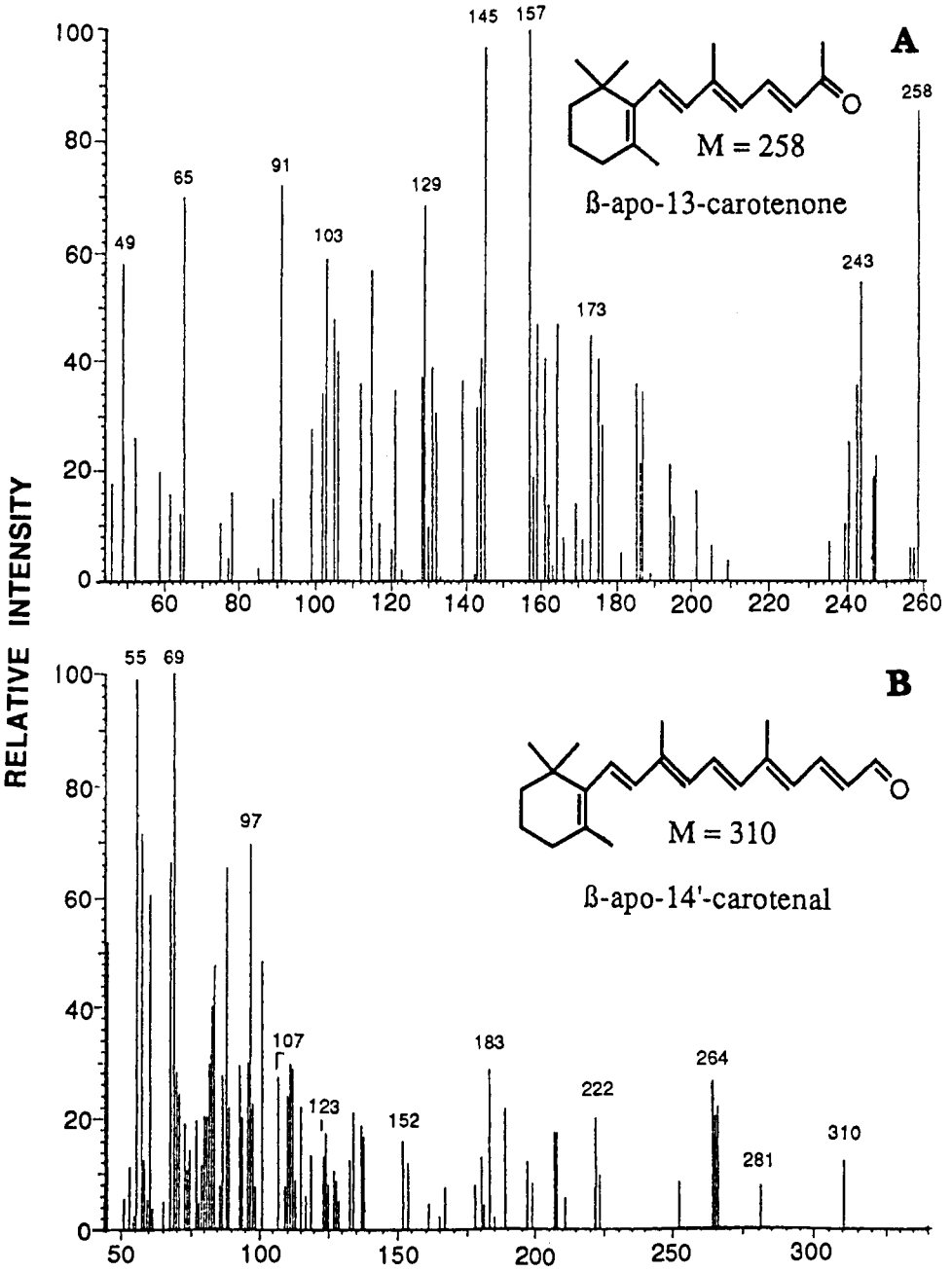

FIGURE 5: Mass spectra for peak 2 (from Figure 1A) and peak 4 (from Figure 1B): (A) the mass spectrum for peak 2 showing the existence of molecular ion of β-apo-13-carotenone (m/e = 258); (B) the mass spectrum for peak 4 showing the existence of molecular ion of β-apo-14′-carotenal (m/e = 310).

carotenone and β-Apo-14′-carotenal. The production of β-apo-13-carotenone and β-apo-14′-carotenal was inhibited completely when 2 mM disulfiram was added to the standard incubation mixture (Wang et al., 1991), which indicated the involvement of a sulfhydryl-containing enzyme(s) in the conversion of β-carotene to β-apo-13-carotenone and β-apo-14′-carotenal. To characterize this enzymatic process, incubations of rat intestinal homogenates and β-carotene was carried out at various incubation times. The time course for the formation of β-apo-13-carotenone and β-apo-14′-carotenal showed that the production of these two carbonyl products of β-carotene metabolism is essentially linear for 60 min.

