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Oxidative Degradation of β-Carotene and β-Apo-8'-carotenal

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Abstract. In the self-initiated oxidation of β-carotene with molecular oxygen the rate of oxygen uptake was shown to depend on the oxygen partial pressure Epoxides, dihydrofurans, carbonyl compounds, carbon dioxide, oligomeric material, traces of alcohols, and probably carboxylic acids were formed. The main products in the early stages of the oxidation were shown to be 5,6-epoxy-β-carotene, 15,15'-epoxy-β-carotene, diepoxides, and a series of β-apo-carotenals and -carotenones As the oxidation proceeded uncharacterised oligomeric material and the carbonyl compounds became more important and the epoxides degraded In the final phase of the oxidation the longer chain β-apo-carotenals were themselves oxidized to shorter chain carbonyl compounds, particularly β-apo-13-carotenone, β-ionone, 5,6-epoxy-β-ionone, dihydroactinidiolide and probably carboxylic acids. The effect of iron, copper and zinc stearates on the product composition and proportions was studied, as was the effect of light The oxidation was inhibited by 2,6-di-*t*-butyl-4-methylphenol and α-tocopherol The oxidations of β-apo-8'-carotenal and retinal under similar conditions were studied briefly, and the main products from the former compound were characterized The initiation, the formation of the epoxides, the β-apo-carotenals and -carotenones, the successive chain shortening of the aldehydes to the ketones, and the formation of dihydroactinidiolide are explained in terms of free radical peroxidation chemistry

INTRODUCTION

Interest in the oxidation of β-carotene (pro-vitamin A) has been rekindled by a number of recent discoveries First, β-carotene oxidations in tissue homogenates were found to yield a range of β-apo-carotenals and β-apo-carotenones from scission of the carotene chain at many sites[1,2,3] It had previously been generally supposed that β-carotene oxidation under "biological conditions" was enzyme-mediated and resulted solely in scission of the 15,15' bond to produce two molecules of retinal (vitamin A) Clearly, these results have far-reaching implications regarding the true mechanism by which vitamin A is formed from β-carotene *in vivo* Second, β-carotene[4,5,6], retinol[7] and various synthetic retinoids[7] have been shown to retard the development of certain experimentally induced animal tumours Third, there is epidemiological evidence that blood retinol levels and carotenoid intake correlate inversely with the incidence of certain human cancers[7] These discoveries immediately raise a very important question Is the anticancer action of carotenoids due to some action of the carotenoids themselves *or to some action of one or more of their oxidation products*? To answer this question properly a necessary first step is to identify all the products of carotenoid oxidation With this idea in mind, we have embarked on a program to characterize the entire slate of products formed in the non-enzymatic autoxidation of β-carotene We have also attempted to establish the mechanism(s) of product formation, the effects of reaction conditions, and the effect of potential pro- and antioxidants on the rate of

reaction and the distribution of the products (In connection with the last it is interesting to note that β-carotene can become an antioxidant at the low oxygen partial pressures found in most tissues[8])

Early work on β-carotene autoxidation indicated that epoxides were produced[9,10]. The "heat treatment" of β-carotene both in the presence and in the (purported) absence of oxygen yielded β-apo-carotenals and β-apo-carotenones, together with epoxides[11,12,13,14,15,16] This work has recently been summarized[17]. The more recent application of high performance liquid chromatography (HPLC) to the analysis of the oxidation products of β-carotene has provided a fairly detailed picture of their composition Thus, Krinsky and co-workers[17] have identified β-apo-carotenals and epoxides while Liebler and Kennedy[18] have identified both 5,6- and 15,15'-epoxy-β-carotene In the present paper we describe in more detail than heretofore[19] our studies on the products formed during the autoxidation of β-carotene under a variety of experimental conditions These products were identified by a combination of analytical procedures (FT-IR, HPLC, CG-MS) and by comparisons wherever possible with authentic materials

RESULTS

Oxygen Uptake, β-Carotene Consumption and Carbonyl Compound Formation

β-Carotene oxidations were carried out in two principal ways, (i) by dissolving ca. 10 mg of purified substrate in 2 ml of tetrachloromethane and continuously shaking in the dark under one atmosphere of oxygen, which was periodically renewed in an otherwise closed system and (ii) by continuously bubbling oxygen through a benzene solution of similar concentration also in the dark In the sealed system experiments, the oxygen uptake was measured with a pressure transducer[20] Small samples were withdrawn from time to time and FT-IR spectra were obtained Oxygen uptake became measurable after a short induction period, of usually less than 1 h The rates of oxygen uptake, $d[O_2]/dt$, at different times during the oxidation, are given for a typical run in Table 1 These rates increased with reaction time for about the first 20 h of oxidation and then decreased to zero uptake by *ca* 50 h reaction The ratio of the number of moles of oxygen consumed per mole of initial β-carotene was found to be 6 ± 1 When air was used as the oxidant the rates of oxygen uptake were always lower than with 760 Torr of oxygen and, at the maximum, the ratio of the $d[O_2]/dt$ value under air to that under oxygen was 0 47 The timescale of the oxidation with air was consequently increased

The FT-IR spectrum of β-carotene in CCl_4 showed a sharp absorption at 966 cm^{-1}, due to the C-H out of plane bending of *trans* double bonds This was integrated to monitor the β-carotene consumption Similar absorption bands were present in some of the products, so this method underestimated the rate of disappearance of β-carotene During the oxidation this peak steadily decreased in intensity, complex changes occurred in the 1000-1500 cm^{-1} region, and a set of overlapping absorptions developed in the carbonyl region (1650-1800 cm^{-1}) An estimate of the total growth in carbonyl compounds was made by comparing the integrated total carbonyl signal with that from known amounts of retinal (Table 1) The concentration of carbonyl compounds in the final oxidation mixture (48h) was about twice the initial β-carotene concentration, i e *ca* two carbonyl groups were produced (together with non-carbonyl compounds) for each molecule of β-carotene oxidized The β-carotene was entirely consumed in $\leq$ 30 h, but oxygen uptake continued for *ca* 15 h further After oxygen uptake had ceased the carbonyl absorption continued to rise for about another 10 h In a separate experiment the final mixture, after 48 h oxidation, *i e* after cessation of oxygen uptake, was completely freed of oxygen by degassing on a high vacuum line, sealed under vacuum, and heated at 80°C for 24 h

Table 1 Autoxidation of β-Carotene at 30°C in CCl_4; Oxygen Uptake, β-Carotene Consumption and Carbonyl Group Formation

Time/ h	10^5[β-carotene]/[a] mol	10^5[carbonyl][b]/ mol	$10^{10}d[O_2]/dt$ mol s^{-1} [mol β-carotene[c]]$^{-1}$
0 00	2 09	0 00	--
1 25	2 09	0 14	0 20
2 75	1 90	0 17	0 28
5 58	1 77	0 75	0 39
7 75	1 57	1 00	0 44
17 75	0 75	2 88	0 46
29 75	0 15	4 33	n d [d]
48 0	0 00	n d	0 0
77[e]	0 00	6 28	--

[a] Approximation only, see text. [b] Total carbonyl estimated from the integral of the 1650-1800 cm^{-1} region compared to retinal, see text [c] Initial concentration of β-carotene [d] n d. = not determined [e] After heating the deoxygenated sample at 80°C for 24 h.

