A Proposed Mechanism for the Hydroxylation of Carotenoids on Micro-Cel C

Robert P. Ritacco, George Britton, and Kenneth L. Simpson*

Further investigation of Micro-Cel C mediated hydroxylation of carotenoids is discussed. A Micro-Cel C water content of about 4% has been shown to give optimum yield of 4-hydroxy-β-apo-8'-carotenal. The adsorbent can be recycled if first oven-dried to remove all traces of the solvent and if water is added back prior to exposure of the carotenoids. The hydroxylation reaction occurred only when water was present, while molecular oxygen was essential for the other reaction products. The reaction was not limited to Micro-Cel C but also occurred on synthetic silica. Both the 10'- and 12'-β-apocarotenals react with Micro-Cel C to yield products analogous to those of the 8'-β-apocarotenal reaction. Addition of methanol (10%) to dry Micro-Cel C gave the 4-methoxy derivative, and the use of $H_2{}^{18}O$-enriched water gave the 4-hydroxy compound also enriched in oxygen-18. This confirms the origin of the hydroxy function to be water and not molecular oxygen. Also discussed is the preparation of 3,4-dehydro-β-apo-8'-carotenal and its reaction with Micro-Cel C to give the 4-hydroxide. Finally, a mechanism for the hydroxylation reaction is proposed.

In previous studies, our laboratory reported on the reactivity of Micro-Cel C (a common chromatographic adsorbent) with various carotenoids to yield hydroxides, epoxides, and, in one noted case, cleavage products, when exposed in the presence of a nonpolar solvent (Rodriguez et al., 1976; Ritacco et al., 1984). The hydroxides produced were specific to the 4-position of the cyclic carotenoids and epoxidation predominantly occurred between the 5,8- and 5,6-carbons in the cyclic and acyclic carotenoids, respectively. The noted exception to this is that lycopene gave the 5,6-dihydroxy derivative, which is believed to have subsequently cleaved to give the 6-aldehyde.

We have continued work in this area in order that more insight might be gained concerning the reaction mechanism(s) involved. This present study is an attempt to determine the mechanism involved in the hydroxylation reaction.

EXPERIMENTAL SECTION

Apparatus. A high-pressure liquid chromatograph (HPLC) (Water Associates, Milford, MA) with a μ-Porasil, 10 μm, silica column was used in some experiments to aid in the determination of reaction products. Mass spectra were obtained by using a type AEJMS12 mass spectrometer, ionization by electron impact (ionizing voltage 70 eV; ion source temperature 180–200 °), and use of a direct insertion probe.

Materials. Crystalline β-carotene, β-apo-8'-carotenal, β-apo-10'-carotenal, and β-apo-12'-carotenal were provided by F. Hoffmann-La Roche and Co., Ltd. (Basel, Switzerland). Retinal was purchased from Eastman Kodak Co. (Rochester, NY). The oxygen-18 isotope of water was purchased from Monsanto Research Corp., Mound Laboratory (Miamisburg, OH).

The β-apo-8'-carotenal and β-carotene required purification prior to use [preparative TLC as previously described (Ritacco et al., 1984)]. Retinol was prepared by the sodium borohydride reduction of retinal (Davies, 1976).

Except in the retinol reaction, the Micro-Cel C was oven-dried at 130 °C for a minimum of 24 h and either left untreated or pretreated with specified amounts of H_2O, CH_3OH, or $H_2{}^{18}O$, 30 min prior to use. All reactions were carried out at room temperature and under subdued light.

Exposure to Micro-Cel C. A measured amount of carotenoid was dissolved in petroleum ether (PE) and exposed to Micro-Cel C as previously described (Rodriguez et al., 1976). The products were extracted with acetone and isolated by preparative TLC (Ritacco et al., 1984). Chemical tests as described by Davies (1976) were carried out when necessary for functional group confirmation.

Effects of Water Content. Six 5-g portions of Micro-Cel C were placed in stoppered flasks. Varying amounts (0–18%) of water were added. Equal volumes (50 mL) of a β-apo-8'-carotenal solution (each containing about 0.5 mg of carotenoid) were then added to the flasks, and the reaction was allowed to proceed. The products were extracted and transferred to 8-mL portions of PE. The samples were then injected (10 μL) into the HPLC (7.5% acetone in hexane, v/v; flow rate of 1.5 mL/min), and the peak areas of the resulting chromatograms were determined.

Recycling Micro-Cel C. Micro-Cel C was recycled by first air-drying (to remove acetone) and finally oven-drying (130 °C for 24 h), followed by the addition of 1 mL of distilled water to 9 g of the dry adsorbent. The remaining procedures were as previously described.

