β-Carotene-15,15′-dioxygenase (*EC* 1.13.11.21) isolation reaction mechanism and an improved assay procedure

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β-Carotene-15,15′-dioxygenase (*EC* 1.13.11.21; β-carotene dioxygenase) activity in extracts from guinea-pig intestinal mucosa was assayed by supplying [15,15′-$^{14}C_2$]- or [15,15′-$^{3}H_2$]β-carotene dissolved in Tween 80. Methods were developed to minimize the breakdown of labelled β-carotene and β-carotene cleavage products during the isolation procedure. Antioxidants and unlabelled carriers were added to extracting solvents and C_{18} Sep-Pak cartridges were used to isolate the remaining β-carotene and retinaldehyde, which was the only cleavage product detected. The labelled material produced by the enzyme was analysed by either normal-phase TLC or reversed-phase HPLC and characterized chemically as retinaldehyde. The lack of other labelled *apo*-carotenals isolated in these experiments and the formation of between 1·5 and 2 mol retinaldehyde/mol β-carotene consumed confirm the central cleavage mechanism for the enzyme's action. More β-carotene dioxygenase activity was obtained from guinea-pig mucosa than from chicken or pig intestinal mucosa. The β-carotene dioxygenase was obtained as a soluble enzyme which was partially purified by gel filtration and ion-exchange chromatography to a specific activity of 0·6 nmol retinaldehyde formed/mg protein per h. The formation of a lipid–protein aggregate containing the β-carotene dioxygenase activity, which has been reported to be present in the exclusion volume of Sephadex columns, was avoided if the mucosal scrapings were homogenized in buffer at a proportion of 1:4 (w/v).

β-Carotene dioxygenase: Retinaldehyde: Vitamin A

Plant carotenes provide an important source of dietary retinol, particularly in developing countries where they contribute approximately 80% of the nutritional retinol requirements (Simpson, 1983). β-Carotene is converted into retinaldehyde by the enzyme β-carotene-15,15′-dioxygenase (*EC* 1.13.11.21; β-carotene dioxygenase). This enzyme has been detected in extracts of mucosa from small intestines of a number of animals (Goodman & Huang, 1965; Olson & Hayaishi, 1965; Goodman *et al.* 1966; Lakshmanan *et al.* 1968, 1972; Fidge *et al.* 1969; Singh & Cama, 1974; Sharma *et al.* 1976; Sklan, 1983*a*), although the reports of its solubility and mechanism of action differ.

Experiments using ultracentrifugal density gradients led Goodman *et al.* (1966) and Fidge *et al.* (1969) to conclude that β-carotene dioxygenase was a soluble, cytosolic enzyme. Sklan *et al.* (1982, 1983*b*) and Sklan & Halevy (1984) reported that β-carotene dioxygenase was not a soluble enzyme but was associated with a high-molecular-weight lipid–protein aggregate which eluted in the void volume from a Sepharose 6B column (LPA). Sklan (1983*b*) states that this lipid–protein aggregate is associated with cellular retinol-binding protein and retinyl esters and suggested the possibility of a single particle (LPA) that was able to combine carotene cleavage, retinyl ester hydrolysis activity and transport.

Since Goodman *et al.* (1966), Olson (1961) and Olson & Hayaishi (1965) performed *in*

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vivo and *in vitro* studies on the mechanism of β-carotene dioxygenase, it had been accepted that the cleavage of β-carotene occurs only at the central 15,15′-double bond to yield two molecules of retinaldehyde. This view was challenged by Sharma *et al.* (1977) and Ganguly & Sastry (1985), who stated that the available evidence was also compatible with random cleavage of the polyene chain and that retinaldehyde was produced from *apo*-carotenals by a series of stepwise oxidase reactions. Hansen & Maret (1988) have stated that their preparations from rat intestine did not cleave β-carotene to yield retinaldehyde or *apo*-carotenals *in vitro*, and emphasized the need to re-evaluate the existing theory of conversion of β-carotene into retinol.

We have investigated the partial purification, solubility and the reaction mechanism of β-carotene dioxygenase. The products of β-carotene cleavage were analysed by reversed-phase HPLC and characterized chemically. Previously, the extraction of the incubation mixture has been achieved by a partitioning method (Folch *et al.* 1957) followed by normal-phase chromatography. This procedure is lengthy, requires large volumes of solvent and can expose the unstable carotenoids and retinoids to a variety of destructive conditions. Commercial prepacked cartridges, which do not bind hydrophilic compounds while retaining hydrophobic ones, have been shown to provide a convenient and gentle extraction procedure (Kozub *et al.* 1988).

We have been able to reproduce and improve on earlier work on β-carotene-15,15′-dioxygenase activity by using commercial reversed-phase cartridges as an alternative extraction procedure for the enzyme assay. In addition, a modified procedure for the isolation of the dioxygenase enzyme has been developed. We have been able to demonstrate, unambiguously, the action of the enzyme *in vitro* and to establish β-carotene is cleaved centrally. Possible reasons why Hansen & Maret (1988) obtained their results are discussed in the light of the present work.

MATERIALS AND METHODS

Male, short-haired, English guinea-pigs (400 g) and Wistar rats (200 g) were obtained from the University of New South Wales Animal Breeding Unit, where they were fed on multi-purpose guinea-pig pellets based on lucerne (*Medicago sativa*; Doust and Raddish, Concord West, NSW, Australia). They were killed by cervical dislocation. Gut samples of chickens and pigs were obtained from animals which had been freshly killed commercially and were transported to the laboratory on ice.

All chemicals used were of the highest purity available commercially and organic solvents were of HPLC grade (Mallinkrodt, Clayton, Victoria, Australia) except *t*-butylmethyl ether which was analytical grade (Fluka, Buchs, Switzerland) and was used in preference to diethyl ether as it is less prone to form peroxides. The *n*-hexane and *t*-butylmethyl ether were dried before use by being held over a type 13 × molecular sieve (Union Carbide, CT, USA). The antioxidant BHT (2,6-di-*tert.* butyl-4-methyl phenol; Sigma, St Louis, MO, USA) was added to all solvents before use (1 mg/l). Protease inhibitors, phenylmethylsulphonylfluoride (PMSF) and *Nα-p*-tosyl-L-lysine chloromethyl ketone (TLCK) were obtained from Aldrich (Milwaukee, WI, USA). All glassware was cleaned by soaking in detergent, rinsed, then placed in HNO_3 (250 ml/l) for 12 h. After this it was washed in Milli Q deionized water. Any residual acidic surfaces or acid in microcracks were neutralized by soaking the glass in 0·8–1·6 M-NH_4OH for 12 h before being rinsed again in distilled water and dried at 100°.

Extraction and partial purification of β-carotene dioxygenase

Intestine samples from directly below the stomach of rat, guinea-pig and pig (0·06, 0·12 and 0·10 M respectively) and below the gizzard of chickens (0·01 M) were used in the animal

studies. The small intestine was flushed with isotonic saline (9 g NaCl/l), cooled to 0°. The intestinal mucosa was prepared and the scrapings were homogenized as described by Goodman & Olson (1969). Alternative extraction procedures tested included a preparation with scrapings–buffer (1:4 w/v) using the same buffer and homogenization procedure and sonication of the homogenate using a microtip probe and a β-12 Sonifier (Branson Sonic Company, Danbury, CT, USA) set at 60 W. The preparation was sonicated four times for 5 s and cooled for 30 s between sonication periods in an ice–salt mixture.