The FT-IR of this heated mixture showed a 45% increase in the carbonyl absorption, compared to the spectrum prior to heat treatment This demonstrated that carbonyl compounds could also be formed by decomposition of primary products, even in the absence of oxygen Before heat treatment the 48 h oxidation mixture tested positive for peroxides with the N,N-dimethyl-*p*-phenylenediamine reagent

The FT-IR spectra also showed the development of a sharp absorption band at 2364 cm^{-1} due to carbon dioxide The formation of CO_2 could not be quantified in this way because its escape was not controlled during the oxidation and sampling However, CO_2 production was more important in the later stages of the oxidation and the most pronounced increase occurred during the heat treatment described above After about 6 h oxidation a broad weak absorption, centred at *ca* 3500 cm^{-1}, became evident This probably signified the production of minor amounts of alcohols/enols and/or carboxylic acids After heat treatment, a very broad band in the 3500-2600 cm^{-1} region was observed This region of the product spectrum was similar to that from retinoic acid, *i e* heat treatment initiated, or accelerated, carboxylic acid formation Oxidations were also carried out at 45 °C With pure oxygen the maximum rate of O_2 uptake was 1 5 x 10^{-10} mol s^{-1} mol(β-carotene)$^{-1}$, *i e* 3 3 times the maximum rate at 30 °C The FT-IR spectra and HPLC chromatograms showed the development of the same products in the 45° C oxidations as in the 30° C oxidations, but at a faster rate

Product Characterization

The β-carotene oxidations gave soluble materials with molecular weights less than ca 600, plus some polymeric/oligomeric material which frequently deposited out of solution, particularly in the later stages of oxidation The polymer/oligomer is currently under investigation and will be reported on in a future article

The soluble materials were separated by both normal and reverse phase HPLC Reverse phase conditions with a Spherisorb ODS2 5μ column were found to be best with a quaternary solvent system of $H_2O/CH_3OH/CH_3CN/CH_2Cl_2$ Figure 1 shows the chromatogram of the products in the early stages (6h) of the oxidation at 30 °C Essentially the same range of products was obtained with dioxygen or air as the oxidant. UV-Vis spectra in the range 210-600 nm were obtained for all the major products The mixtures were also examined at various stages of the oxidation by coupled LC-MS using the same column and solvent system The total ion current (TIC) chromatograms were similar to those obtained in the analytical HPLC (except that peak intensities differed) and the two sets of results were easily correlated Much better resolution of the long retention time (R_t), non-polar components was achieved with the analytical HPLC

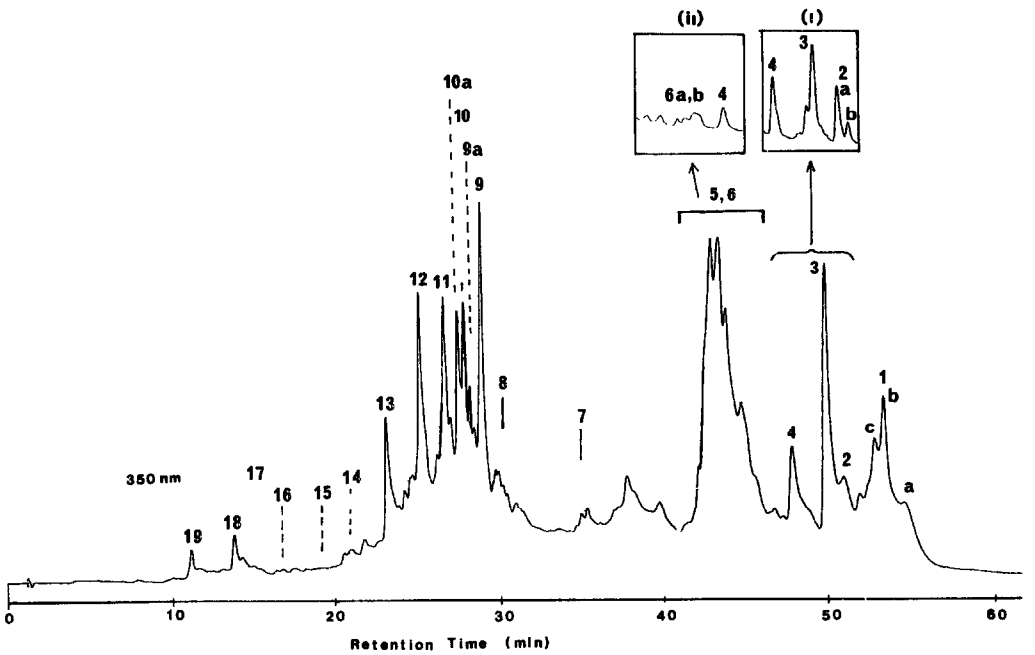
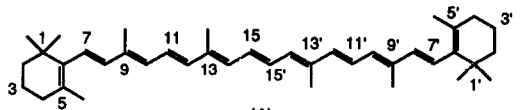


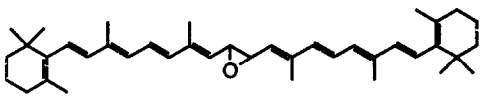
Figure 1 HPLC chromatogram of the oxidation products from β-carotene after 6 h reaction in benzene The column was an ODS2 5μ Spherisorb type with a quaternary solvent system (see text) Note that the peak numbers correspond to the structure numbers Inset (i) shows peaks 2 to 4 under higher resolution Inset (ii) shows the group of peaks 4 to 6 after 24 h oxidation

The longest R_t peaks, 1a-c, were unreacted β-carotenes The central component, 1b, was shown to be *trans*-β-carotene (**1**) by comparison of its R_t with authentic material These peaks were not resolved on the LC-MS chromatogram, but the MS showed only the β-carotene molecular ion across the whole broad peak The UV spectra of 1a, 1b and 1c were very similar and all showed the band at 340 nm which is characteristic of *cis*-carotenes[21] It is probable therefore that the shoulders 1a and 1c correspond to *cis*-isomers of β-carotene The fact that 1b also showed the 340 nm band can be attributed to the extensive peak overlap which led to spectral mixing Peaks 2 and 3 (Figure 1) were also not resolved on the LC-MS chromatogram which showed only the molecular ion for epoxy-β-carotene in this region Better resolution of this sector of the chromatogram was achieved with a larger diameter column (with the same ODS2 packing) (see Figure 1, inset

