CHARACTERIZATION OF METABOLITES OF ASTAXANTHIN IN PRIMARY CULTURES OF RAT HEPATOCYTES

ERICH WOLZ, HANS LIECHTI, BRIGITTE NOTTER, GOTTFRIED OESTERHELT, AND ANDREAS KISTLER

Vitamins and Fine Chemicals, Human Nutrition and Health (E.W., H.L., A.K.) and Pharma Research (B.N., G.O.), F. Hoffmann-La Roche Ltd., Basel, Switzerland

This paper is available online at http://www.dmd.org

ABSTRACT:

The metabolism of the nonprovitamin A carotenoid astaxanthin was investigated in primary cultures of rat hepatocytes. In a time course study based on HPLC and gas chromatography-mass spectrometry analyses, one main metabolite, (*rac*)-3-hydroxy-4-oxo-β-ionone, was found. This metabolite was conjugated mainly into glucuronides, as demonstrated by glusulase treatment of the conjugates under sulfatase-inhibiting conditions. Within 24 h more than 50% astaxanthin was metabolized and conjugated. Deconjugation of the polar conjugates with glusulase and analyses with HPLC and gas chromatography-mass spectrometry identified two metabolites, (*rac*)-3-hydroxy-4-oxo-β-ionone and its reduced form (*rac*)-3-hydroxy-4-oxo-7,8-dihydro-β-ionone, indicating that the former was reduced in the conjugated form. We confirmed that the ketocarotenoid astaxanthin induces xenobiotic-metabolizing enzymes in rat liver in vivo. However, there were no differences in the metabolism of astaxanthin in cultured hepatocytes from rats that were pretreated with astaxanthin and, thus, with induced cytochrome P-450 systems compared with control hepatocytes. Neither liver microsomes from astaxanthin-pretreated nor control rats metabolized astaxanthin. These results indicated that the cytochrome P-450 enzymes were not involved in the metabolism of astaxanthin in rat hepatocytes. We conclude that astaxanthin was metabolized in primary cultures of rat hepatocytes into (*rac*)-3-hydroxy-4-oxo-β-ionone and its reduced form (*rac*)-3-hydroxy-4-oxo-7,8-dihydro-β-ionone independent of the xenobiotic-metabolizing enzymes induced by astaxanthin.

Carotenoids are the most widespread group of naturally occurring pigments in nature. Astaxanthin is the major pigment in most aquatic animals. Although the conversions of β-carotene and other provitamin A carotenoids into retinal and retinoic acid are well documented in rat and humans (Goodman and Huang, 1965; Olson and Hayaishi, 1965; Goodman et al., 1966; Brubacher and Weiser, 1985; Ganguly and Sastry, 1985), little information is available on the metabolism of nonprovitamin A carotenoids like astaxanthin. Recently, it was reported that although the carotenoids astaxanthin, canthaxanthin, and β-apo-8′-carotenal induce xenobiotic-metabolizing enzymes in rat liver, other carotenoids like β-carotene, lycopene, and lutein do not (Astorg et al., 1994; Gradelet et al., 1996a,b).

The goal of the present study was to investigate metabolites of astaxanthin in primary cultures of rat hepatocytes obtained from animals pretreated with astaxanthin to induce xenobiotic-metabolizing enzymes.

Materials and Methods

Chemicals. Fetal calf serum, streptomycin/penicillin, insulin, dexamethasone, Hanks' balanced salt solution, and William's E medium without L-glutamine were obtained from Gibco BRL (Basel, Switzerland) and collagenase type 2 from Worthington Biochemical Corporation (Lakewood, NY.). [^{14}C]Astaxanthin (specific activity 4.8 MBq/mg = 132.5 μCi/mg) labeled at positions 6,7,6′, and 7′, reference substances used as HPLC and gas chroma-

tography-mass spectrometry (GC-MS)[1] standards and commercially available astaxanthin preparations (carophyll pink 8%) and placebo carophyll pink (containing the same ingredients as the carophyll pink 8%, but no astaxanthin) for administration to rats were obtained from F. Hoffmann-La Roche (Basel, Switzerland). BSA, ethoxyresorufin, glucose 6-phosphate, resorufin, NADP and β-NADPH were purchased from Sigma (Buchs, Switzerland), glucose 6-phosphate dehydrogenase (grade II) was obtained from Boehringer Mannheim (Mannheim, Germany), Folin-Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany), and glusulase (β-glucuronidase and sulfatase; helix pomatia juice) was purchased from IBF (Paris, France).

Animals and Induction of Xenobiotic-Metabolizing Enzymes in Rat Liver. Male Wistar rats (Han Brl:WIST, 3–5 weeks old), obtained from Biological Research Laboratories (Füllinsdorf, Switzerland) were fed commercial rodent diets (30–343-4, KLIBA, Kaiseraugst, Switzerland) containing 3.75 g carophyll pink 8%/kg diet to provide 300 mg astaxanthin/kg diet (corresponding to about 30 mg astaxanthin/kg body weight/day) for 4 or 5 days. Control animals received diet containing 3.75 g placebo carophyll pink. At the end of the treatment, rats were starved for 18 h before killing by decapitation. Livers were rapidly removed, weighed, and immediately frozen in liquid nitrogen and stored at −80°C. These livers were used for preparation of microsomes and determination of astaxanthin content.

Preparation of Rat Liver Microsomes. Rat livers were thawed and microsomal fractions were prepared as described (Lu et al., 1972).