The homogenates were centrifuged (104000 *g* for 60 min at 4°). An alternative purification of the supernatant fraction by precipitation induced by the dropwise addition of 0·2 M-acetic acid to pH 5·2 was also tested. After recentrifugation (30000 *g* for 15 min at 4°) the supernatant fraction and pellet were each adjusted to pH 7·8 before being assayed for β-carotene dioxygenase activity.

In separate experiments the fat-free infranatant fraction (below the fatty surface and above the pellet) obtained from the ultracentrifuged supernatant fraction was incubated with [15,15′-$^3H_2$]β-carotene (5·0 μg) and then concentrated by $(NH_4)_2SO_4$ (0–60 g/l) precipitation or concentrated by ultrafiltration under N_2 using an Amicon YM membrane with a molecular cut-off of 10000 (200 kPa). The concentrate was fractionated on Sepharose 6B as described by Sklan (1983*b*). The active β-carotene dioxygenase fraction obtained from the Sepharose 6B column was dialysed overnight against phosphate buffer (10 mM, pH 7·8) and fractionated by ion-exchange chromatography on DEAE Sepharose as described by Fidge *et al.* (1969).

Carotene cleavage assay

Synthetic [15,15′-$^3H_2$]β-carotene (40·1 Ci/mol) and [15,15′-$^{14}C_2$]β-carotene (20·4 Ci/mol) were obtained from Hoffmann La Roche & Co. Ltd, Basle, Switzerland. Samples were stored in individual, sealed ampoules, in benzene, held in 1 ml amber-glass vials, shielded from light, filled with N_2 and stored at −20°. β-carotene was separated from isomeric forms and breakdown products by HPLC routinely just before use, often while the enzyme preparation was being centrifuged. The sample was passed through a silica guard column and then chromatographed in *n*-hexane (3 ml/min) on a Techsil 10 silica column (250 × 8 mm i.d.). The hexane was evaporated under N_2, the β-carotene was taken up in Tween 80 in acetone (10 mg/ml), the acetone was evaporated under N_2 and K_2PO_4 buffer (0·1 M, pH 7·8) was added to give a concentration of β-carotene of 5–10 μg/ml and Tween 80 at 10 mg/ml. The mixture was dispersed by agitation in a Vortex mixer. Portions (100 μl) of this solution were then added to the enzyme preparation (1·9 ml) and mixed quickly to initiate the reaction. Triplicate or duplicate portions (100 μl) were counted separately for each experiment to determine total amounts of radioactivity added. The enzyme reactions (slightly staggered initiation times) were incubated at 37° for 1 h with gentle shaking (40 cycles/min). The incubation mixture (2·0 ml) and conditions were as described by Fidge *et al.* (1969) except that the β-carotene was dispersed in 1 mg Tween 80 instead of Tween 40 and no phospholipids were added.

Extraction of products from incubation mixtures

The reaction was stopped by the addition of unlabelled β-carotene (15 μg) retinaldehyde (30 μg) and methanol (2 ml). These quantities were approximately fifty times the amount of labelled material present. In assays which contain large amounts of protein (above 2 mg and up to 20 mg/2 ml) the addition of methanol (2 ml) caused precipitation of the proteinaceous material, which was removed by centrifugation (1000 *g*, 30 s). The clear supernatant fraction was decanted and the methanol–water mixture was adjusted from 1:1 (v/v) to 1:5 (v/v) by the addition of more methanol before loading onto the activated

C_{18} Sep-Pak column of three C_{18} cartridges connected, in series, by small glass sleeves. The C_{18} Sep-Pak cartridges had been activated previously by washing with methanol (3 ml) and water (3 ml). Water-soluble materials were then washed from the cartridges with water (10 ml) and the non-polar components were then extracted into *t*-butylmethyl ether (10 ml) followed by acetone (4 ml) and dichloromethane (4 ml). These organic eluates were combined, dried over anhydrous Na_2SO_4 and concentrated under N_2 at 35° within 30 min. All tubes were wrapped in aluminium foil to shield the contents from light. The concentrated total organic extract was then subjected to TLC or HPLC.

TLC

The concentrated organic eluate from the Sep-Pak cartridges was routinely separated by silica gel TLC using hexane–acetone (97:3, v/v). The basal 20 mm of the silica gel was deactivated by an application of BHT (1 mg/ml) in methanol (0·5 ml) before the solutions containing β-carotene or retinoids were put onto the plates (200 × 50 × 0·25 mm Kieselgel F_{254}; Merck, Darmstadt, Germany). BHT was present in the sample solution and in the solvent used to develop the plates. When the solvent front was 10 mm from the top the zones carrying β-carotene (R_f 0·99) and retinaldehyde (R_f 0·4) and the origin (R_f0–0·1) were scraped into scintillation vials and counted directly in PPO (5 g/l; Packard Instruments Co., Downers Grove, IL, USA), toluene (660 ml/l; HPLC grade; Mallinkrodt), Triton X-100 (335 ml/l; Sigma). Evidence of identification of the labelled product formed from [^{14}C]β-carotene as retinaldehyde was obtained by its co-chromatography with authentic, synthetic material on silica gel TLC plates. [^{14}C]retinaldehyde was obtained by oxidation of [^{14}C]retinol (Amersham, North Ryde, NSW, Australia) using standard techniques. Labelled [^{14}C]retinaldehyde samples mixed with authentic, unlabelled material were co-chromatographed on silica gel TLC plates. The exact coincidence of zones of labelled material and unlabelled standards was established by autoradiography after the corners of the dried plates had been marked with radioactive ink. The developed X-ray films were placed over the silica gel plates which were viewed under screened u.v. (254 nm) light so that the distribution of exposure of the film by a ^{14}C-labelled compound could be compared with the position and intensity of quenching of the u.v.-induced fluorescence from the plate caused by the presence of the unlabelled retinaldehyde. The data shown in Figs. 2 and 3 were calculated from the measurements of radioactivity in β-carotene and retinaldehyde extracted from the reaction mixtures. The amount of β-carotene isolated from the test reaction mixture at the end of the incubation period was subtracted from the amount obtained similarily from a boiled control to give the amount of β-carotene degraded. Retinaldehyde production was calculated as the difference between the amount of retinaldehyde obtained from the test reaction mixture and the amount obtained similarily from a boiled control. To allow for the differences found in the percentage recovery of radioactivity, all values were corrected to give 100% recovery, and then used for calculation. For an example of the procedure, see the legend of Fig. 2.