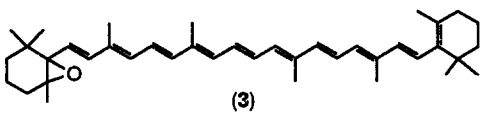
(i)) The main component, peak 3, was shown to be 5,6-epoxy-β-carotene, (**3**), by comparison of its R_t and UV spectrum with authentic material The minor peaks, 2a, b had the same UV maximum (330 nm) as reported for *trans*- and *cis*-15,15'-epoxy-β-carotene[18] (**2**), and we attribute them to these compounds Figure 1 (inset (i)) shows several additional, minor components in this region which suggested that other epoxides were also formed Peak 4 was too weak for observation by LC-MS, but comparison of its R_t and UV spectrum with authentic material showed this to be 5,6,5',6'-diepoxy-β-carotene, (**4**) The complex group of peaks 5, were short-lived compounds They were absent in the later stages of the oxidation (> 24 h), did not survive mild heating to 45 °C, or storage, and did not withstand the thermospray inlet of the LC-MS Their UV spectra had maxima in the visible with featureless tails extending into the UV This, together with their retention times, suggested that they were long-chain di-oxygenated carotenes, possibly hydroperoxides or cyclic peroxides (**5**) The group of peaks 6a,b, which remained after decomposition of this transient material (Figure 1, inset (ii)), were shown to include the dihydrofurans (**6a**) and (**6b**), 5,8-epoxy- and 5,8,5',8'-diepoxy-β-carotene respectively, by R_t and UV comparisons with authentic compounds



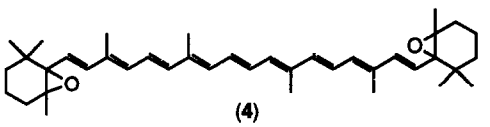
(**1**)



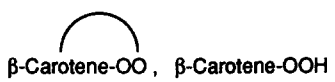
(**2**)



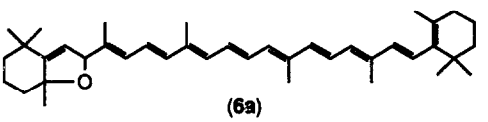
(**3**)



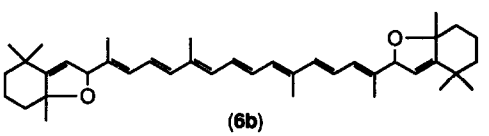
(**4**)



(**5**)



(**6a**)



(**6b**)

Succeeding peaks, up to no 9, were minor at all wavelengths and in the TIC chromatograms, of these the group of peaks labeled 7 was shown to probably contain β-apo-8'-carotenal, by R_t and UV comparisons with an authentic specimen Peaks 9 to 14 were shown to be β-apo-12'-carotenal (**9**), β-apo-14'-carotenal (**10**), retinal (**11**), β-apo-13-carotenone (**12**), β-ionylidene acetaldehyde (**13**), and β-ionone (**14**) respectively from their MS, UV, and R_t data A plot of logR_t vs C-number, for the series of mono-carbonyl compounds was a smooth curve (Figure 2) The R_t of β-apo-10'-carotenal (**8**), which, was interpolated from this curve, indicated that it was probably one of the group of minor peaks labeled 8 on Figure 1 The HPLC chromatogram showed a large peak at 10a with λ_m = 390 nm, however, the LC-MS TIC chromatogram showed nothing significant at this R_t and it is likely that this unknown is a minor product with a large extinction coefficient at the monitoring wavelength (350 nm)

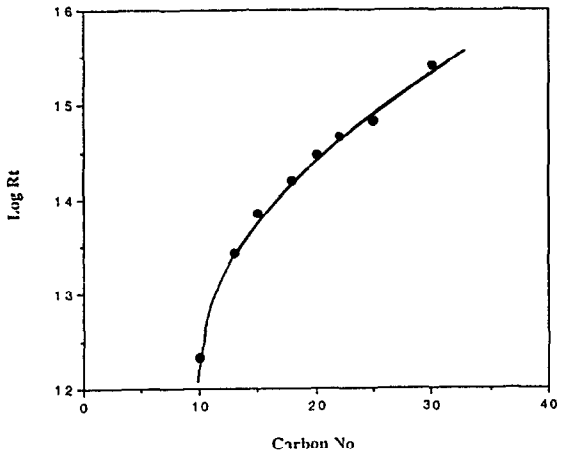
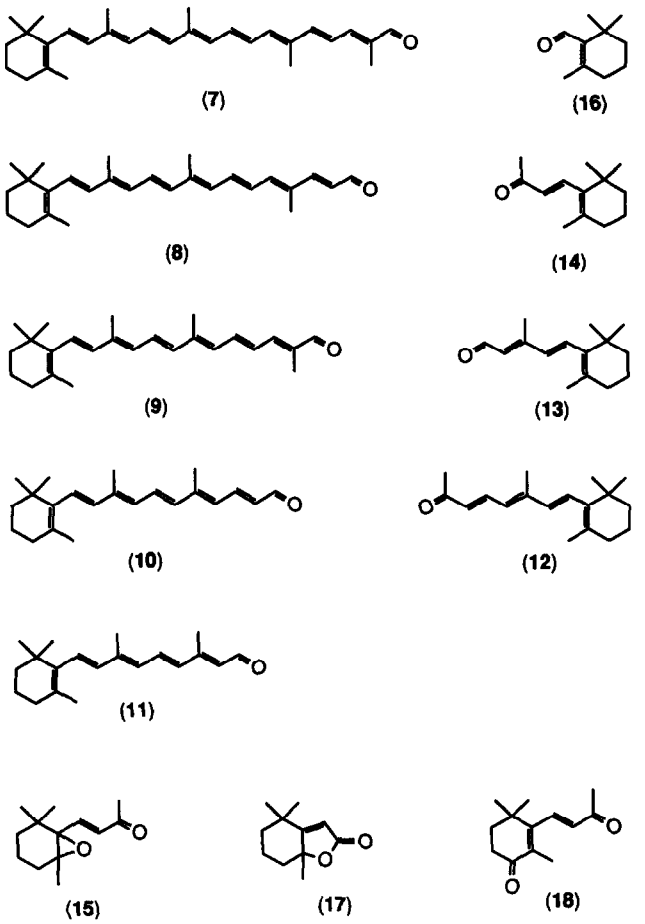


Figure 2 Plot of log R_t, derived from the HPLC chromatogram, vs carbon number for the series of β-apo-carotenals and -carotenones

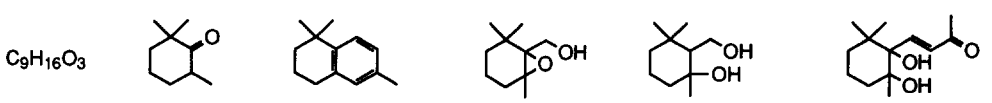


Several short chain di- and tri-oxygenated compounds were identified in the R_t = 21 to 11 min. region 5,6-Epoxy-β-ionone (**15**) was identified from its MS and by comparison of its R_t and UV spectrum with authentic material Product (**15**) appears insignificant in the chromatogram shown in Figure 1, but shorter wavelength detection and the LC-MS data showed that it is an important product The same was true for β-cyclocitral (**16**), dihydroactinidiolide (**17**), 4-oxo-β-ionone (**18**), and peak 19, all of which became important in the later stages of the oxidation Peak 19 had λ_m = 330 nm and M^+ = 172, which was consistent with $C_9H_{16}O_3$, but full characterization was not possible