Protein Determination and Enzyme Assay. Protein was measured by the Lowry assay (Lowry et al., 1951) using BSA as the standard. Ethoxyresorufin O-deethylase (EROD) activity was determined by the fluorometric method of Burke and Mayer (1974). Reactions were carried out in fluorometric cuvettes at 37°C using a Model SLM4048/4048S spectrofluorometer (Spectronic Instruments, Rochester, NY). The reaction mixture (final volume 2 ml), contain-

Send reprint requests to: Dr. Erich Wolz, Ph.D., Vitamins and Fine Chemicals, Bldg. 72/38, F. Hoffmann-La Roche Ltd., 4070 Basel, Switzerland. E-mail: erich.wolz@roche.com

[1] Abbreviations used are: GC-MS, gas chromatography-mass spectrometry; EROD, ethoxyresorufin O-deethylase.
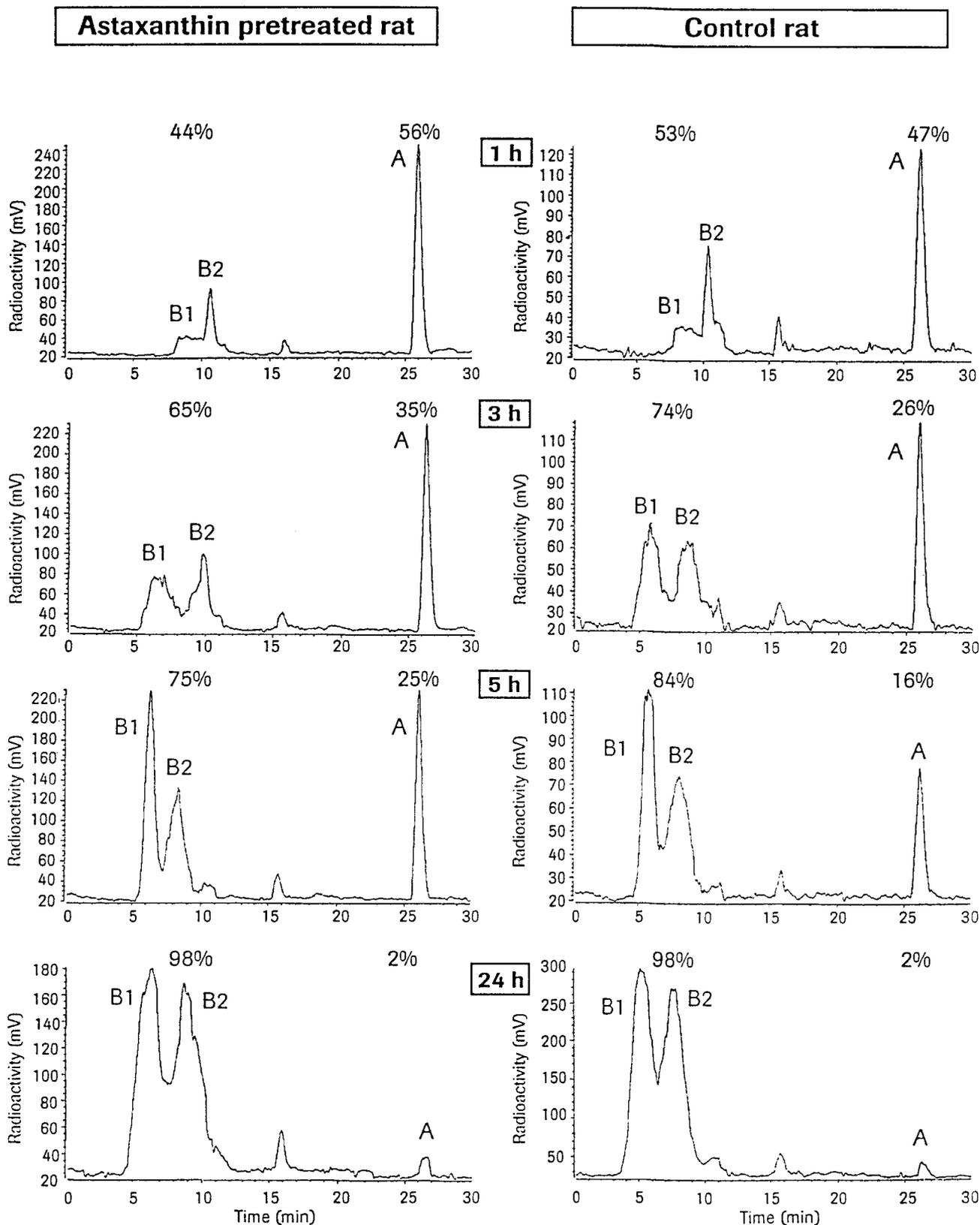
Astaxanthin pretreated rat

Control rat

FIG. 1. *Time course of HPLC radioactivity pattern in the ethanol-soluble fraction from primary cultures of rat hepatocytes incubated with [^{14}C]astaxanthin.*

Rats were pretreated with astaxanthin for 4 days. Note that peak A appeared early and disappeared during the incubation period, whereas peaks B1 and B2 became more prominent during incubation. Within 24 h more than 50% of the applied astaxanthin was metabolized and conjugated. The retention time for astaxanthin-containing peaks was about 35 min. [^{14}C]astaxanthin's stability was tested by incubation in medium without cells for 24 h. Less than 3% of the radioactivity eluted at the same retention time as peak A, the remainder eluted at astaxanthin's retention time. HPLC elution was performed with a Prontosil column and a flow rate of 0.55 ml/min and solvents A (water), B (methanol-acetonitrile-tetrahydrofuran, 70:15:15, by volume), and C (acetone). Gradient elution: 100% A for 0.5 min, 5% B for 5 min, 5 to 62.5% B lasting 23 min, and 100% C for 12 min.
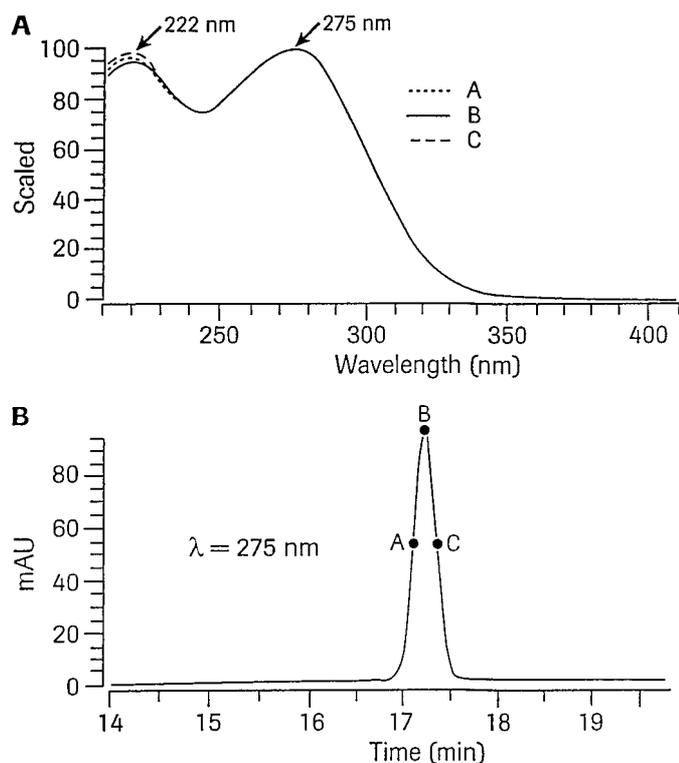
FIG. 2. *Analysis of peak A from the time course experiment shown in Fig. 1.*