HPLC of β-carotene, β-8-apo-carotenal, retinaldehyde and retinol

A Waters' HPLC system comprised a Model 510 pump, a model M45 pump, a U6K injector and a model 660 solvent programmer (Millipore-Waters, Milford, MA, USA). Effluent was monitored by an HP 1040A Diode Array Detector (Hewlett-Packard, Waldbronn, Germany). The procedure of Roberts *et al.* (1978) was adapted whereby a mixture of the standards (20 μg each) and 1 mg Tween 80 was dissolved in 100 μl acetonitrile. The mixture was injected into a C_{18} guard column and an analytical Spherisorb 5 ODS column (250 × 5 mm; Philips, Cambridge, Cambs.) which had been equilibrated for 15 min with solvent A (acetonitrile–water; 4:1, v/v) at 1 ml/min. The HPLC was then run

isocratically with solvent A; retinol was eluted at 12·6 min and retinaldehyde at 15 min. After the retinaldehyde was collected a linear gradient of solvent B (propan-2-ol–acetonitrile (1:1, v/v); 1 ml/min) was run for 10 min and the solvent B isocratically. Retention times (min) were: retinaldehyde 15, β-8′-*apo*-carotenal 30·9, β-carotene 33·7. Although these compounds were always well separated there was some day-to-day variation in retention times probably caused by a carry-over of Tween 80 (extracted by the ether) and, hence, water in the solutions.

The extracted and concentrated β-carotene dioxygenase assay samples were also injected in 100 μl acetonitrile and the same HPLC system was used. Incubation mixtures contained 1·5 mg of the ultracentrifuged supernatant fraction while control incubations contained boiled supernatant protein. Triplicate test and control incubations were extracted with and without the addition of unlabelled standards (β-carotene 10 μg, retinaldehyde 30 μg). The assay samples were extracted using the C_{18} Sep-Pak system and concentrated under N_2 at 35°. Due to the high recovery of the organic components of the assay samples (including lipids from supernatant fraction) only a subsample (30%) of each extract was chromatographed in the Spherisorb 5 ODS (reversed-phase) HPLC column. This precaution was necessary to prevent overloading of the HPLC column and to maintain effective chromatographic separation. Protein was determined using the method of Bradford (1976).

Exchange of the oxygen atom of retinaldehyde

Samples of synthetic retinaldehyde and retinol were dissolved for 14 d in ethanol (10 μl) and $H_2{}^{18}O$, (98% atoms enrichment; Novachem Pty, S. Yarra, Victoria, Australia) (1 ml). They were then extracted into *t*-butylmethyl ether, dried with anhydrous $MgSO_4$ and evaporated to a small volume which was coated onto the probe of a Finnegan Quadrapole Mass spectrometer (model 3200) interfaced to a Finnegan Model 6115 data system. The source pressure 107–120 Pa and chemical ionization spectra of labelled and unlabelled materials were obtained with CH_4 as the ionizing gas.

RESULTS

Handling procedures and measurements

Because β-carotene and, to a lesser extent, retinaldehyde are unstable in the presence of air, water, light and particularly acidic conditions, a considerable effort was expended in perfecting handling techniques so that the small amounts of substrate [^{14}C]- and [^{3}H]β-carotene and the enzymic product retinaldehyde could be recovered almost quantitatively. A protocol was developed in which every piece of glassware was soaked in 1 M-NH_4OH, all solvents were dried and gassed with N_2, all possible manipulations were carried out in a cold room and the solutions shielded from light. The antioxidant BHT was present in all organic solvents and glutathione was present in aqueous solutions. The number of sample vials in each experiment was restricted to six and the starting times for the initiation of the reactions were staggered, thereby enabling the solutions to be extracted into *t*-butylmethyl ether within less than 10 min from the end of incubation.

Fidge *et al.* (1969) noted that β-carotene dispersed in Tween 40 was converted into retinaldehyde at a faster rate than when added to the enzyme preparation in acetone. The same result was obtained using Tween 80 in these experiments. Singh & Cama (1974) dispersed all substrates tested in Tween 20 to ensure complete solubilization and claimed that differences in dioxygenase activity observed could not be attributed to differences in solubility. However, the inclusion of non-ionic detergents such as Tween (acylpoly-oxyethylene sorbitan esters) reduces the efficiency of the partitioning techniques and this

Table 1. *β-carotene-15,15'-dioxygenase* (EC *1.13.11.21*) *activity in ultracentrifuged supernatant fractions of intestinal extract*

(The results are the means of duplicate experiments)

Extraction procedure*	Specific activity of enzyme preparation (nmol [^{14}C]retinaldehyde/mg protein per h)			
	Rat	Guinea-pig	Chicken	Pig
Goodman *et al.* (1967)	0·008	0·025	na	0·006
Goodman *et al.* (1967) and sonication of homogenate	na	0·010	0·029	na
Buffer–scrapings (4:1, w/v) and then homogenized	0·022	0·055	0·026	0·010

na, not assayed.

* All preparations were assayed using the incubation conditions of Fidge *et al.* (1969). For details, see p. 399.

could have affected the results. Reversed-phase, prepackaged (Sep-Pak) columns were investigated to overcome this problem in the experiments reported here and they provided an effective and convenient method for the isolation of carotenoids and retinoids from solutions containing non-ionic detergents and eliminated the need for partitioning in large volumes of organic solvents. The three Sep-Pak cartridges could retain the organic constituents of an incubation mixture which contained up to 2 mg protein and then the carotenoids and retinoids could be extracted with 85 (SE 5)% (*n* 12) recovery of total added radioactivity. Amounts of protein above 2 mg required precipitation before binding onto the cartridges and this resulted in lower recoveries of β-carotene and retinoids (75 (SE 5)%; *n* 21). However, the major advantage of the C_{18} Sep-Pak system over conventional partitioning methods was that each test sample could be extracted in less than 5 min. Breakdown of the unstable molecules during extraction, therefore, was minimized. Also, the presence of small quantities of non-ionic detergents did not reduce the efficiency of the extractions of β-carotene or retinoids. After extraction the separated fractions could be quickly dried and stored in conditions which minimize breakdown (within 0·5 h of the end of the incubation).

Another major factor responsible for the high percentage recoveries of β-carotene and retinaldehyde reported in these experiments is the addition of unlabelled carrier at the end of the incubation period. As noted later (p. 408) the presence of added retinaldehyde increased the amount of radioactive retinaldehyde detected by 200%. However, it is probably the combination of all the previously mentioned procedures that enabled the high percentage recoveries of these unstable molecules and, hence, maintained stoichiometry of the reaction mixture.

Isolation of carotene dioxygenase

The results in Table 1 show that the guinea-pig is the most active source of β-carotene dioxygenase. Sonication of the crude homogenates did not increase the specific activity of the β-carotene dioxygenase from either rat or guinea-pig. The original method of Goodman *et al.* (1967) for the isolation of enzyme was performed by adding 1 ml buffer per 100 mm gut scraped. This gave less activity than when the scrapings were placed in ice-cold phosphate buffer (weighed) and then extra buffer was added to give 1 unit mucosa:4 units buffer, before being dispersed in a Potter-Elvejhem homogenizer. An increase in buffer:scrapings ratio almost doubled the specific activity of the enzyme obtained from rat, guinea-pig and pig. As the guinea-pig again provided the most active source of enzyme it was used in all subsequent experiments.