Reaction under Nitrogen. Oven dried Micro-Cel C (10 g, 130 °C for 24 h) was placed in a vacuum flask and held under high vacuum for 24 h, after which time nitrogen was allowed to flow into the flask until normal atmospheric pressure was reached. Deoxygenated, distilled water (1 mL, vacuum filtered and nitrogen bubbled) was carefully added to the Micro-Cel C, under a nitrogen atmosphere. The mixture was shaken and allowed to equilibrate for 30 min. β-Apo-8'-carotenal in deoxygenated PE was then added and the mixture allowed to stand another 30 min. The reaction products were analyzed by HPLC (7.5% acetone in hexane; 1.5 mL/min).

Reaction on Synthetic Silica. Dry silica (10 g; Syloid 65, Davison Chemical Co., Baltimore, MD; dried for 24 h at 130 °C) was held under vacuum for 24 h as described above after which time 1 mL of distilled, deoxygenated water was added. The carotenoid in PE was then added with the remaining workup as previously described. The products were analyzed by HPLC.

Addition of Methanol. Methanol, 20 g (10%), was added to 180 g of Micro-Cel C. A total of 11 mg of β-apo-8'-carotenal in PE was then added and the products were isolated for analysis (absorption spectroscopy and mass spectrometry.

University of Rhode Island, Department of Food Science & Technology, Nutrition & Dietetics, Kingston, Rhode Island 02881 (R.P.R. and K.L.S.), and University of Liverpool, Department of Biochemistry, Liverpool L6938X, England (G.B.).

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Table I. Peak Area Comparison of the Chromatograms Obtained from Reactions of β-Apo-8′-carotenal on Micro-Cel C of Varying Water Content

% H_2O	peak area of 4-hydroxide, mm^2	total peak areas	area % 4-hydroxide
0.00	9.7	135.4	7
1.96	27.2	82.5	33
3.85	27.4	69.7	*39*
7.41	23.0	79.2	29
10.71	22.1	99.6	22
18.03	20.4	101.6	20

Addition of $H_2{}^{18}O$. A 45% enrichment of $H_2{}^{18}O$ in water (2 mL) was added to 18 g of Micro-Cel C; 1 mg of β-apo-8′-carotenal in PE was then added. The 4-hydroxy derivative was isolated (TLC) for mass spectral analysis.

Preparation of 3,4-Dehydro-β-apo-8′-carotenal. The procedure of Melton and McMurry (1975) for dehydration of alcohols was employed in the production of 3,4-dehydro-β-apo-8′-carotenal, with the following modifications. After dissolving 5 mg of 4-hydroxy-β-apo-8′-carotenal (isolated from the Micro-Cel C Reaction) in 40 mL of dichloromethane (DCM) and lowering the temperature to 0 °C, 50 mg of methanesulfonyl chloride in 20 mL of DCM was added dropwise with continual stirring. Trimethylamine was employed as the base; 25 mL was added in one portion. (**Caution**! Trimethylamine should be handled at 0 °C.) The mixture was allowed to stand at 0 °C for 2 h after which time it was transferred to a separatory funnel containing a mixture of ice and water. Washing followed as described. The DCM layer was removed, dried over anhydrous $MgSO_4$, and evaporated to dryness on a rotary evaporator. Acetone and PE were then added, followed by partitioning with water. The PE layer was isolated and the products separated by preparative TLC (10° acetone/PE, v/v). The 3,4-dehydro derivative was isolated and the product was used in the following Micro-Cel C reaction.

Reaction of 3,4-Dehydro-β-apo-8′-carotenal. The 3,4-dehydro derivative was exposed to Micro-Cel C containing 10% water. Workup was as in previous experiments. Identification of products was by cochromatography and absorption spectroscopy.

RESULTS AND DISCUSSION

Effect of the Water Content of Micro-Cel C. Substitution of the hydroxy function at the allylic 4-position of the carotenoid β-ring has been shown to be dependent upon the presence of water with Micro-Cel C (Ritacco et al., 1984). Table I shows that optimum reactivity for β-apo-8′-carotenal occurs at a water content of about 4%, with good reactivity at levels of from 2 to about 10%. Micro-Cel C was shown to lose about 7% of its weight when placed in an oven at 130 °C for 24 h (Ritacco et al., 1984). However, this observation was for 1 specific day and the water content may actually vary with atmospheric humidity causing a subsequent variation in reactivity.