The material in peak A at each time point was pooled for analysis. Absorption spectrum (A); HPLC chromatogram (B). Note that the absorption spectra were similar at the three positions A, B, and C, indicating a single compound. HPLC elution was performed with an Inertsil column, a flow rate of 1.0 ml/min, and solvents A (water) and B (methanol-acetonitrile, 50:50, v/v). Gradient elution: pre-equilibration with 15% B for 15 min before data acquisition, and 15 to 35% B lasting 20 min.

FIG. 3. *Analysis of deconjugates appearing after glusulase treatment of material in peaks B1 and B2.*

Absorption spectra (A); HPLC chromatogram (B). Note that the absorption spectra were different at the left shoulder (A and B) from that at the peak position (C) and the right side (D) of the peak, indicating two different compounds. HPLC elution was performed with a Prontosil column, a flow rate of 0.75 ml/min, and solvents A (water) and B (methanol-acetonitrile, 50:50, v/v). Gradient elution: 100% A for 0.5 min, 5% B for 4.5 min, and 5 to 49% B lasting 40 min.

ing 50 to 130 μg microsomal protein, 5 μM substrate (10 μl of a 1 mM solution of ethoxyresorufin in dimethylsulfoxide), and 0.1 M phosphate buffer (pH 7.4) was equilibrated for 1 min at 37°C. The reaction was then started by the addition of 250 μM NADPH (50 μl of a 10 mM solution). The reaction rate was measured directly as reflected by the increasing fluorescence of the reaction mixture that was recorded in chart form.

Primary Cultures of Rat Hepatocytes. Hepatocytes were isolated from control or astaxanthin-pretreated rats by a two step collagenase perfusion method (Seglen, 1976). A rat was anesthetized with pentobarbital and the liver was perfused in situ via the portal vein with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution containing 0.5 mM EGTA. The liver was then removed and perfused for 10 min with the above perfusion buffer containing Ca^{2+} (5 mM) and collagenase (120 I.U./ml). Cell viability was determined by erythrosin B exclusion and, if lower than 75%, a Percoll purification step was performed. 10^6 rat hepatocytes were seeded in collagen-coated culture wells in 1 ml of William's E medium containing 10% fetal calf serum. After 2 h to permit attachment, supernates were aspirated and 1 ml of radiolabeled or unlabeled astaxanthin (2 μM) in serum-free William's E medium supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), insulin (10^{-7} M), and dexamethasone (10^{-7} M) was added to each well; incubation lasted for the indicated times. Incubation was stopped by the addition of 1 ml of 50% ethanol in water (v/v) to each culture. The contents of replicate cultures were pooled and centrifuged to separate ethanol-insoluble and -soluble fractions. The ethanol-soluble fraction was extracted with 2 volumes of *n*-hexane always containing 0.05% (w/v) butylhydroxytoluol. The ethanol-insoluble fraction was washed with *n*-hexane. The combined *n*-hexane extracts were evaporated to dryness under N_2 stream at 37°C and dissolved in *n*-hexane; this is termed the *n*-hexane fraction. Finally, the ethanol-insoluble fraction was dissolved in 0.1 N NaOH by heating at 50°C for 1 h. Distribution of radioactivity in the three fractions was determined by liquid scintillation counting.
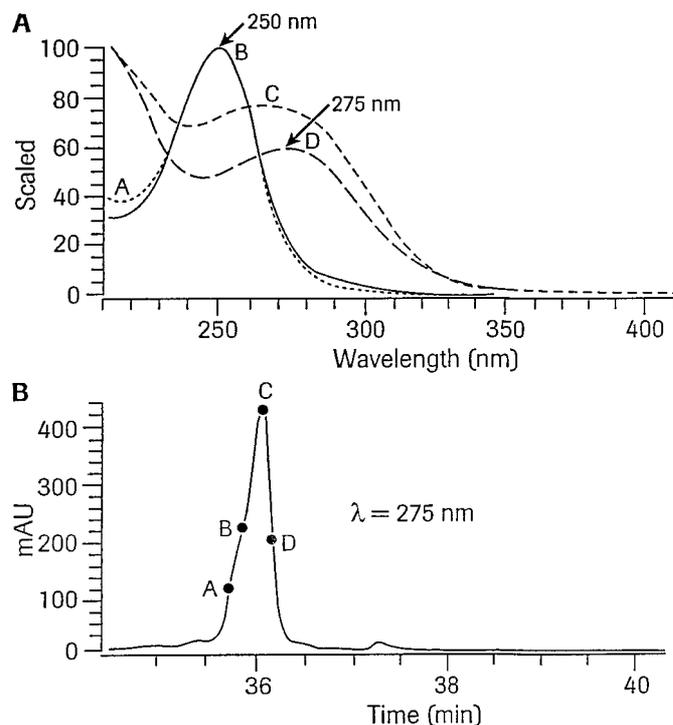
Astaxanthin Determination in Rat Livers. Three livers each from astaxanthin-treated and control rats were homogenized, exhaustively extracted with acetone, and analyzed for astaxanthin by normal phase HPLC (Schüep and Schierle, 1995).