Table 2. *Subcellular distribution of β-carotene-15,15′-dioxygenase* (EC *1.13.11.21*) *in extracts of guinea-pig intestinal mucosa**

(Values are means with their standard errors from three separate experiments)

Fraction (nmol [^{14}C]retinaldehyde/h)		Composition	Specific activity (nmol [^{14}C]retinaldehyde /mg protein per h)		Total activity (nmol [^{14}C]retinaldehyde/h)
			Mean	SE	
30000 *g*, 30 min: Supernatant		Soluble protein lipoproteins, aggregates and microsomes	0·08	0·03	30
Pellet		Nuclei, membranes, mitochondria	0·02	0·01	6
104000 *g*, 60 min: Supernatant		Soluble protein and lipoprotein aggregates	0·08	0·03	25
Pellet		Microsomes	0·06	0·01	4
Pellet mixed with its supernatant			0·08	0·02	29
Supernatant with phospholipids added			0·05	0·01	12
Supernatant acid precipitated at pH 5·2 recentrifuged:	Supernatant	Soluble proteins	0·07	0·02	16
	Pellet	Lipoprotein aggregates	0·08	0·02	6
Sepharose CL-6B					
Void volume		Insoluble proteins	—	—	—
Included volume		Soluble proteins	0·19	0·05	19

* All fractions were assayed using the conditions of Fidge *et al.* (1969) except the Sepharose CL-6B fractions which were assayed with only 1 mg protein and no phospholipids were added.

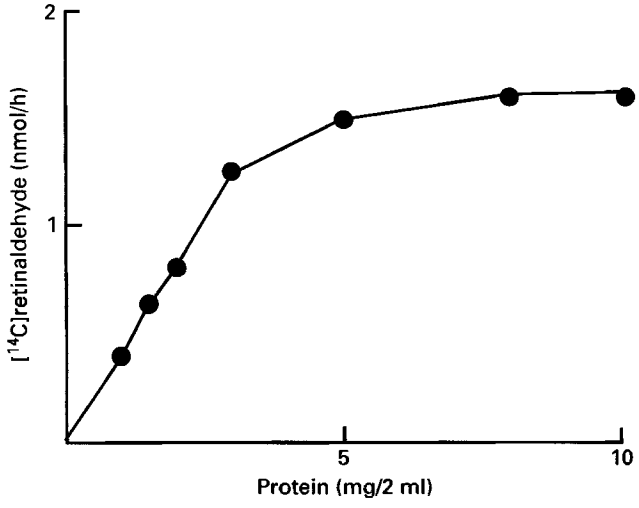

Fig. 1. The effect of protein concentration on the conversion of β-carotene into retinaldehyde. The assay system was that described by Fidge *et al.* (1969) except that the guinea-pig 104000 ***g*** supernatant fraction (2·0 mg protein) was prepared with buffer–scrapings (4:1, w/v) and [15,15′-^{14}C]β-carotene (1·0 μg) was dispersed in Tween 80. The reaction mixture was extracted by the Sep-Pak procedure and the components of the total organic extract were separated by TLC. The mean recovery was 80 (SE 12)% and the results are the means of duplicates from a single experiment. This experiment was carried out three times and gave similar results. For further details of procedures, see pp. 399–400.

Table 2 also indicates that dioxygenase activity was not substantially precipitated by acidification and that the enzyme activity was present in the included volume of a Sepharose CL-6B column. These findings indicate that the guinea-pig β-carotene dioxygenase, as described, is a soluble enzyme. The results in Table 2 show that most of the β-carotene dioxygenase activity is present in the 104000 ***g*** supernatant fraction and that there was no stimulation by the addition of the microsomal pellet or a phospholipid mixture so neither of these materials was added in later experiments. The effects of varying substrate, bile and protein concentrations and the duration of incubation were tested using the ultracentrifuged supernatant fraction as a source of β-carotene dioxygenase. The results in Fig. 1 show that the reaction rate was directly proportional to the amount of protein in the range 0–3 mg/incubation mixture (2 ml) when this fraction was prepared from a homogenate using the defined proportions (4:1) of buffer–scrapings. This preparation was more active than those reported previously (Goodman *et al.* 1966; Fidge & Goodman, 1969; Singh & Cama, 1974; Sklan, 1983*b*) where between 5 and 15 mg protein from an ultracentrifuged supernatant fraction was required to give a measurable reaction. Reaction rate was found to be approximately proportional to the amount of β-carotene in the range 0–0·6 μg/2 ml (Fig. 2). The optimum sodium glycocholate concentration was found to be 6 μmol/ml; both these values agree with those of Goodman *et al.* (1967) and Fidge *et al.* (1969). However, the reaction rate was almost constant over 90 min (Fig. 3); whereas activity decreased with time in earlier reported work.

Purification of β-carotene dioxygenase

Purification of the guinea-pig β-carotene dioxygenase was attempted using the methods described by Sklan (1983*b*) and Fidge *et al.* (1969). The results in Table 3 show that the procedures gave little purification of the enzyme. Losses of activity occurred during concentration by ultrafiltration or $(NH_4)_2SO_4$ (0–600 g/l) precipitation as well as during

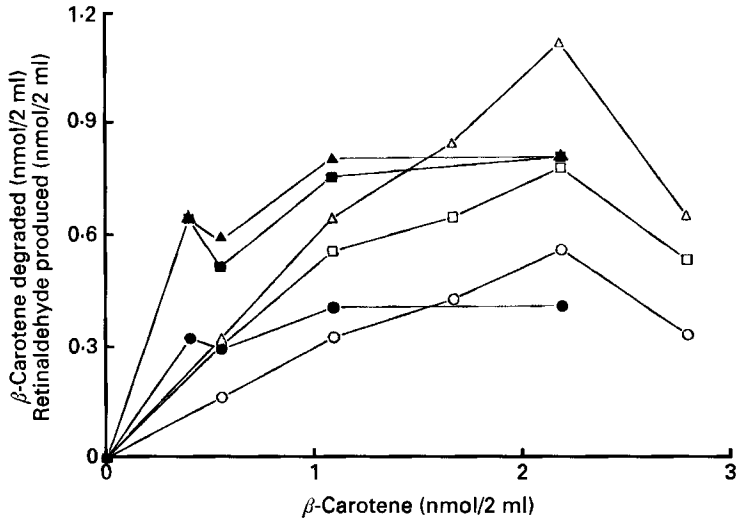

Fig. 2. Effect of concentration of β-carotene on yield of retinaldehyde. Incubation conditions were those described in Fig. 1 except that [15,15′-^{14}C]β-carotene was used at the concentrations shown. Each point represents the mean of duplicates from a single experiment. Values were calculated as follows, using 0·56 nmol carotene as an example. To both the test assay and a boiled enzyme control was added 0·56 nmol β-carotene (26310 disintegrations/min (dpm)). The total extracted radioactivity at the end of the incubation was 71% of this value for the control and 72% for the test sample. The amount of β-carotene degraded in the test sample was calculated by subtracting the amount of radioactivity in the β-carotene fraction of the test sample (uncorrected value 7042 dpm, corrected to 100% recovery as 9780 dpm) from that in the corresponding fraction of the control (uncorrected value 12399 dpm, corrected to 100% recovery as 17463 dpm), i.e 17463 − 9780 = 7683 dpm which is equivalent to 0·17 nmol β-carotene. The amount of retinaldehyde formed was calculated by subtracting the amount of radioactivity in the retinaldehyde fraction in the boiled control (uncorrected value 1754 dpm, corrected to 100% extraction as 2470 dpm from that in the corresponding fraction in the test solution (uncorrected value 6847 dpm, corrected to 100% extraction as 9509 dpm), i.e. 9509 − 2470 = 7039 dpm, which is equivalent to 0·30 nmol retinaldehyde. Boiled enzyme controls were made up with 0·56 and 2·8 nmol β-carotene. Test samples containing intermediate amounts of β-carotene were calculated using the mean of boiled controls. Theoretical, expected values for retinaldehyde were calculated from the amounts of β-carotene degraded on the basis of 2 mol retinaldehyde per mol β-carotene. (○, □, △) and (●, ■, ▲), represent data from two experiments respectively. (○, ●), β-carotene degraded; (□, ■), retinaldehyde formed; (△, ▲), theoretical amounts of retinaldehyde calculated from the amounts of β-carotene degraded, on the basis of 2 mol retinaldehyde per mol β-carotene.