Recycling Micro-Cel C. Previous attempts to recycle Micro-Cel C (Ritacco et al., 1984) were unsucessful. A revised method is now reported. Table II shows a comparison of the reaction of β-apo-8′-carotenal on both used and unused Micro-Cel C that has been dried and rewet with 10% water. In both cases, the percent yields of 4-hydroxy-β-apo-8′-carotenal were nearly identical, suggesting that the only part of the adsorbent used up in the reaction is water and, therefore, the Micro-Cel C is acting as a catalytic support.

β-Carotene upon exposure to used Micro-Cel C treated in the same manner, gave similar results (Table III). The

Table II. Comparison of the Reactions of β-Apo-8′-carotenal (1.01 mg) on New and Recycled Micro-Cel C

	9.8 g of new Micro-Cel C + 1.1 mL of H_2O		9.0 g of used Micro-Cel C + 1.0 mL of H_2O	
compound	mg	%	mg	%
a, β-apo-8′-carotenal	0.189	45	0.288	55
b (5,6-epoxy-β-apo-8′-carotenal)	0.019	5	0.022	4
c, 5,8-epoxy-β-apo-8′-carotenal	0.047	11	0.020	4
d, unidentified	0.101	24	0.111	21
e, unidentified mixture				
f, 4-hydroxy-β-apo-8′-carotenal	0.063	15	0.080	15

Table III. Products from the Reaction of β-Carotene (3.0 mg, $E_{1cm}{}^{1\%} = 2505$) with Recycled Micro-Cel C (100 g, Dry, + 11 mL of H_2O)

fraction	λ_{max}, nm (hexane)	mg	%	compound
1	442, 466, 496	0.078	8	3,4-dehydro-β-carotene
2	427, 448, 474	0.051	5	β-carotene
3	426, 453, 469	0.023	3	echinenone
4	428, 445, 468	0.172	18	unidentified
5	423, 442, 466	0.104	11	unidentified
6	428, 449, 477	0.510	*54*	(isocryptoxanthin)

yield of 54% is directly comparable to those obtained on the unused adsorbent (Ritacco et al., 1984; Rodriguez et al., 1976). Identification of the major components was accomplished by absorption spectroscopy and mass spectrometry. Echinenone was confirmed by cochromatography with authentic material (Eastman Chromatogram Sheet 13179).

The molecular ion for fraction 1 (Table III), $M\cdot^+$, was at m/z 534 (100%, $C_{40}H_{54}$) with very few characteristic fragment ions except at m/z 442 (20%, M − toluene) and m/z 428 (20%, M − *m*-xylene). The TLC properties were consistent with those of a hydrocarbon and the 18-nm increase in λ_{max} indicates an additional conjugated double bond over β-carotene, all consistent with the assigned structure.

The fraction identified as isocryptoxanthin showed an apparent molecular ion, $M\cdot^+$, at m/z 534 (100%, $C_{40}H_{54}$) but a weak peak was seen at m/z 552 (5.8%, $C_{40}H_{56}O$), indicating that the peak at m/z 534 could be due to the very easy loss of water (552 − H_2O), however, no meta stable was observed for this. Other diagnostic peaks were at m/z 442 (15%, 534 − 92) and m/z 428 (3.7%, 534 − 106; the relative intensity of m/z 442 to m/z 428, approximately 3:1, is characteristic of a bicyclic carotenoid. The adsorption properties (TLC) and absorption spectral data are consistent with the monohydroxycarotenoid, isocryptoxanthin. The hydroxyl function being in the allylic C-4 position could be very easily lost. The structure assigned is likely but certainly not proven.

Reaction of β-Apo-8′-carotenal under Nitrogen. The elimination of molecular oxygen from the reaction system had a very marked effect on the overall reaction (Figure 1). Normal production of the 4-hydroxy compound was observed, while formation of the other products was inhibited. This clearly demonstrates that there are at least two mechanisms in progress simultaneously, one requiring molecular oxygen (epoxidation) and the other, hydroxylation, requiring only the presence of water.

Conversion on Synthetic Silica. Exposure of β-apo-8′-carotenal to synthetic silica resulted in a reaction similar to that observed with Micro-Cel C. The 4-hydroxy derivative is again the major product. This suggests that