There was some evidence for the formation of a series of 4-oxo-carbonyl compounds in minor concentrations In order of increasing R_t these were (**18**) (identified by comparisons with authentic material), 4-oxo-β-ionylidine acetaldehyde, i e 4-oxo-**13**, (R_t ~ 18 min), 4-oxo-β-apo-13-carotenone, i e 4-oxo-**12** (R_t ~ 21 min), and 4-oxo-β-apo-10'-carotenal, i e 4-oxo-**8**, (R_t ~ 29 min , peak 9a), the latter three structures were tentatively assigned on the basis of the MS data Members of this series with molecular weights intermediate between that of 4-oxo-**8** and 4-oxo-**12**, if present, would be hidden under the "hump" from R_t = 25 to 30 min

The products of the β-carotene oxidations were also studied by GC-MS We found that components with molecular weights greater than that of retinal did not elute from the capillary column and therefore were not detectable with this technique However, the method gave well-resolved chromatograms of the lower molecular weight products and served as a useful cross-check on their identification Thus, the formation of the following compounds was confirmed by comparison of their electron impact spectra with the literature In decreasing order of elution they were retinal (**11**), β-apo-13-carotenone (**12**), β-ionylidene acetaldehyde (**13**), 4-oxo-**13**, dihydroactinidiolide (**17**), 5,6-epoxy-β-ionone (**15**), β-ionone (**14**), and β-cyclocitral (**16**) Naturally, the retention order was different from that of the HPLC chromatograms, however, this independent analytical technique confirmed that the main low molecular weight components had been correctly identified and that other important products had not been missed The GC-MS results also showed that minor amounts of 2,2,6-trimethylcyclohexanone (**20**), and 1,1,6-trimethyltetrahydronaphthalene (**21**) were also formed Several minor components remained unidentified

The analysis did not reveal any alcohols as major products That this class of compound was not hidden under other HPLC peaks was checked by comparing the R_t of authentic retinol and β-ionol with the oxidation chromatograms Both these alcohols were resolved from the corresponding carbonyl peaks which indicated that major amounts of alcohols were not formed However, trace quantities of compounds (**22**) - (**24**) were shown to be present from their mass spectra and by R_t comparisons with authentic materials



(**19**) (**20**) (**21**) (**22**) (**23**) (**24**)

Although some *cis*-isomers of β-carotene were partly resolved from the all-*trans* substrate, it was not generally possible to resolve *cis/trans* isomers of the carbonyl products by HPLC For example, *cis*-9-retinal and *cis*-13-retinal both had the same R_t as *trans*-retinal and could not be distinguished Thus, the present analytical techniques were not able to establish the extent of this type of isomerisation amongst the products

Product Proportions

The diode array HPLC detector response was calibrated, by using solutions of known concentration, for all the products which could be made or bought The accuracy of quantification was limited by the incomplete resolution on the chromatograms Attempts to arrive at a complete material balance were unsuccessful for this reason and because the amount of oligomer was unknown, and because of the lack of calibration factors for some of the products However, approximate values of the relative concentrations of most of the lower molecular weight products, are given in Table 2 for various oxidation times

Table 2 Relative Molar Proportions[a] of the Major Low Molecular Weight Products from β-Carotene Autoxidation in Benzene at 30 °C

Time of Oxidation/h	3	6	24	48
Compound				
β-Carotene, (**1**)[b]	0 60	0 35	0 03	<0 001
15,15'-Epoxy-β-carotene, (**2**)[c]	0 085	0 10	0 071	<0 001
5,6-Epoxy-β-carotene, (**3**)	0 21	0 33	0 38	<0 001
5,6,5',6'-Diepoxy-β-carotene, (**4**)	0 085	0 13	0 25	<0 001
β-Apo-10'-carotenal, (**8**)	<0 001	<0 001	<0 001	<0 001
β-Apo-12'-carotenal, (**9**)[d]	0 001	0 016	0 001	<0 001
β-Apo-14'-carotenal, (**10**)	0 001	0 012	0 004	0 006
Retinal, (**11**)	0 001	0 012	0 004	0 009
β-Apo-13-carotenone, (**12**)	0 005	0 009	0 058	0 13
β-Ionylidene acetaldehyde, (**13**)	0 002	0 004	0 020	0 072
β-Ionone, (**14**)	0 008	0 012	0 071	0 29
5,6-Epoxy-β-ionone, (**15**)	0 004	0 024	0 071	0 29
β-Cyclocitral, (**16**)	0 001	0 001	0 007	<0 001
Dihydroactinidiolide, (**17**)	<0 001	0 017	0 034	0 20

[a] Molar proportions as a fraction of the sum of total measured products and unreacted β-carotene [b] Unreacted β-carotene

[c] Detector response assumed to be the same as for (**3**) [d] Detector response assumed to be the same as for (**11**)

Carbonyl compounds were present after very short (1 h) oxidation times (not shown in Table 2) At somewhat longer times (>24 h) the main products were the epoxy-carotenes, but these steadily decreased in importance

and were undetectable beyond *ca* 30h oxidation. It is probable that these longer chain materials were further oxidized to give lower molecular weight carbonyl-containing components The longer chain β-apocarotenals (**8**, **9** and **10**) also decreased in importance with oxidation time. After *ca*. 48h oxidation the main products remaining were β-ionone (**14**), and its epoxide (**15**), β-apo-13-carotenone (**12**), β-ionylidene acetaldehyde (**13**) dihydroactinidiolide (**17**), the unidentified tri-oxo-component (peak 19), together with smaller amounts of other short chain carbonyl compounds

The HPLC results were in substantial agreement with the results of the FT-IR analyses. In addition to the carbonyl compounds, the latter technique showed that alcohols and/or carboxylic acids were produced in the later stages of the oxidation The minor hydroxyl components (**22**), (**23**) and (**24**) were characterized from their HPLC R_t data and UV spectra It is possible that peak 19 and other minor, unidentified, components are carboxylic acids, and/or peracids

Table 3 Relative Molar Proportions of the Major Products from Autoxidations of β-Carotene[a] in the Presence of Additives, Light, or Methyl caprylate

Additive/ Conditions	Fe(III) stearate	Cu(II) stearate	Zn stearate[b]	Methyl caprylate[c]	hν[d]
10^4 mol	1 8	1 7	1 5	--	--
Compound					
β-Carotene (1)[e]	0 19	0 18	0 34	0 494	0 032
15,15'-Epoxide (**2**)	0 10	0 04	0 097	0 087	0 039
5,6-Epoxide (**3**)	0 44	0 44	0 24	0 291	0 834
5,6,5',6'-Diepoxide (**4**)	0 24	0 31	0 194	0 087	0 088
β-Apo-12'-carotenal (**9**)	0 001	<0 001	0 002	0 001	<0 001
β-Apo-14'-carotenal (**10**)	0 001	0 001	0 005	0 001	<0 001
Retinal (**11**)	0 001	0 001	0 005	0 001	<0 001
β-Apo-13-carotenone (**12**)	0 01	0 01	0 048	0 006	0 003
β-Ionylidene acetaldehyde (**13**)	0 002	0 002	0 019	0 003	0 001
β-Ionone (**14**)	0 015	0 006	0 048	0 029	0 004