dialysis. The inclusion of protease inhibitors PMSF, TLCK and the reducing agent mercaptoethanol in all buffers did not reduce these losses.

When the fat-free residual fraction was incubated with 15,15′-$^{3}H_2$]β-carotene (5 μg), as described by Sklan (1983*b*), the preparation concentrated, and then chromatographed on a Sepharose CL-6B column, over 90% of the radioactivity was found in the void volume corresponding to the elution volume of dextran blue (molecular weight 2×10^6 Da). This distribution of radioactivity agrees closely with that described by (Sklan, 1983*b*). However, when the protein peaks were assayed no β-carotene dioxygenase activity was found in the void volume (Fig. 4). The enzyme activity was in the first major protein peak following the void volume and the specific activity of the β-carotene dioxygenase had increased 1·5–2-fold. This differs markedly from the result reported by Sklan (1983*b*) where enzyme activity was found in the void volume and an apparent purification of 20-fold was reported. (However, Sklan (1983*b*) cites values as g retinaldehyde/h per ng protein, obviously a misprint; it was assumed that this should have been ng retinaldehyde/mg protein per h.) When the active zone from a Sepharose CL-6B column was dialysed overnight, as described by Fidge *et al.* (1969), a loss of 20% of total protein occurred and enzyme activity was

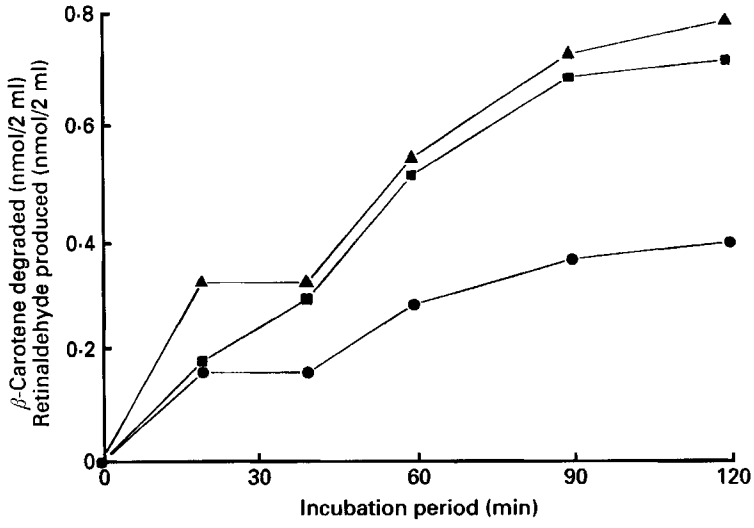

Fig. 3. Effect of incubation time on β-carotene conversion into retinaldehyde. The incubation conditions were those described by Fidge *et al.* (1969) except that 1·5 mg protein from the guinea-pig 104000 *g* supernatant fraction and [15,15′-^{3}H]β-carotene (0·3 μg) were used. The assay mixture was extracted by the Sep-Pak procedure and the components of the total organic extract separated by TLC. All data shown were corrected to 100% recovery. (●), Amount of [15,15′-^{3}H]β-carotene degraded, calculated as described on p. 405, except a 60 min boiled control was used; (■), observed production of [15-^{3}H] retinaldehyde; (▲), expected production of retinaldehyde. The amounts of retinaldehyde expected were calculated from the amounts of β-carotene consumed on the basis of 2 mol retinaldehyde per mol β-carotene. For further details of procedures, see pp. 398–401.

Table 3. *Summary of purification procedures for guinea-pig β-carotene-15,15′-dioxygenase.* (EC *1.13.11.21*)*

(Values are means with their standard errors from three separate experiments)

Fraction	Specific activity (nmol [^{14}C]retinaldehyde /mg protein per h)		Total activity (nmol [^{14}C]retinaldehyde/h)
	Mean	SE	
Cytosol	0·38	0·10	56·5
Ultrafiltration concentrate	0·37	0·09	44·5
Sepharose CL-6B (pooled active fractions)	0·60	0·05	28·0
Dialysate	0·30	0·03	14·0
DEAE Sepharose (pooled active fractions)	0·60	0·05	10·0

* All fractions assayed as described by Fidge *et al.* (1969) except that 1–2 mg protein, 0·6–1·2 μg [15,15′-$^3H_2$]β-carotene were used and no phospholipids were added.

reduced by 50%. When the dialysed sample was applied to the DEAE Sepharose column little enzymic activity was found in the void volume. The enzyme activity eluted in the first major peak after the salt gradient was started and a 2-fold purification was obtained. These results agree with those of Fidge *et al.* (1969), who report a 3-fold purification using similar ion-exchange chromatography. These results also indicate that the guinea-pig β-carotene dioxygenase is a somewhat unstable enzyme.

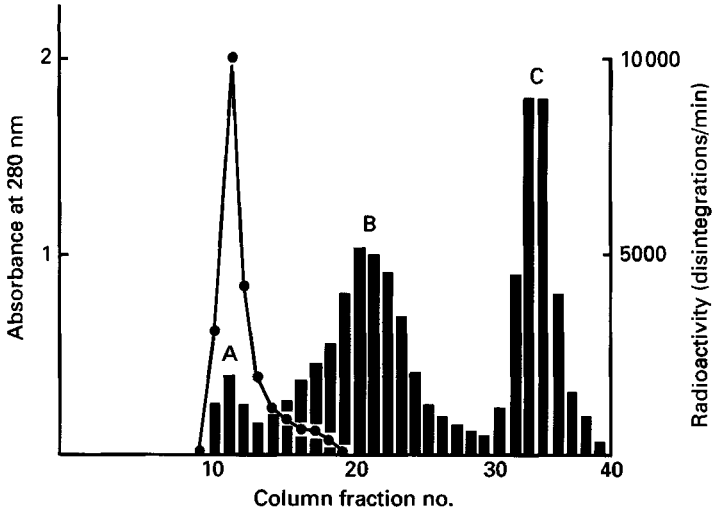

Fig. 4. Elution of a guinea-pig mucosal enzyme preparation from a Sepharose CL-6B column. The redissolved, dialysed $(NH_4)_2SO_4$ pellet (100 mg protein) was stirred with [15,15′-^{3}H]β-carotene (5 μg added as a dispersion in Tween 80) at 4° for 60 min, pH 7·8 before chromatography. The ^{3}H-labelled β-carotene recovered (●) was measured in each fraction for the absorption of the protein in each fraction (■) was monitored at 280 nm. Combined fractions were assayed for β-carotene dioxygenase (*EC* 1.13.11.21) activity as: (A), fractions 10–14; (B), fractions 15–29; (C), fractions 30–40. All [^{3}H]β-carotene appeared in the void volume fraction. β-Carotene dioxygenase activity was confined to fractions 15–29 (B), which exhibited a rate of formation of 23·2 nmol retinaldehyde/h. Total activity of the protein before chromatography was 29 nmol retinaldehyde formed/h. For further details of procedures, see pp. 398–401.