Incubation of Astaxanthin with Rat Liver Microsomes. Each reaction mixture contained 1.5 mg microsomal protein and 2 μM [^{14}C]astaxanthin in a total volume of 1 ml of 0.1 M sodium phosphate buffer (pH 7.4) and was incubated for 2 to 3 min at room temperature. Enzymatic oxidation was started by the addition of 10 μl of NADPH-regenerating system (20 mM NADP, 100 mM glucose 6-phosphate, and 50 mM $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.4; before use, 2 U/ml of glucose 6-phosphate dehydrogenase were added), followed by incubation for 30 min at 37°C under air in a shaking water bath. The reaction was stopped by adding 2 volumes of ice-cold ethanol, centrifuged, and the supernatants were analyzed by reversed phase HPLC.

HPLC Analysis. HPLC analysis of ethanol-soluble fractions was carried out by reversed phase chromatography for astaxanthin and metabolites, whereas *n*-hexane fractions were analyzed by normal phase chromatography for astaxanthin; a HP-1090 system (Hewlett Packard, Urdorf, Switzerland) was used. In the effluent, UV/VISIBLE absorbance and radioactivity were monitored continuously using a HP-diode array detector (range 210–410 nm) and an LB 507A on-line radioactivity detector (Berthold, Regensdorf, Switzerland). Individual peaks and fractions were collected and pooled for further characterization.

Reversed phase HPLC systems. Analytical analyses were done using a Prontosil ODSAQ column (250 × 4 mm, 120 Å, 5 μm; Bischoff GmbH, Leonberg, Germany) and an Inertsil ODS-2 column (250 × 4.6 mm, 150 Å, 5 μm; GL Sciences, Tokyo, Japan); a Stagroma Spherisorb ODS-2 column (250 × 8 mm, 80 Å, 5 μm; Stagroma, Wallisellen, Switzerland) was used for preparative analysis.

Normal phase HPLC. For normal phase HPLC, a Spheri-5 Silica column (220 × 4.6 mm, 80 Å, 5 μm; Brownlee Laboratories, San Jose, CA) was used and eluted at a flow rate of 1 ml/min with solvents A (*n*-hexane) and B
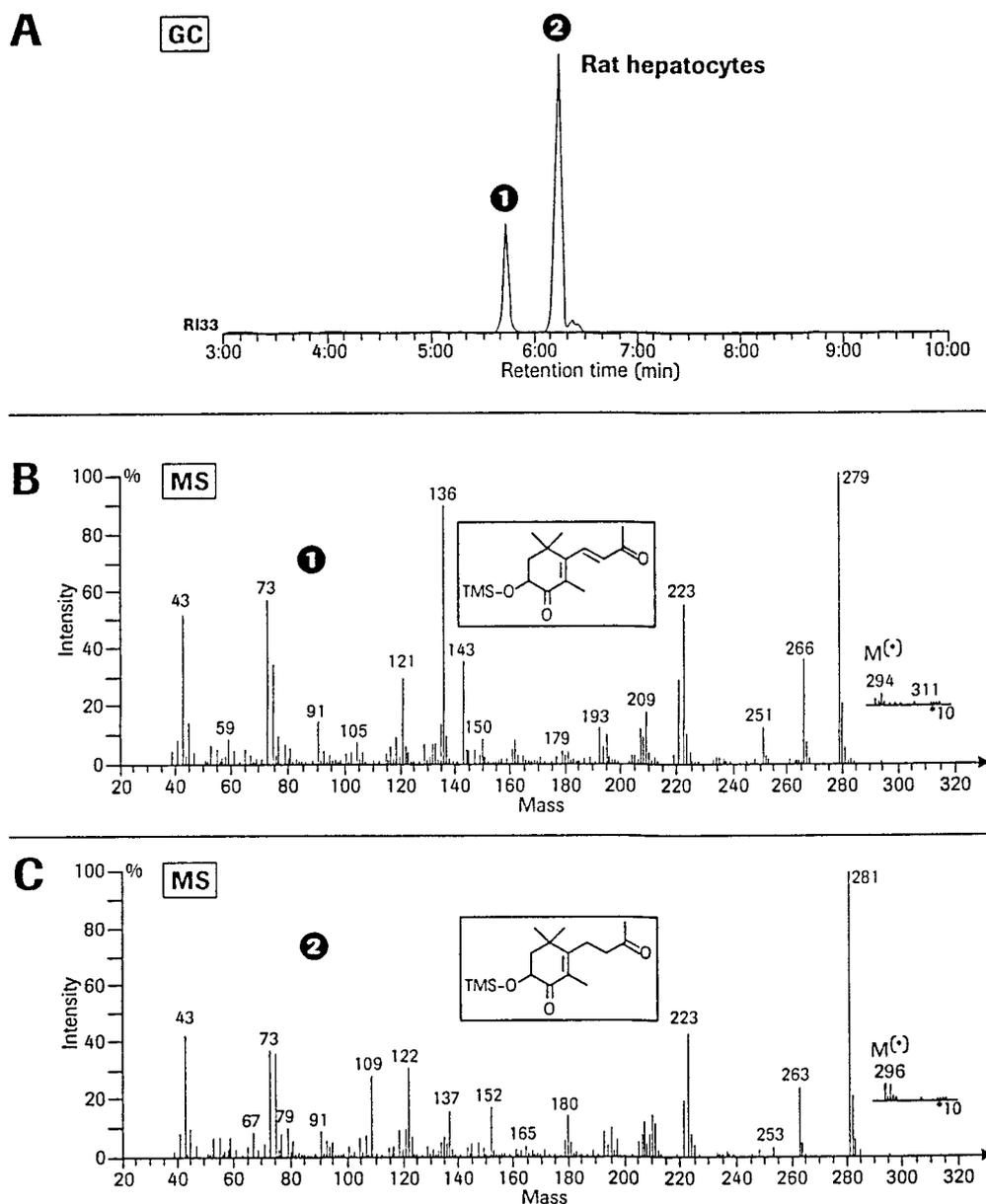
FIG. 4. *GC-MS of the purified peak A compounds shown in Fig. 3.*

Preparative HPLC elution was performed with a Stagroma column, a flow rate of 2.3 ml/min, and solvents A (water) and B (methanol/acetonitrile, 50:50, v/v). Gradient elution: 100% A for 0.5 min, 3% B for 6.5 min, and 3 to 38% B lasting 35 min. Then analytical HPLC elution was performed as indicated in the text to Fig. 3. Compound 1 was (*rac*)-3-hydroxy-4-oxo-β-ionone with absorption maxima at 222 and 275 nm and compound 2 was (*rac*)-3-hydroxy-4-oxo-7,8-dihydro-β-ionone with an absorption maximum at 250 nm.