[a] Oxidation of β-carotene (0 2 x 10^{-4} mol) in benzene at 30°C for 6 h (unless otherwise noted), molar proportions as a fraction of the sum of the total measured products and unreacted β-carotene [b] Product distribution after 24 h oxidation [c] Reaction in liquid methyl caprylate [d] Reaction illuminated with a tungsten lamp [e] Unreacted β-carotene

The autoxidation was accelerated when 2,2'-azo-bis(2-methylpropionitrile) (AIBN) was added, as shown by the more rapid development of the product range The known free radical chain-breaking antioxidants 2,6-di-*t*-butyl-4-methylphenol (BHT) and α-tocopherol (vitamin E) very effectively inhibited product formation α-Tocopherol was more effective than BHT, as would be expected[20]. For example, one molar equivalent of α-tocopherol completely suppressed product formation from β-carotene in solution for ca. 72 h, whereas a similar amount of BHT was effective for only ca 24 h

The autoxidation of β-carotene at 30°C in benzene in the dark was also carried out in the presence of iron(III), copper(II) and zinc stearates since such metal carboxylates are potential oxidation catalysts and may be present in food The range of products formed under these conditions (Table 3) was similar to that obtained in the absence of metal ions (Table 2) A similar slate of products was also obtained when β-carotene was subjected to self-initiated autoxidation in the dark in methyl caprylate as solvent (Table 3). This solvent was chosen to imitate the conditions which might be encountered in a lipid environment but, unfortunately, it gave rather broad and poorly resolved HPLC chromatograms (which is the reason most work was done in benzene)

By way of contrast, the product distribution was perturbed when β-carotene was subjected to oxidation in benzene with irradiation by the light from a tungsten lamp The oxidations were strongly accelerated and epoxides became almost the only products in the 6h oxidation mixtures (Table 3)

Oxidation of β-Apo-8'-Carotenal and Retinal.

Table 2 shows that the epoxy-carotenes, and the longer chain carbonyl compounds, decreased in relative concentration as the oxidation proceeded It seemed likely therefore that the higher molecular weight materials, with long conjugated chains, were themselves undergoing oxidative degradation The synthetic 5,6-epoxy-β-carotene (**3**) was found to rapidly oxidize giving a complex range of products The amounts of β-apo-8'-carotenal (**7**) and β-apo-10'-carotenal (**8**) formed were extremely small under all conditions To check if these longer chain aldehydes autoxidised at a rate comparable to that of β-carotene, the oxidation of (**7**) was examined. The rate of the self-initiated autoxidation of (**7**) at 30°C in benzene solution was approximately half that of the self-initiated autoxidation of β-carotene, nevertheless, after 24 h an extensive range of products had developed (Figure 3) Half the original (**7**) was consumed in *ca* 30 h and (**7**) became undetectable on the chromatograms (< 1% starting concentration) after 72 h oxidation The HPLC chromatogram of the products showed a series of carbonyl compounds similar to that obtained from (**1**) A cluster of peaks with R_t values similar to that of (**7**) suggested that *cis/trans* isomerisation of the reactant occurred The chromatogram also showed a major product with an R_t intermediate between that of the reactant and the main carbonyl compounds, we attribute this to 5,6-epoxy-β-apo-8'-carotenal, although definite identification was not possible Following this was a series of carbonyl compounds including (**9**), (**10**), (**11**), (**12**), (**13**) and (**14**) In addition, several important short R_t components were observed, these were probably poly-oxygenated, but were not structurally characterized

The self-initiated autoxidation of retinal was briefly examined In benzene solution, at 30°C, retinal is significantly more resistant to oxidation than β-carotene, but minor quantities of oxidation products were observed after prolonged reaction (> 70 h) and when oxidations were carried out at higher temperatures

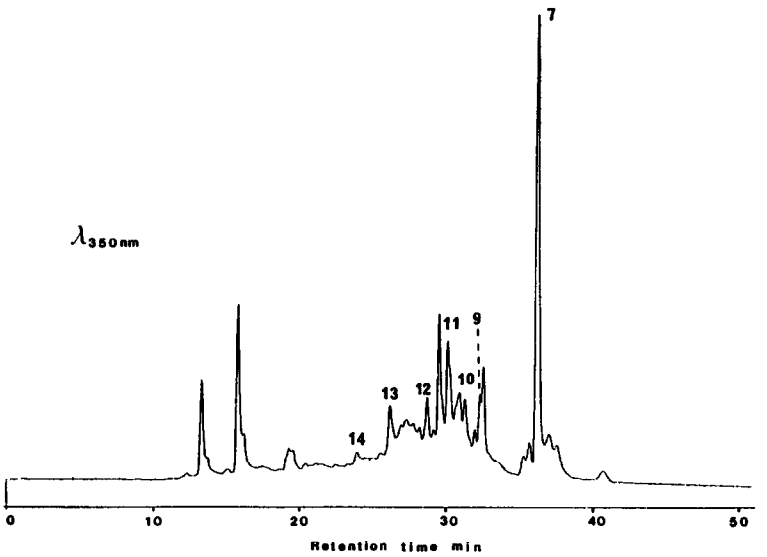
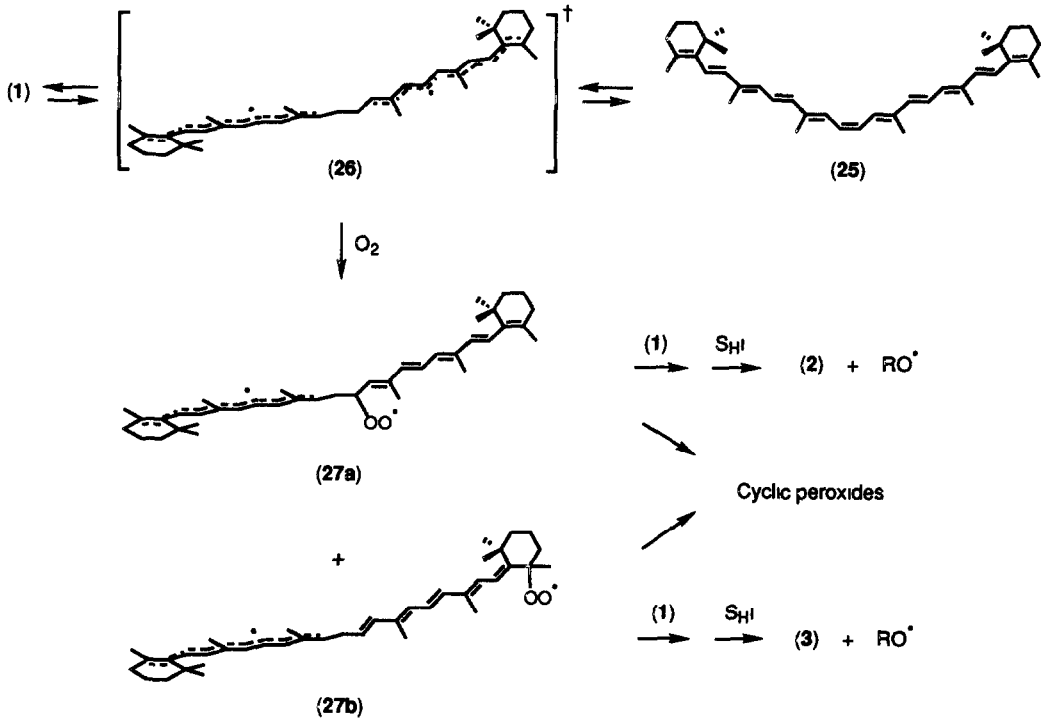