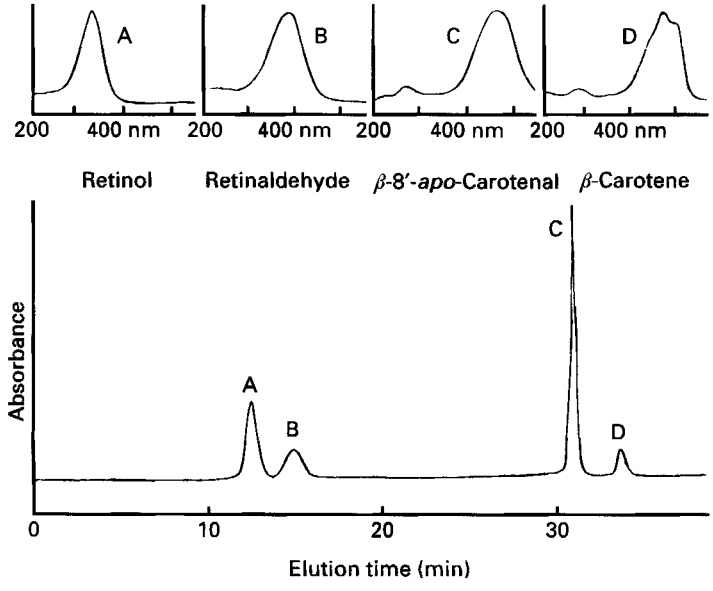

Fig. 5. Chromatogram showing separation of standard (approximately 10–20 μg) retinol (12·5 min), retinaldehyde (15 min), β-8′-*apo*-carotenal (31 min) and β-carotene (34 min) using reverse-phase HPLC. the chromatographic conditions are described on pp. 400–401. The absorbance was recorded at 325 nm with a band-width of 4 nm using 580 nm as a reference for the initial 30 min. The remainder of the chromatogram was recorded at 450 nm with a bandwidth of 4 nm and a reference of 580 nm.

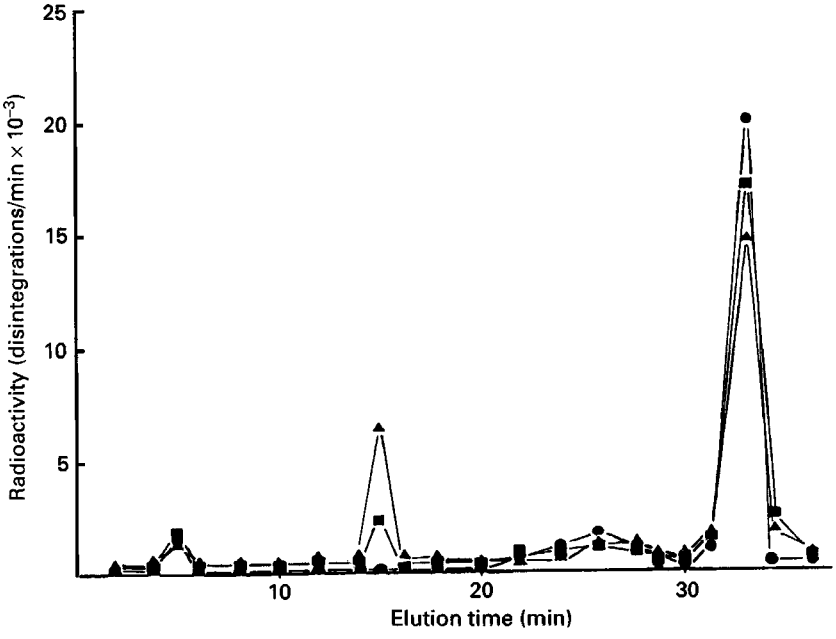

Fig. 6. Elution profile of the recovered radioactivity of the β-carotene-15,15′-dioxygenase (*EC* 1.13.11.21; β-carotene dioxygenase) assay components which were extracted with and without the addition of unlabelled standards. The assay profile corresponds to a 30% subsample of a Sep-Pak-extracted β-carotene dioxygenase assay. The extracted components were separated by HPLC. The test assay was that of Fidge *et al.* (1969) except that the guinea-pig 104000 *g* supernatant fraction (2 mg protein) and 0·6 μg [15,15′-^{3}H]β-carotene were used. (●) Boiled enzyme control; (▲), unlabelled carriers added, (■), no carrier added. For further details of procedures, see pp. 400–401.

β-carotene dioxygenase reaction mechanism

The HPLC trace from the diode-array detector is shown in Fig. 5. This system provides baseline separation of retinol and retinaldehyde and also completely separates β-8′-*apo*-carotenal and β-carotene and it was used to investigate the products of the β-carotene dioxygenase reaction. The elution profile of the test assays, which were incubated for 60 min (Fig. 6), correspond to the profiles of the controls (boiled enzyme) except for a peak radioactivity at elution time 14–16 min (retinaldehyde) which was absent from the control. A similar profile was obtained for assays extracted without the addition of unlabelled standards except that the (retinaldehyde) peak contained only 2000 disintegrations/min (dpm) as opposed to 6000 dpm in the presence of unlabelled retinaldehyde.

This HPLC retinaldehyde fraction also was then reduced as described by Goodman & Olson (1967). The product was subjected to HPLC, whereupon most of the radioactivity emerged as a single peak at 12–14 min (identical with that for standard retinol). In two experiments (with and without the addition of unlabelled β-carotene and retinaldehyde) there was no radioactive compound between retinaldehyde (retention time 15 min) and β-carotene, indeed the background counts in this zone were lower than those for the control sample obtained after incubation with boiled enzyme for 60 min. There was no formation of labelled retinaldehyde in the control sample nor was there any formation of labelled *apo*-carotenals running between retinaldehyde and β-8′-*apo*-carotenal (retention time 31 min; Fig. 5) where *apo*-carotenals of between C_{20}–C_{30} would be expected. *Apo*-carotenals of less than C_{20} would not be detected, of course, as the ^{14}C and ^{3}H atoms were at the centre of the molecule. The formation of retinaldehyde as the only labelled product indicates that

β-carotene dioxygenase cleaves the substrate centrally. It should be noted that due to the problems of overloading HPLC columns, Fig. 6 is a chromatogram of sub-samples of assay extracts and represents the formation of retinaldehyde and the recovery of the added β-carotene (not the amount of β-carotene consumed).