(acetone/dichlormethane/*tert*-butylmethylether/chloroform, 54:23:13:10, by volume). Gradient elution: 100% A for 0.5 min, 8% B for 6 min, 8 to 15% B lasting 4 min, 15% B for 3 min, 15 to 50% B lasting 12 min, 50% B for 20 min.

Enzymatic Digestion of Conjugates. Conjugates of astaxanthin metabolites were isolated and purified by reversed phase HPLC; details are given in the figure legends. Aliquots of dissolved fractions (about 50,000 dpm) were evaporated to dryness under N_2 gas and dissolved in 2 ml 0.1 N ammonium acetate (pH 5.0). After addition of 100 μl glusulase, the mixtures were incubated for 18 h at 37°C. The sulfatase activities were selectively inhibited by incubation in 0.2 M sodium phosphate buffer, pH 6.6. The deconjugation products were isolated and purified by reversed phase HPLC analysis.

GC-MS Analysis. The residues of the HPLC fractions were dissolved in 50 μl silylating reagent (pyridine + bis(trimethylsilyl)trifluoroacetamide, 50:50, v/v) and analyzed by GC-MS after standing at room temperature for approximately 30 min. The silylated products were injected into a model 5890A gas chromatograph set at 270°C (Hewlett-Packard), which was coupled to a model 5989B mass spectrometer (Hewlett-Packard). The stationary phase was phenyl (50%)-methyl (50%)-silicon (DB-17, film thickness 0.25 μm) in a fused silica column (15 m × 0.25 mm). Helium was used as the carrier gas at a flow rate of 0.5 m/s. A heating program was applied to the column (150–320°C, heating rate 4°C/min). Ionization was performed by electron impact at 70 eV and 250°C.

Results

Analysis of Microsomes from Rat Liver Cells. Liver microsomal fractions prepared from rats treated with astaxanthin for 4 days and food-deprived for 18 h had a 17-fold increase in EROD activity, which is accompanied by an elevation of cytochrome P-4501A1 isoform level (Gradelet et al., 1996a,b). EROD activities in control and astaxanthin-treated rats were 52.0 ± 4.6 and 890.2 ± 13.4
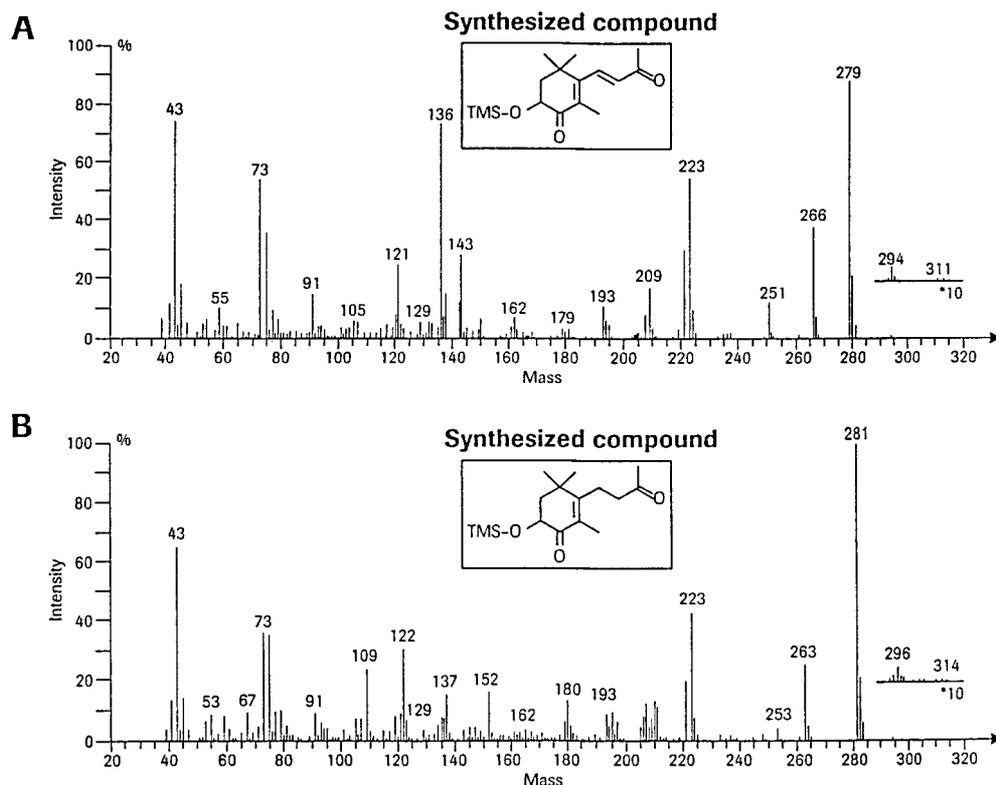
FIG. 5. *GC-MS analysis of the chemically synthesized (rac)-3-hydroxy-4-oxo-β-ionone (A) with absorption maxima at 222 and 275 nm (data not shown) and (rac)-3-hydroxy-4-oxo-7,8-dihydro-β-ionone (B) with an absorption maximum at 250 nm (data not shown).*

pmol/min/mg protein, respectively, whereas liver astaxanthin content was 0.23 ± 0.06 mg/kg ($n = 3$) in astaxanthin-pretreated rats and below the detection limit of 0.1 mg/kg in control rats. [^{14}C]Astaxanthin incubated with liver microsomes from control or astaxanthin-pretreated rats was not metabolized (data not shown) in contrast with cultured hepatocytes from both astaxanthin-pretreated and control rat livers where astaxanthin was metabolized (see below).