Figure 3 HPLC chromatogram of the oxidation products obtained from the β-apo-8'-carotenal (7) oxidation after 24 h The instrumental parameters were similar to those used in the β-carotene analyses The numbering of the peaks is the same as in the β-carotene oxidation

DISCUSSION

The oxygen uptake and FT-IR data confirmed that, in the absence of antioxidants, and without added radical initiators, purified β-carotene oxidises very rapidly in solution in the dark The actual rate of consumption of (1) depended on oxygen pressure, but changing the solvent from benzene to tetrachloromethane to methyl caprylate had little effect The oxygen uptake curves were sigmoidal which is consistent with an autocatalytic reaction Other evidence that the oxidation proceeded by a free radical chain reaction was its inhibition by BHT and α-tocopherol and its acceleration by AIBN The main stable products were (i) a higher molecular weight component of unknown constitution, (ii) epoxy-β-carotenes, (iii) a series of β-apo-carotenals and -carotenones and, particularly in the late stages of the oxidation, (iv) other low molecular weight di- and tri-oxygenated compounds, probably including carboxylic and/or peracids, and (v) carbon dioxide The oxidation mixtures also contained thermally unstable components which could be degraded to form additional carbonyl containing compounds Since peroxides were demonstrated to be present we assume that they are the thermally unstable materials The autocatalytic nature of the oxidation can probably be attributed to thermal decomposition of these peroxides

The HPLC chromatograms showed that from the earliest stages of the dark reaction, isomerisation of the all-*trans* reactant to *cis*-isomers took place, and this may well have preceded formation of the carbonyl compounds and epoxides

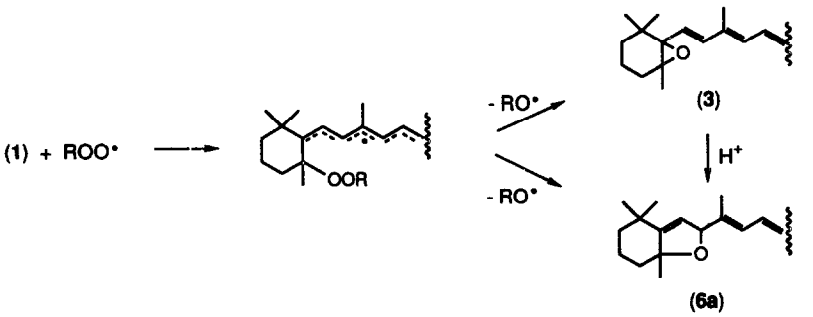


Scheme 1

The carotene isomers were not sufficiently well resolved to be identified with certainty, but the UV-vis spectra were consistent with an isomerisation giving mainly the 15,15'-*cis*-isomer (**25**) Recent work by Doering and coworkers has implied that the isomerisation of (**1**) to 15,15'-*cis*-β-carotene can occur in solution at temperatures < 40 °C[22] The reaction is facile because the *singlet* biradical (**26**) (Scheme 1) is thermodynamically stabilised by extensive delocalization of the two unpaired electrons The facile thermal isomerization of *trans* (**1**) to *cis* (**25**) β-carotene (and *vice versa*) via the biradical (**26**) provides a ready explanation for the rapid self-initiation of β-carotene's autoxidation That is, during twisting of the β-carotene "backbone" unpaired spin density will develop in each half of the molecule, reaching a maximum (one free spin in each half) in the perpendicular transition state (**26**) It seems likely that unpaired spin can be "captured" by oxygen even at twist angles considerably below 90°C This will produce carbon-peroxyl *triplet* biradicals (**27**) which being triplets are presumably relatively long-lived and hence may act as the initiators of the autoxidation chain reaction (Scheme 1) These biradicals may collapse to (thermally unstable) cyclic peroxides or they may add to a second molecule of β-carotene following which a well-precedented[23] intramolecular homolytic substitution (S_{HI}) will yield epoxide (e g , (**2**) and (**3**)) and a carotene alkoxyl radical, RO• (see Scheme 1) The much less facile self-initiated autoxidation of compounds containing fewer conjugated double bonds than β-carotene (e g β-apo-8'-carotenal and retinal) can simply be attributed to a much less facile *trans/cis* thermal isomerization and hence to a much slower rate of initiation

The main product in the early stages of the oxidation of (**1**) in the dark was 5,6-epoxy-β-carotene (**3**) which is probably formed only in part in the initiation process (Scheme 1) A more important route to this

product is probably the chain propagating step in which a peroxyl radical ROO• adds to C(5) followed by an $S_{H}1$ reaction to produce the epoxide (Scheme 2) Peroxyl radical addition to C(5), at the terminus of the conjugated system of (**1**), will give an intermediate radical with a greater extent of electron delocalisation than would be the case for non-terminal addition It follows that terminal addition will give the lowest energy intermediate which probably explains the preponderance of (**3**) and (**4**)



Scheme 2

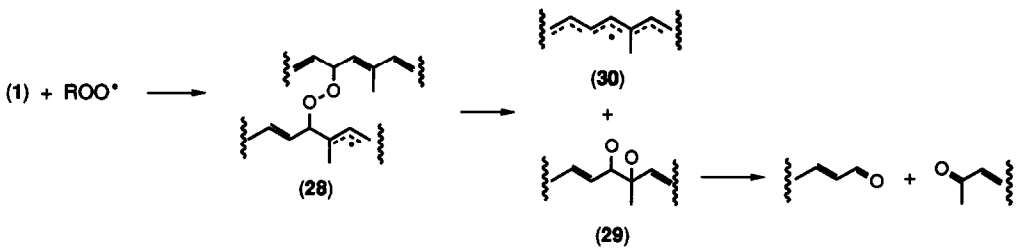
The five-membered cyclic ethers (**6a,b**) may be formed in a similar way to the epoxides by homolytic substitution from the radical centre at C(8) (Scheme 2) However, it is well established[24] that 5,8-epoxy-β-carotene (**6a**) can be formed by acid catalysed rearrangement of the 5,6-epoxide, (**3**) Since we have shown that the oxidation probably produces carboxylic acids, it is possible that (**6a,b**) are formed in this way either additionally or exclusively

The 15,15'-epoxides (**2** *trans* and *cis*) were next in importance as initial products They are probably formed in part during the initiation step (Scheme 1) but, more importantly, by peroxyl radical addition at C(15) during chain propagation Of course, addition to C(15) will give a much less delocalised carbon radical than addition at C(5) and, other things being equal, would therefore be a much slower reaction However, the 15,15'-bond is sterically the most exposed and this should facilitate peroxyl radical addition