DISCUSSION

Chemical identification of carotene-cleavage products

When the retinaldehyde fraction from an incubation (without added unlabelled retinaldehyde) was analysed by HPLC using a C_{18} reversed-phase column, the retention time of the labelled material was identical with that of a sample of authentic material. Furthermore, the u.v. spectrum detected by a diode-array detector coupled in series with the HPLC column was identical with that of authentic material. The retinaldehyde was reduced to retinol by the method of Goodman & Olson (1967) and, on rechromatography by HPLC, it had the same retention time and u.v. spectrum as authentic retinol. Retinaldehyde formed from β-carotene by mucosal enzymes has also been characterized as a semi-carbazone derivative (Goodman & Huang, 1965) and as an O-ethyloxime (Lakshmanan *et al.* 1989).

Isolation and partial purification of β-carotene-15,15′-dioxygenase

It can be seen from Table 1 that the guinea-pig tissue extracted by our modified procedure gives a more active preparation than any previously reported preparation. The properties of the enzyme were similar to those reported by Goodman *et al.* (1966, 1967) and Fidge *et al.* (1969), except 2 mg protein from a homogenate of guinea-pig mucosa is required to give measurable enzyme activity and phospholipids were inhibitory rather than stimulatory (Table 2). They concluded that β-carotene dioxygenase was a soluble enzyme but Sklan (1983*b*) challenged this and reported that activity was associated with a high-molecular-weight lipid–protein aggregate. The experiments described here conflict with Sklan's (1983*b*) report and are in agreement with the earlier findings that β-carotene dioxygenase is soluble.

Sklan (1983*b*) incubated [15,15′-$^3H_2$]β-carotene with an enzyme preparation from chicken gut and the mixture was then applied to a Sepharose 6B column. Over 90% of the radioactivity and the enzyme activity eluted at the void volume. We repeated Sklan's (1983*b*) procedure, but with a guinea-pig cytosol fraction extracted at 1 vol. mucosa:4 vol buffer and concentrated by ultrafiltration or $(NH_4)_2SO_4$ precipitation; 90% of the labelled β-carotene eluted in the void volume, as found by Sklan (1983*b*). However, this fraction contained no β-carotene dioxygenase activity, which was found within the included volume. These results indicate that the β-carotene was dissolved in an insoluble lipoprotein aggregate while the β-carotene dioxygenase was soluble, well separated from the aggregate and was found in the column's included volume (Fig. 4).

It should be noted that Sklan (1983*b*) prepared tissue homogenates by adding 1 vol. buffer per unit weight of tissue while we used 4 vol. buffer per unit weight of scraped mucosa. The small proportion of buffer may favour binding of the dioxygenase to lipid–protein aggregates. Sklan (1983*b*) suggested that the aggregate he isolated could be a specific vitamin A complex but did acknowledge it could be associated with the lipid–protein aggregate in both rat liver (Sklan *et al.* 1982) and chick intestinal mucosa. When investigating retinyl palmitate esterase (*EC* 3.1.1.21) activity in rat liver, Prystowsky *et al.* (1981) and Harrison *et al.* (1979) noted an unusual distribution; retinyl palmitate esterase activity appeared in the void volume of a Sepharose 6B column except when sodium cholate had been added. Retinyl palmitate esterase activity eluted as a single peak within

the included volume when cholate was present. They interpreted these observations as the adsorption of the enzyme to membraneous components and Prystowsky *et al.* (1981) suggested that the formation of complexes or aggregates could occur after homogenization because of the hydrophobic properties of the enzymes.

Although β-carotene dioxygenase is considered to be a soluble enzyme its purification by conventional procedures for soluble enzymes has been largely unsuccessful. Goodman *et al.* (1967), Fidge *et al.* (1969) and Singh & Cama (1974) have all reported difficulties in purifying the enzyme by column chromatographic techniques. The intractability of β-carotene dioxygenase and its stimulation by bile salts indicates that it may be amphiphilic. This would tend to make it unstable in aqueous solutions and difficult to purify by techniques appropriate for soluble enzymes (Renswoude & Kempf, 1984). However, the possible direct stimulatory effects of bile salts or detergent on enzyme activity cannot be separated from the simultaneous effects on the solubilization of the highly lipophilic substrate. Although the enzyme can be found in the soluble fraction it may occur in the cell loosely bound to a membrane (Hjelmeland & Chrambach, 1984) from which it is displaced during isolation. These observations were also noted by Goodman *et al.* (1967) who suggested that the β-carotene cleavage reaction takes place *in vivo* at an extracellular, membraneous interface, despite the fact that the enzyme involved is a soluble protein. The difficulty in isolating and purifying an active preparation of this enzyme may partially explain the lack of activity as described by Hansen & Maret (1988), who performed experiments with a dialysed $(NH_4)_2SO_4$ preparation but no mention is made of the enzyme activity in the starting material.

Carotene cleavage reaction mechanisms: central cleavage v. *random cleavage*

The cleavage of β-carotene by the dioxygenase has been accepted to occur at the central, 15,15′-double bond with the formation of two molecules of retinaldehyde because the latter compound is the only product which has been identified. Similarly, the maintenance of the same ^{14}C:^{3}H ratio in the retinaldehyde as that in the original [15,15′-^{14}C,^{3}H]β-carotene also excludes a conventional β-oxidation mechanism as the cleavage between alternate C atoms of the polyene chain would be expected to leave a CoA thioester at C-15 which would produce a molecule without ^{3}H at what had been C-15 of the β-carotene if it were then converted into retinaldehyde. As noted earlier, Goodman *et al.* (1966) found no loss of ^{3}H relative to ^{14}C during the formation of retinol from [15,15′-$^{14}C_2$,$^{3}H_2$]β-carotene.

Ganguly & Sastry (1985) challenged the established view by claiming the evidence favoured a random cleavage mechanism in which retinaldehyde would be only one possible product in a series of *apo*-carotenals. They marshalled four lines of evidence to support this proposal: biopotency studies, imperfect stoichiometry, the presence of *apo*-carotenals in animal extracts and the reported ability of the β-carotene dioxygenase to cleave *apo*-carotenals (Lakshmanan *et al.* 1972; Sharma *et al.* 1977). Hansen & Maret (1988) identified three products as 8′-, 10′- and 12′-*apo*-carotenals in a preparation from rat intestine but these compounds were also formed in blank samples without enzyme.

In the experiments reported here an active preparation of β-carotene dioxygenase was incubated with [15,15′-$^{3}H_2$]β-carotene and the products analysed by HPLC in an attempt to detect labelled products other than retinaldehyde. The largest labelled product that can be produced by a random cleavage process is 8′-*apo*-carotenal, while retinaldehyde (15-*apo*-carotenal) is the smallest. These eluted at 15 and 30 min from the HPLC column; thus, any other *apo*-carotenals of intermediate size would be expected to chromatograph between these times (Fig. 6).