Protein concentration of the rat intestinal homogenates was varied between 0 and 4 mg, and the production of both β-apo-13-carotenone and β-apo-14′-carotenal from β-carotene was linear up to 1.5 mg of protein/mL. However, the production of these two metabolites did not increase further when the protein concentration was increased to 4 mg/mL.

The effect of varying β-carotene concentration from 0.3 to 3 μM on the formation of β-apo-13-carotenone and β-apo-14′-carotenal is presented in Figure 6. The results indicate that the yield of β-apo-13-carotenone increased up to 2 μM β-carotene, while the yield of β-apo-14′-carotenal only increased moderately.

Differences between Four Species in the Production of β-Apo-13-carotenone and β-Apo-14′-carotenal from β-Carotene. The comparison of these metabolites from the incubation of 2 μM β-carotene with homogenates of intestinal mucosa of human, monkey, ferret, and rat is shown in Table I. The yields of β-apo-13-carotenone differed in each species investigated, with the monkey producing the largest amount. However, the yields of β-apo-14′-carotenal were similar in monkeys and humans and similar in rats and ferrets. The identification and quantitation of β-apo-14′-carotenal increased

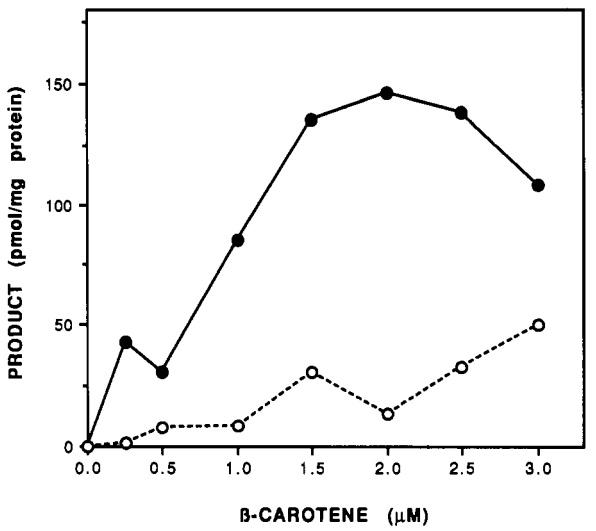

FIGURE 6: Effect of β-carotene concentration on the formation of β-apo-13-carotenone and β-apo-14′-carotenal after incubation of β-carotene with rat intestinal mucosa homogenates. Incubations were conducted for 60 min with 1 mg of protein at pH 7.35. Data are the means of duplicates. A filled circle represents β-apo-13-carotenone and an open circle represents β-apo-14′-carotenal.

Table I: Formation of β-Apo-13-carotenone and β-Apo-14′-carotenal after a 60-min Incubation of β-Carotene (2 μM) with Intestinal Homogenates of Four Species[a]

intestinal homogenate	β-apo-13-carotenone [pmol/(h·mg of protein)]	β-apo-14′-carotenal [pmol/(h·mg of protein)]
human (n = 5)	190.7 ± 10.4[b]	65.5 ± 8.7[f]
monkey (n = 3)	367.1 ± 26.1[c]	53.6 ± 5.7[f]
ferret (n = 3)	252.7 ± 10.3[d]	26.8 ± 4.0[g]
rat (n = 7)	123.3 ± 15.7[e]	18.4 ± 3.3[g]

[a] Data are represented as mean ● SEM. Data in a column not sharing a common superscript letter are significantly different at $p < 0.05$.

the total yield of long-chain β-apocarotenals from β-carotene to 22.3% for humans, 14.6% for monkeys, 4.3% for ferrets, and 4.2% for rats, from the results previously published (Wang et al., 1991). By adding the yield of retinoids (retinal and retinoic acid) from β-carotene, the percent conversion of β-carotene was 23.6% for humans, 16.5% for monkeys, and 4.7% for both ferrets and rats.

Discussion

As the major unidentified enzymatic oxidation products of β-carotene in our HPLC analysis, peak 2 and peak 4 have been separated (Figure 1), analyzed, and identified. Their maximum absorption (Figure 2) showed opposite (toward short or long wavelengths) but equal (27–29 nm) shifts from retinal, a polyene with six conjugated double bonds. These absorption differences indicate that peak 2 and peak 4 have chromophores of five double bonds and seven double bonds, respectively (Das & Becker, 1978). Their spectral properties and retention times are identical to authentic samples of β-apo-13-carotenone and β-apo-14′-carotenal. Reduction by KBH_4 and derivatization with *O*-ethylhydroxylamine showed the existence of conjugated carbonyl groups, on the basis of the spectral changes accompanying derivatization (Figure 3). Moreover, the mass spectrum from GC–MS (Figure 5) of putative β-apo-13-carotenone provided evidence of the existence of molecular ion at m/e 258, as well as the fragments corresponding to a loss of a methyl group at m/e 243 and groups of fragments at m/e 173, m/e 145, m/e 131, and m/e 91 resulting from the C-10 polyene transient ion and the cyclohexenyl ring. The mass spectrum from GC–MS of putative β-apo-14′-carotenal yielded the molecular ion m/e 310, as well as the fragments corresponding to a loss of a terminal CHO at m/e 281 and groups of fragments m/e 123, m/e 107, and m/e 55 from the cyclohexenyl polyene structure. Furthermore, mass spectra from chemical ionization demonstrated the M + 1 peak at m/e 259 for peak 2 and the M + 1 peak at m/e 310 for peak 4 (data not shown). These mass spectra results are in agreement with earlier analyses of these compounds (Ouyang et al., 1980; Handelman et al., 1991). Even though the mass spectra included some background fragments, presumably due to coelution of material from the extraction of biological material, the mass spectra of two unknowns provided direct structural information for confirming the existence of β-apo-13-carotenone and β-apo-14′-carotenal. The identification of these two products demonstrates that β-apo-13-carotenone and β-apo-14′-carotenal (Figure 4) are in vitro metabolites of β-carotene and indicates that the β-carotene molecule can be enzymatically cleaved at the 13,14 or 13′,14′ double bond.