Analysis of Metabolites from Cultured Rat Hepatocytes. Rat hepatocytes were cultured with [^{14}C]astaxanthin and ethanol-insoluble, ethanol-soluble, and *n*-hexane fractions were prepared. Radioactivity decreased with time in the *n*-hexane and ethanol-insoluble fractions but increased in the ethanol-soluble fractions; this pattern was identical for hepatocytes obtained from control and astaxanthin-pretreated animals (data not shown). The HPLC chromatograms of ethanol-soluble fractions from hepatocytes from control and astaxanthin-pretreated rats were almost identical; three main polar peaks were found in each case (Fig. 1). Peak A appeared within 30 min (data not shown) and after purification it had an absorption spectrum with two maxima at 222 and 275 nm (Fig. 2A). Glusulase treatment of peaks B1 and B2 (including the shoulder or small peak at the lipophilic side) resulted in their complete disappearance and the reappearance of peak A (see deconjugation results below) but now with a shoulder detected by both spectral photometry (Fig. 3B) and by radioactivity monitoring (data not shown). Absorption spectrum analysis revealed two different spectra, one with a maximum at 250 nm and the other with the above maximum at 275 nm (Fig. 3A). Further investigations by GC-MS revealed that the metabolite with a spectral maximum of 250 nm was (*rac*)-3-hydroxy-4-oxo-7,8-dihydro-β-ionone and the other was (*rac*)-3-hydroxy-4-oxo-β-ionone (Fig. 4). These structures were confirmed by HPLC and GC-MS analyses (Fig. 5) of synthesized standards.

Results from glusulase treatment of the combined fractions of peaks

B1 and B2 under conditions inactivating sulfatase, 0.2 M phosphate buffer pH 6.6, demonstrated that the substances in fractions B1 and B2 were deconjugated. This indicated that these substances were glucuronides. A small amount (less than 5%) remained at the origin and was deconjugated by subsequent treatment with glusulase without sulfatase-inactivating conditions (data not shown), demonstrating that it was sulfated metabolites; this deconjugated material was more polar than the metabolites in peak A, but was not further characterized because of insufficient amounts. Separate deconjugation of the material in peaks B1 and B2 with glusulase (Fig. 6) and subsequent characterization of the products by absorption spectroscopy confirmed that peaks B1 and B2 contained conjugates of the metabolites (*rac*)-3-hydroxy-4-oxo-7,8-dihydro-β-ionone and (*rac*)-3-hydroxy-4-oxo-β-ionone, respectively.

The radioactive material in the *n*-hexane fraction was evaporated, resolubilized in ethanol, and analyzed by reversed phase HPLC; based on HPLC retention times, about 2% was (*rac*)-3-hydroxy-4-oxo-β-ionone, whereas about 98% was astaxanthin.

The ethanol-insoluble fractions were re-extracted with acetone and the acetone extracts were evaporated, resolubilized in 5% ethanol, and analyzed by reversed phase HPLC. More than 95% of the radioactivity was astaxanthin and its isomers, whereas only about 5% was unidentified.

To evaluate its stability, [^{14}C]astaxanthin was incubated for 24 h in culture medium without fetal calf serum and without hepatocytes; less than 3% of the applied radioactivity localized to the same position as peak A in Fig. 1. Because of the small amount of this compound, it could not be identified. In an attempt to identify this compound, unlabeled astaxanthin was exposed to O_2 for 12 h followed by collection of possible oxidation products by washing the carotenoid with 10% ethanol. Purification and characterization of the main com-
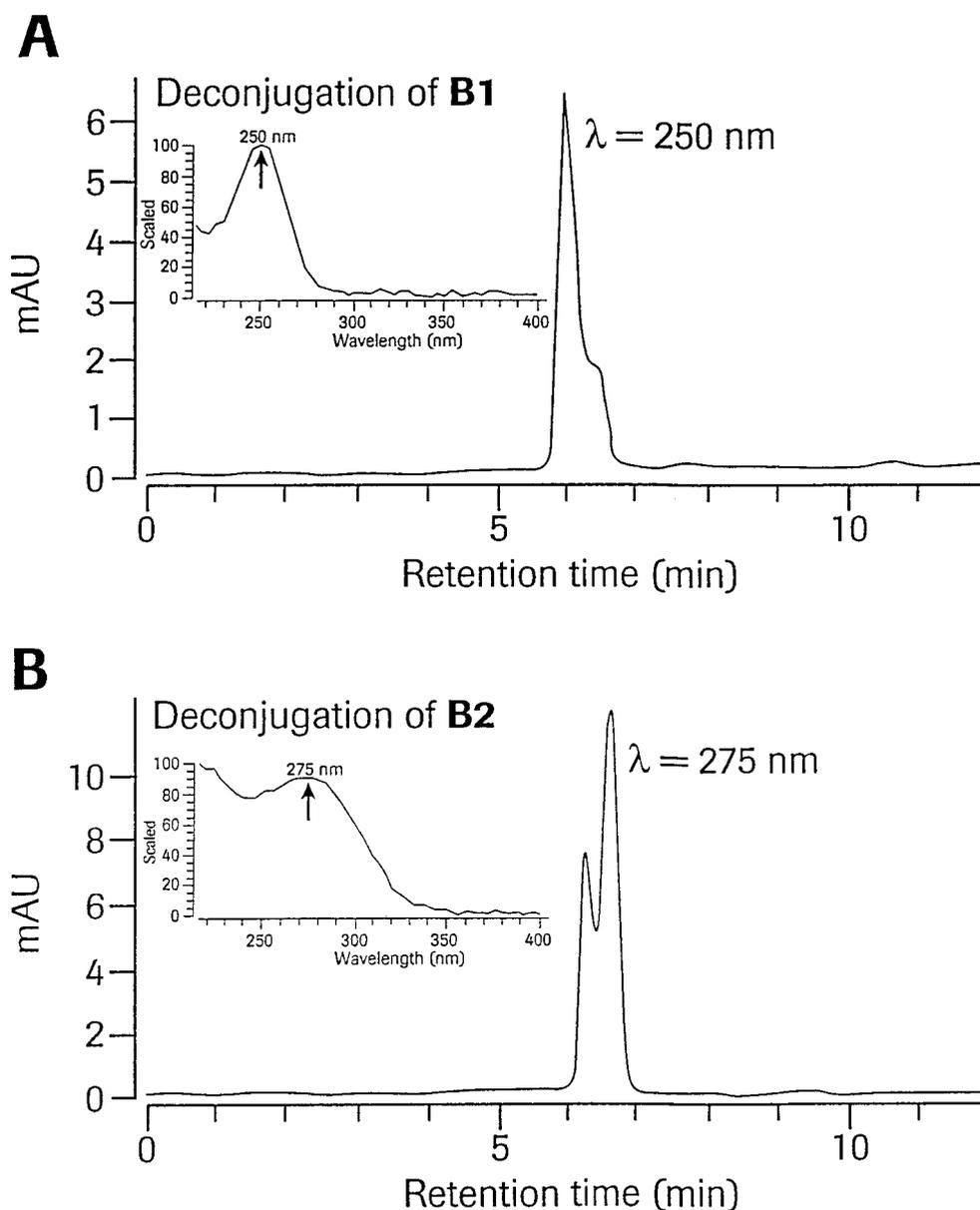
FIG. 6. *Separate deconjugation of material in peaks B1 (A) and B2 (B) and HPLC analysis and absorption spectra of the ligands.*