This experiment was repeated three times and retinaldehyde was the only labelled product. Another set of assays was extracted without the addition of unlabelled

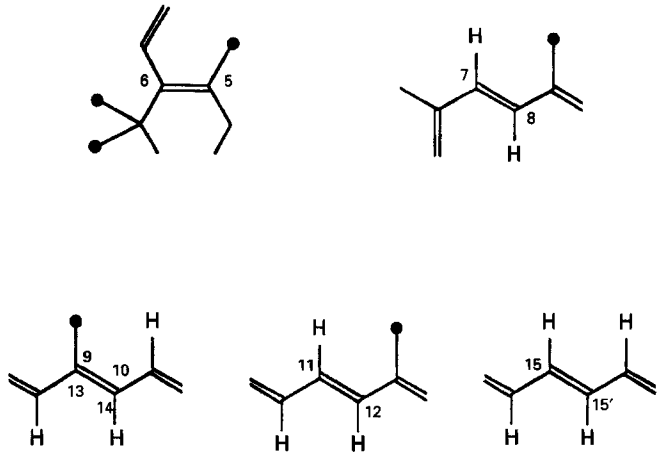

Fig. 7. The environment of substituent groups adjacent to the various double bonds of β-carotene. The 15,15′-double bond is unique in having no methyl group (●) on its C atoms or an adjacent C atom.

retinaldehyde and β-carotene because small quantities of unlabelled β-*apo*-carotenals could suffer losses while labelled retinaldehyde would be protected by the added carrier. Again, no *apo*-carotenals were found although traces of other breakdown products from β-carotene were detected, but an identical pattern was seen in controls with boiled enzyme. The labelled retinaldehyde collected after HPLC was completely reduced with borohydride and retinol was the only labelled product. These results, and the ratio, 1 mol β-carotene degraded: approximately 2 mol retinaldehyde produced (Figs. 2 and 3), are consistent with the central cleavage mechanism of β-carotene to retinaldehyde and exclude the possibility of random cleavage. Asymmetric cleavage of β-carotene gives a maximum of one molecule of retinaldehyde per molecule of β-carotene. Central cleavage will yield two molecules of retinaldehyde. Consequently, any demonstration of a retinaldehyde:β-carotene ratio satisfactorily greater than 1 is indicative of central cleavage while a ratio of 2 excludes anything but central cleavage. The specificity of the dioxygenase for the central 15,15′-double bond is entirely understandable in terms of the ability of the enzyme to interact with the unique environment of functional groups at this site position (Fig. 7).

Oxygen atom at C-15

Experiments with $^{18}O_2$ showed that O_2 from air, but not from water, is incorporated into the terminal alcohol group of retinol *in vivo* (Vartapetian *et al.* 1966, cited by Olson, 1968). The argument for this is the requirement by dioxygenases for O_2 while the O atom inserted by hydration of a double bond is derived from water. Hence, any *apo*-carotenal of more than C_{20} formed from β-carotene and cut down to the C_{20} retinaldehyde by a β-oxidation type of mechanism would not carry ^{18}O derived from $^{18}O_2$ but would carry ^{18}O derived from $H_2{}^{18}O$.

These results with ^{18}O do not exclude the possibility of cleavage of the β-carotene elsewhere in the chain followed by cleavage at the 15,15′-double bond. Olson (1989) pointed out that enzymic cleavages of carotenoids other than at the central 15,15′-double bond undoubtedly occurs in plants and micro-organisms but there is no unambigous evidence of this activity in mammals. The reported ability of the β-carotene dioxygenase to cleave $> C_{20}$ *apo*-carotenals (Lakshmanan *et al.* 1972; Singh & Cama, 1974; Sharma *et al.* 1977) to yield retinaldehyde is entirely understandable. The pattern of substituents around the 15,15′-double bond is unique in β-carotene (Fig. 7) and so the β-carotene dioxygenase

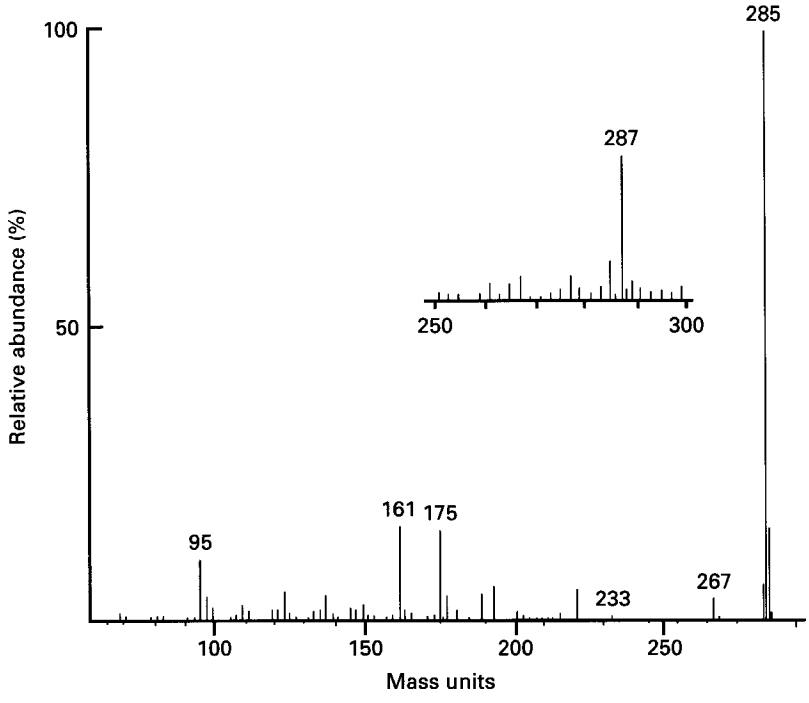

Fig. 8. Mass spectrum (positive ion, CH_4 chemical ionization, sample on probe) of synthetic retinaldehyde (56384 ion events). Also shown is the parent ion region of a similar sample after incubation for 14 d in $H_2{}^{18}O$ (98 atoms%, 24640 ion events).

would be expected to recognize this structural pattern when it occurs in β-carotene or in a large *apo*-carotenal.

We have found that most of the O of retinaldehyde exchanges with water during 14 d in $H_2{}^{18}O$ (Fig. 8). The hydroxyl atom of retinol using identical conditions does not exchange. The presence of ^{18}O in the retinol extracted from rats breathing $^{18}O_2$ (Vartapetian *et al.* 1966) indicates the dwell time for retinaldehyde must have been relatively short so that the ^{18}O was retained until the molecule was reduced to retinol. Similarly, in the reciprocal experiment cited by Vartapetian *et al.* (1966) there was, presumably, insufficient time for the retinaldehyde to take up ^{18}O by exchange from the $H_2{}^{18}O$ in the rats before it was converted into retinol. The absence of ^{18}O from $H_2{}^{18}O$ in the retinol also supports the involvement of a dioxygenase mechanism *in vivo* which is able to incorporate molecular O into retinaldehyde.

In summary, due to the unstable chemical nature of β-carotene and retinaldehyde as well as the fragility of β-carotene dioxygenase, experiments to demonstrate the enzymic conversion of β-carotene into retinaldehyde are technically difficult and require a great deal of care. These studies using a very active preparation of β-carotene dioxygenase support the hypothesis that this enzyme cleaves the 15,15′ central double bond of β-carotene. Previous biopotency studies and the ability of β-carotene dioxygenase to cleave *apo*-carotenals to yield retinaldehyde are also in accord with the hypothesis that these molecules are cleaved at the C-15 double bond.

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