The production of these two compounds was essentially linear for 60 min except that the production of β-apo-13-carotenone at 45 min was lower than that at 30 min. The reason for this is unclear. Increasing the protein concentration to 1.5 mg/mL resulted in a linear increase in the production of β-apo-13-carotenone and β-apo-14′-carotenal. The formation of these two compounds was also dependent on the concentration of β-carotene, although the effect was more pronounced on the production of β-apo-13-carotenone than on that of β-apo-14′-carotenal (Figure 6). Moreover, in all incubations, the yields of β-apo-13-carotenone were considerably greater than that of β-apo-14′-carotenal. Also, in the four species we investigated, the intestinal formation of β-apo-13-carotenone from β-carotene was 3.0–9.4 times greater than that of β-apo-14′-carotenal (Table I). One possibility is that these two compounds are not formed exclusively from a single molecule of β-carotene but could be produced from the metabolism of β-apo-12′-, 10′-, and 8′-carotenals which are also metabolites of β-carotene in this system (unpublished experiments). Another possible explanation is that the further metabolism of β-apo-13-carotenone and β-apo-14′-carotenal which may be formed from one molecule of β-carotene are different. This will be addressed by using β-apocarotenals as substrates in in vitro incubations in future experiments.

The quantitation of these two newly identified metabolites increased the total yield of cleavage products (retinoids and carotenoids) to 665.0 pmol/(h·mg of protein) in human intestinal homogenates and to 216.4 pmol/(h·mg of protein) in rat intestinal homogenates. In our previous paper, β-apo-14′-carotenal had not been identified and was not included in the total production of β-carotenals (Wang et al., 1991). By including the formation of β-apo-14′-carotenal, the production rate of total β-apocarotenals (8′, 10′, 12′, and 14′) formed from β-carotene by intestinal homogenates is 446.0 pmol/(h·mg of protein) for humans, 292.7 pmol/(h·mg of protein) for monkeys, 85.8 pmol/(h·mg of protein) for ferrets, and 84.0 pmol/(h·mg of protein) for rats. There are statistically significant differences between the primate species, human and monkey, and the carnivores and rodents, ferret and rat (Table I). However, there is no correlation between species in the production of β-apo-13-carotenone. This may imply that the ketonic carbonyl in β-apo-13-carotenone is less readily metabolized, in contrast to the aldehydic carbonyls in β-apo-14′-, 12′-, 10′-, 8′-carotenals.

The degradation of β-carotene in chemical and radical processes has been widely investigated [reviewed by Krinsky (1989)]. The reactivity of β-carotene with oxygen in the presence of a free radical initiator (Hunter & William, 1945;

Petracek & Zechmeister, 1956; Friend, 1958; El-Tinany & Chichester, 1970; Handelman et al., 1991) or under high-temperature treatment (Ouyang et al., 1980; Onyewu et al., 1986; Marty & Berset, 1988, 1990) has been demonstrated. In chemical processes, the oxidation of β-carotene resulted in the formation of 4-keto-β-carotene, β-carotene 5,6-epoxide, β-apocarotenals, β-apocarotenols, and β-apocarotenones. Many of these products can also be found in plants as a result of enzymatic processes (Klein et al., 1985). In mammalian tissue, retinal and β-apo-14′-, 12′-, 10′-, and 8′-carotenal are formed as in vitro enzymatic oxidation products of β-carotene and may serve as intermediates in the formation of retinoic acid (unpublished experiment). It is the polyene chain of β-carotene that appears to be most susceptible to attack by a variety of oxidizing agents and enzymes, forming either degradation products or biologically active compounds (with vitamin A activity).

Olson has stated (Olson, 1989) that the evidence for excentric cleavage "clearly requires unambiguous direct supporting evidence". With our identification of β-apo-13-carotenone and β-apo-14′-carotenal arising from β-carotene metabolism in mammalian tissues, we feel that this supporting evidence is now in hand.

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