The overall autoxidation process in the dark may be divided into two main phases First, the formation of epoxides, carbonyl compounds and oligomer Second, degradation of these primary products to shorter chain carbonyl compounds This second phase was accompanied by CO_2 and carboxylic acid formation The β-apo-carotenals underwent more rapid oxidation than the β-apo-carotenones (**12**) and (**14**), which is hardly surprising since the presence of the weaker aldehydic C-H bond means that aldehydes are much more readily autoxidized than ketones As a consequence, the β-apo-carotenones became the major products after long reaction times Although minor amounts of alcohols (**22**) - (**24**) were detected, no regular sequence of alcohols analogous to the carbonyls was produced This indicated that the carbonyl compounds were not formed by peroxyl/peroxyl termination reactions[25]

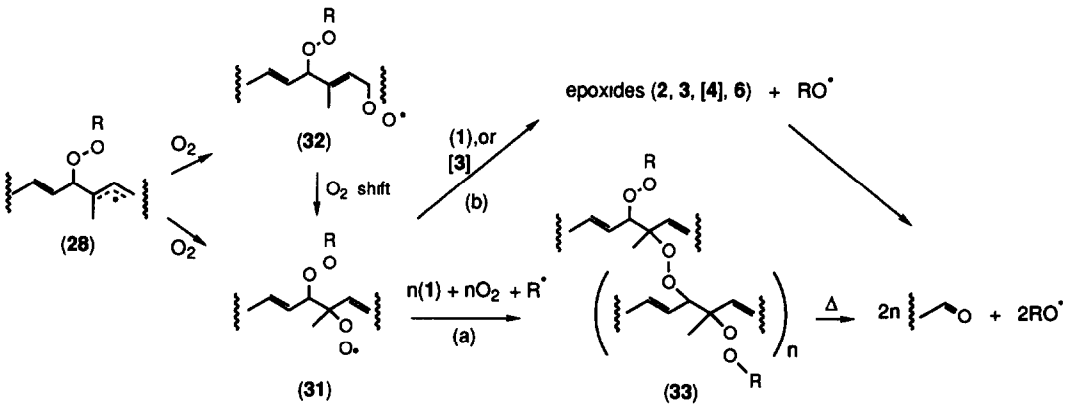
$$RR'CHOO^{\bullet} + RR'CHOO^{\bullet} \rightarrow RR'CHOH + RR'C{=}O + O_2$$

It is likely, therefore, that the carbonyls are formed by oxidative addition of peroxyl radicals to the conjugated system followed by scission There are two main mechanistic possibilities First, the initial peroxyl β-carotene adducts (**28**) might undergo an alternative $S_{H}1$ reaction to produce dioxetanes (**29**) and carotenyl radicals (**30**) (Scheme 3) The dioxetanes will be very unstable and are expected to decompose rapidly to give two carbonyl compounds Although there is no precedent for this type of substitution occurring in alkene and



Scheme 3

diene autoxidations, we note that with carotenes the displaced radical (**30**) is strongly resonance stabilised, which might make this reaction competitive with the usual S_Hi formation of epoxide (Schemes 2 and 3) Second, the carbonyls might arise from decomposition of an oligomeric peroxide (**33**) formed by successive addition of peroxyl radicals to carotene molecules (route (a), Scheme 4) This type of process is well documented for alkene and diene autoxidations[26] Thermal decomposition of (**33**) would then produce the observed carbonyls as the oligomer "unzipped" Alternatively, the "unzipping" might occur *via* formation of epoxides (route (b), Scheme 4) A difficulty with this mechanism is that the initial adduct radical (**28**) is extensively delocalised so that oxygen attachment could occur at many sites down the chain to give other oligoperoxides (cf (**32**)) which cannot decompose so straight forwardly to the observed carbonyls It is possible however that radicals of type (**32**) undergo oxygen migration by the well known and facile allyl peroxyl radical rearrangement[27] to give (**31**) Radical (**31**) is the most stable of these peroxyls because the carotene chain conjugation is least disrupted

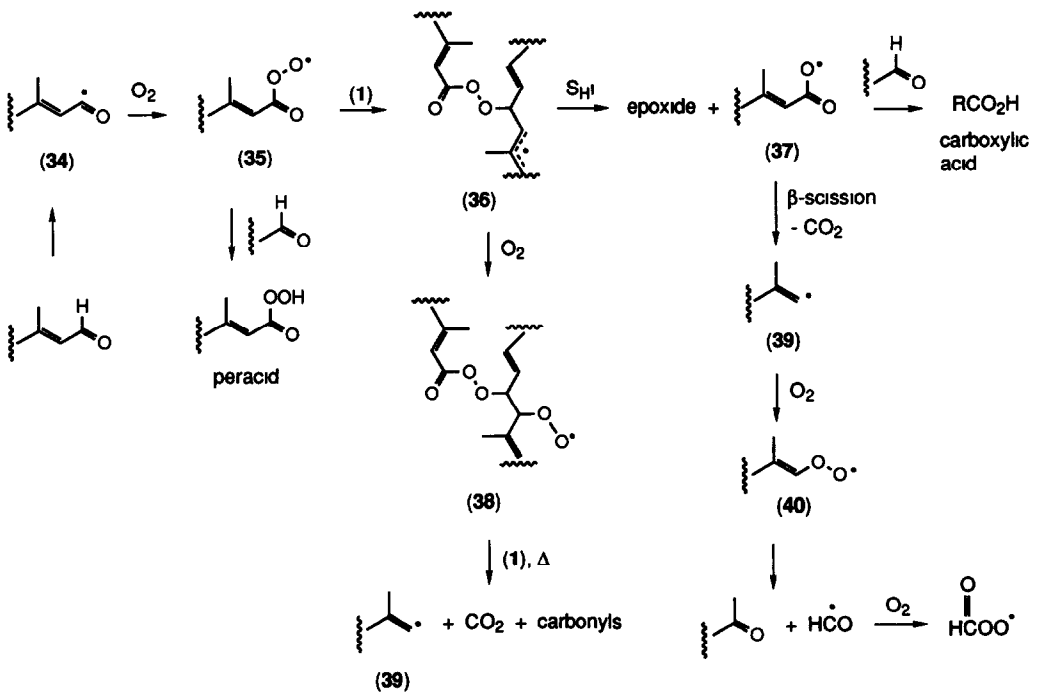


Scheme 4

Both of these mechanistic schemes predict that the scission of each particular double bond should produce equal amounts of the two carbonyl compounds Thus, (**10**) and (**12**) would be expected to be produced in equal amounts, as would (**9**) and (**13**), (**8**) and (**14**), (**7**) and (**16**) In actual fact, the measured yields of the carbonyl compounds do not fit this pattern, even at short oxidation times (see Tables 2 and 3) Obviously, the initial product yield ratios will quickly become distorted, because the longer chain carbonyl compounds, particularly (**7**) and (**8**) will be rapidly degraded, making their concentrations less than that of their partners,