Note that the deconjugates of substances in peaks B1 and B2 had absorption maxima of 250 and 275 nm corresponding to (*rac*)-3-hydroxy-4-oxo-7,8-dihydro-β-ionone and (*rac*)-3-hydroxy-4-oxo-β-ionone, respectively. HPLC elution was performed with a Prontosil column, a flow rate of 0.75 ml/min, and solvents A (water) and B (acetonitrile). Isocratic elution: pre-equilibration with 23% B for 11 min before data acquisition, 23% B for 12 min.

pound in the ethanol wash by reversed phase HPLC and GC-MS showed that it was (*rac*)-3-hydroxy-4-oxo-β-ionone.

Discussion

The present study demonstrated that rat hepatocytes in primary culture metabolized astaxanthin into (*rac*)-3-hydroxy-4-oxo-β-ionone, which was conjugated mainly into glucuronides. Part of the conjugate was reduced to (*rac*)-3-hydroxy-4-oxo-7,8-dihydro-β-ionone. It is noteworthy that in the Japanese catfish *Parasilurus asotus,* two carotenoids, parasiloxanthin and dihydroparasiloxanthin, are also hydrated at C7,8- and C7,8,7',8'-positions (Matsuno et al., 1976). Liver microsomes prepared from rats pretreated with astaxanthin and, thus with induced xenobiotic-metabolizing enzymes (see above and Astorg et al., 1994; Gradelet et al., 1996a,b), did not convert astaxanthin, indicating that xenobiotic-metabolizing enzymes were not involved in the metabolism of this carotenoid. Furthermore, the metabolism of astaxanthin in hepatocytes from rats either pretreated or not pretreated with astaxanthin was similar (Fig. 1), also indicating that cytochrome P-450 systems were not involved in astaxanthin metabolism in rat hepatocytes.

Investigations in vitro with carotenoids are hindered by their low solubility in all biologically suitable solvents. The concentration (2 μM) of astaxanthin used in our in vitro studies was near its solubility point in culture medium. However, this astaxanthin concentration was about 7 times higher than the baseline concentration of the common dietary carotenoid β-carotene, which is about 0.3 μM in human serum (Albanes and Heinonen, 1994; Khachik et al., 1997). In humans, serum β-carotene concentration levels up to 5.6 μM were reached after β-carotene supplementation (20 mg/day) for 3 years (Albanes and Heinonen, 1994).
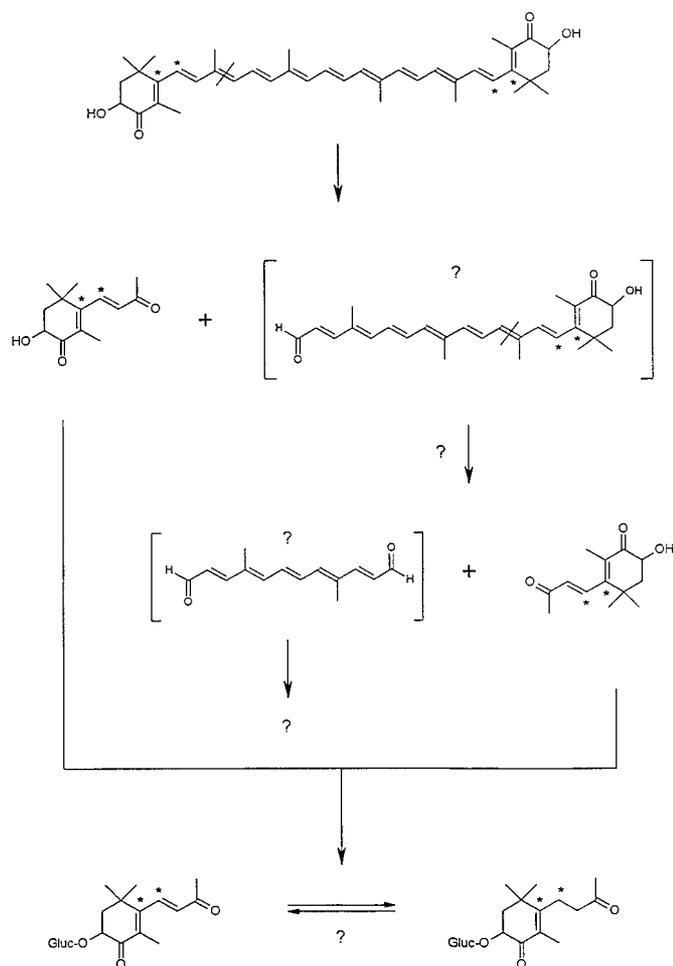
FIG. 7. *Metabolism of astaxanthin in primary cultures of rat hepatocytes.*
Molecules in brackets indicate possible but not identified metabolites.