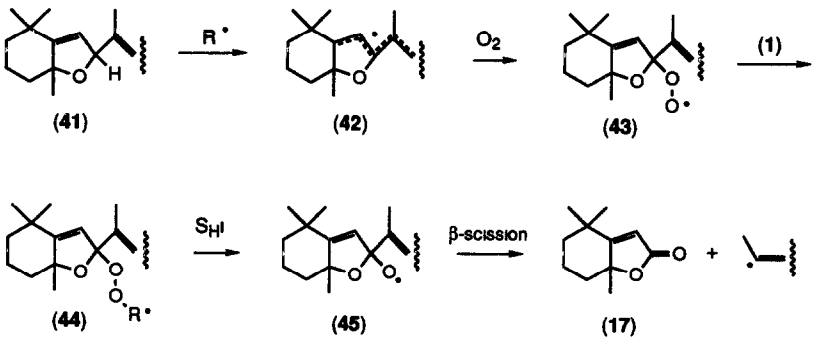
(16) and (14), respectively Furthermore, oxidative degradation of the longer chain carbonyl compounds is bound to produce additional amounts of the shorter chain carbonyls Scrutiny of the relative carbonyl concentrations (Tables 2 and 3) revealed that the methyl ketones, β-ionone (14) and β-apo-13-carotenone (12) predominate over the aldehydes, particularly at longer oxidation times The aldehydes are more susceptible to autoxidation than the ketones because of their labile acyl hydrogens A likely mechanism is outlined in Scheme 5 Abstraction of the aldehydic hydrogen by any radical will produce acyl radicals (34) which will combine with oxygen to give acyl peroxyl radicals (35) Addition of the latter to β-carotene will produce delocalised perester radicals (36) which can react in two ways First, the S_{HI} reaction will give epoxide and an acyloxyl radical (37) which is expected to undergo β-scission with loss of CO_2 to afford vinyl radicals (39) Intermediate (36) may also pick up oxygen to give a peroxyl radical (38) which on combination with more β-carotene will give an oligoperoxide Under the oxidation conditions employed and more particularly on heating under vacuum, this oligoperoxide will decompose to carbonyls, CO_2 and a vinyl radical The vinyl radicals probably combine with oxygen to give peroxyl radicals (40) which are presumably the source of the methyl ketones This process explains the successive chain shortening of the aldehydes, and the formation of CO_2 and epoxides

The fact that carbonyl compounds arising from scission of every double bond (except the ring C(5)-C(6) bond) were observed, indicates that the oxygenation is a fairly random process with not very much selectivity for any particular double bond Scission of the ring bond was not observed, possibly because the intermediate radical of type (28) prefers to undergo the S_{HI} reaction to form the 5,6-epoxide



Scheme 5

An important product, particularly in the later stages of the oxidation in the dark, was dihydroactinidiolide (**17**) which has been reported as a product in several studies of the thermal decomposition of β-carotene[13,28]



Scheme 6

The 5,8-epoxides, (**6**), and shorter chain analogues, contain an extremely labile hydrogen atom which is bisallylic and also activated by the adjacent oxygen functionality Abstraction of this hydrogen will give the delocalised radical (**42**) which would add oxygen to give the peroxyl radical (**43**) (Scheme 6). Addition of this peroxyl to β-carotene would afford the oligoperoxycarotenyl radical (**44**) which could undergo the S_{Hi} reaction to give more 5,6-epoxide, together with the alkoxyl radical (**45**) which on β-scission would yield (**17**)

Oxidation under tungsten lamp irradiation leads to increased yields of epoxides (Table 3). The mechanism by which these are formed is uncertain but we tentatively suggest that irradiation increases the rate of *trans/cis* isomerisation of (**1**) and the epoxides are formed as shown in Scheme 1

In so far as comparison is possible with the partial product analyses from β-carotene oxidations in tissue homogenates[1-3], there appear to be marked similarities to the self-initiated autoxidation of (**1**) in the dark

EXPERIMENTAL

^1H NMR spectra (300 MHz) and ^{13}C spectra (75 MHz) were obtained with a Bruker AM 300 instrument on $CDCl_3$ solutions containing tetramethylsilane as internal standard The oxygen uptake experiments were performed in an apparatus incorporating a pressure transducer, calibrated by AIBN decomposition, similar to that described previously[20] FT-IR spectra were obtained on a Bomem MB 100 Fourier transform spectrometer equipped with a deuterated triglycine sulfate (DTGS) detector For the IR measurements oxidations were run in tetrachloromethane and small samples were introduced into a fixed pathlength 1 mm sealed KBr micro-transmission cell Usually 100 interferograms were co-added and apodization was by cosine wave High performance liquid chromatography (HPLC) was carried out with a Perkin-Elmer 410 pump and LC 235 Diode Array detector, and also with a Hewlett Packard 1090 instrument Elution of the components was monitored at 450, 340, 300 and 280 nm Best separations were achieved with a 250 x 4 6 mm Spherisorb S5 ODS2 5μ column operated with a quaternary solvent system of $H_2O/CH_3OH/CH_3CN/CH_2Cl_2$ The initial solvent mixture was $H_2O/CH_3OH/CH_3CN$, 80/10/10, which was programmed to 100% CH_3CN and finally 100% CH_2Cl_2 Samples, usually 10 μl, were introduced onto the column either in neat benzene, or in $CH_3OH/CH_3CN/CH_2Cl_2$, 2/3/5, v/v/v Coupled LC-MS was carried out

with a Hewlett Packard 5988A-1090 system operated in the thermospray mode A similar Spherisorb ODS2 column and solvent system was employed The GC-MS data was obtained with a Finnigan MAT INCOS 50 instrument, with 70 ev EI ionisation The GC was a Hewlett Packard 5890A fitted with a fused silica capillary column (25 cm x 0 2 mm) coated with cross-linked phenylmethyl silicone

β-Carotene, β-ionone, β-cyclocitral, vitamin A acetate, cis-9-retinal, cis-13-retinal, and β-apo-8'-carotenal were obtained commercially The β-carotene was purified by column chromatography on neutral alumina with tetrachloromethane as eluant It was stored at -20°C and periodically re-purified

5,6-Epoxy-(**3**), *5,6,5',6'-diepoxy-*(**4**), *5,8-epoxy-*(**6a**), *and 5,8,5',8'-diepoxy-β--carotenes-*(**6b**) were prepared by the method of Barber et al [24] *5,6-Epoxy-β-ionone*(**15**) was made in a similar way; δ_H 1 1 (6H,s), 1 4 (2H, m), 1 8 (3H, s), 2 1 (2H, m), 2 3 (3H, s), 6 1 (1H, d), 7 3 (1H, d), δ_C 16 64, 20 50, 25 56, 25 61, 27 56, 29 53, 33 23, 35 26, 65 38, 70 07, 132 43, 142 34, 196 71

4,5-Epoxy-α-ionone[29], *4-Hydroxy-β-ionone*[29], *retinal*,(**11**)[30], *β-ionylidene acetaldehyde*, (**13**)[31], *β-apo-13-carotenone*, (**12**)[31], and *β-apo-14'-carotenal*, (**10**)[32] were made by literature methods

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