Liver drug-metabolizing enzymes are induced by astaxanthin in a dose-dependent fashion in rats (Astorg et al., 1994; Gradelet et al., 1996a,b) with a 34-fold increase in EROD activity at a dose of 300 ppm in the diet. We confirmed this induction and found a 17-fold increase. The dissimilar experimental conditions in our and the previous studies probably accounts for the 2-fold difference.

Provitamin A carotenoids are cleaved at the central C15 = C15′ double bond by β-carotene dioxygenase into vitamin A (Goodman and Huang, 1965; Olson and Hayaishi, 1965). Astaxanthin was not cleaved at this central bond (W.D. Woggon, personal communication). Our results in rat hepatocytes, which focused on polar metabolites of astaxanthin, supported this finding and indicated that astaxanthin was cleaved asymmetrically at the C9 position. We cannot definitively exclude that cleavage of the polyene chain occurred at points other than the C9,C9′ positions. However, the presence of other cleavage points would imply an extremely rapid sequential degradation of the polyene chain because we did not find intermediary products in the time course studies (Fig. 1). In these studies as well as in all other HPLC analyses of the water-soluble fractions, we did not find the "free" unconjugated metabolite (*rac*)-3-hydroxy-4-oxo-7,8-dihydro-β-ionone. This metabolite only appeared after glusulase treat-

ment of the combined fractions of peaks B1 and B2. In the two independent time course studies (Fig. 1) as well as in preliminary studies, HPLC analysis always revealed a symmetrical peak A with an absorption spectrum, indicating a single compound (Fig. 2). After deconjugation of the material in peaks B1 and B2, the resulting peak A had a shoulder as revealed by spectral photometry (Fig. 3B) and radioactivity monitoring. Spectral analysis of this shouldered peak indicated two substances (Fig. 3A). Thus, either this metabolite was immediately conjugated so that it was not detected in HPLC chromatograms of the water-soluble fractions (Fig. 1) or the (*rac*)-3-hydroxy-4-oxo-β-ionone was first conjugated and then reduced in its conjugated form. Furthermore, as shown by HPLC analysis, glusulase treatment of the synthesized standard (*rac*)-3-hydroxy-4-oxo-β-ionone did not affect this compound, excluding the possibility that glusulase converted (*rac*)-3-hydroxy-4-oxo-β-ionone into (*rac*)-3-hydroxy-4-oxo-7,8-dihydro-β-ionone. Moreover, separate deconjugations demonstrated that the conjugates in peaks B1 and B2 contained the metabolites (*rac*)-3-hydroxy-4-oxo-7,8-dihydro-β-ionone and (*rac*)-3-hydroxy-4-oxo-β-ionone, respectively (Fig. 6). These results indicate that rat hepatocytes in primary culture metabolize astaxanthin according to the scheme shown in Fig. 7.

References

Albanes D and Heinonen (1994) The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N Engl J Med* **330:**1029–1035.
Astorg P, Gradelet S, Leclerc J, Canivenc MC and Siess HM (1994) Effects of β-carotene and canthaxanthin on liver xenobiotic-metabolizing enzymes in the rat. *Food Chem Toxicol* **32:**735–742.
Brubacher GB and Weiser H (1985) The vitamin A activity of β-carotene. *Int J Vitam Nutr Res* **55:**5–15.
Burke MD and Mayer G (1974) Ethoxyresorufin: Direct fluorometric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methycholanthrene. *Drug Metab Dispos* **2:**583–588.
Ganguly J and Sastry PS (1985) Mechanism of conversion of β-carotene into vitamin A—Central cleavage versus random cleavage. *World Rev Nutr Diet* **45:**198–220.
Goodman DS and Huang HS (1965) Biosynthesis of vitamin A with rat intestinal enzymes. *Science (Wash)* **149:**879–880.
Goodman DS, Blomsrand F, Werner B, Huang HS and Shiratori T (1966) The intestinal absorption and metabolism of vitamin A and β-carotene in man. *J Clin Invest* **45:**1615–1623.
Gradelet S, Astorg P, Leclerc J, Chevalier J, Vernevaut MF and Siess MH (1996a) Effects of canthaxanthin, astaxanthin, lycopene and lutein on liver xenobiotic-metabolizing enzymes in the rat. *Xenobiotica* **26:**49–63.
Gradelet S, Leclerc J, Siess MH and Astorg PO (1996b) β-Apo-8′-carotenal, but not β-carotene, is a strong inducer of liver cytochromes P4501A1 and 1A2 in rat. *Xenobiotica* **26:**909–919.
Khachik F, Spangler CJ, Smith JC, Canfield LM, Steck A and Pfander H (1997) Identification, quantification, and relative concentrations of carotenoids and their metabolites in human milk and serum. *Anal Chem* **69:**1873–1881.
Lowry OH, Rosebrough NJ, Farr, AL and Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* **193:**265–275.
Lu AYH, Kuntzman R, West S, Jacobson M and Conney AH (1972) Reconstituted liver microsomal enzyme system that hydroxylates drugs, other foreign compounds, and endogenous substrates. II. Role of the cytochrome P-450 and P-448 fractions in drug and steroid hydroxylations. *J Biol Chem* **247:**1727–1734.
Matsuno T, Nagata S and Kitamura K (1976) New carotenoids, parasiloxanthin and 7,8-dihydroparasiloxanthin. *Tetrahedron Lett* **50:**4601–4604.
Olson JA and Hayaishi O (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.
Schüep W and Schierle J (1995) Determination of stabilized, added astaxanthin in fish feeds and pre-mixes, in *Carotenoids, vol. 1A: Isolation and Analysis* (Britton G, Liaaen-Jensen S and Pfander H eds) pp 273–276, Birkhäuser, Basel.
Seglen PO (1976) Preparation of rat liver cells. *Methods Cell Biol* **13:**29–83.