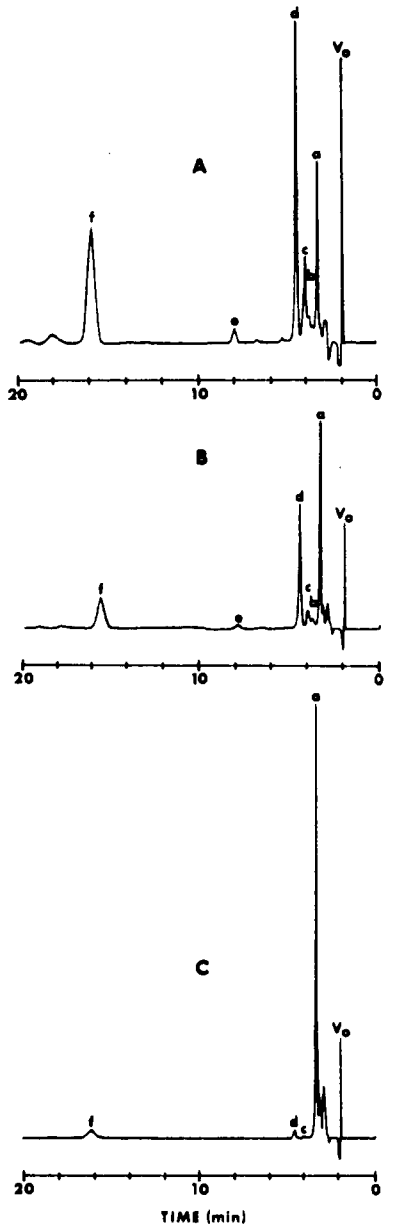

Figure 1. HPLC separation of the products from the reaction of β-apo-8′-carotenal with Micro-Cel C under normal atmosphere [10% H_2O (A)] and under a nitrogen atmosphere [10% H_2O (B) and dry (C)]. The lower case letters correspond to the compounds listed in Table II. Note the nearly complete inhibition of reaction in C and the suppression of components b, c, and d in (B) in comparison to (A). The solvent system was 7.5% acetone in hexane (v/v) with a flow rate of 1.5 mL/min, detector at λ = 450 nm.

possibly a structural similarity exists along the surface of the adsorbents.

Conjugated Chain Length and Reactivity. Retinal was previously shown to remain essentially unchanged after exposure to Micro-Cel C, while β-apo-8′-carotenal readily reacted (Ritacco et al., 1984). In an attempt to determine at which point the reaction is completely inhibited, other carotenoids were examined. Both the β-apo-10′- and -12′-carotenals reacted upon exposure to Micro-Cel C to yield the 4-hydroxy derivatives, epoxides, and other unidentified compounds. The identities of the allylic hydroxides were confirmed by treatment with acidic chloroform (Davies, 1976) and the epoxides by acid treatment (concentrated HCl) to yield a green complex on the TLC plates (Davies, 1976). Retinol did undergo slight transformation, indicated by a very light spot on TLC running below the retinol, but the adsorption was indicative of an epoxide. No functional group tests were done on this compound due to insufficient quantity.

Addition of Methanol. Methanol was added in place of water, to the dry Micro-Cel C prior to exposure of the carotenoid. The course of the reaction changed, and instead of the major product being the 4-hydroxy derivative, it was the 4-methoxy compound [confirmed by MS, $M^{+\cdot}$ at m/z 446, with a strong fragment ion at 414 (metastable at 384.5)]. Hydroxylation was completely inhibited.

Addition of $H_2{}^{18}O$-Enriched Water. When water enriched in $H_2{}^{18}O$ was added to the Micro-Cel C, the 4-hydroxy compound produced was found to be enriched in oxygen-18. A comparison of the high-mass regions of the mass spectra of both the enriched and unenriched hydroxy compounds showed that the peak at m/z 434 was greater in the enriched reaction product. The enrichment was determined to be 20%, about half that expected. This may be explained by the possibility of incomplete removal of water prior to adding the $H_2{}^{18}O$-enriched water. We, therefore, feel that this confirms the origin of the hydroxy function in these reactions to be water.

Reaction of 3,4-Dehydro-β-apo-8′-carotenal. The 3,4-dehydro derivative of β-apo-8′-carotenal reacted upon exposure to Micro-Cel C to give 4-hydroxy-β-apo-8′-carotenal. The absorption spectra of the product showed a change in λ_{max} from 458 nm for the 3,4-dehydro derivative to 451 nm with absorption peaks also at 430 and 478 nm. Regaining the fine structure in the absorption spectrum and the loss of 7 nm coupled with adsorption characteristics on TLC indicated hydration of the 3,4 double bond. The HPLC of authentic 4-hydroxy-β-apo-8′-carotenal and the compound produced in this reaction gave identical retention times (R_T = 6.7).

Catalytic dehydrogenation reactions are well-known for partially unsaturated six-membered rings. The allylic hydrogen is particularly easy to abstract and the presence of the quaternary carbon inhibits aromatization under the mild conditions encountered (e.g., low temperatures).

More conventional methods for the allylic hydroxylation of carotenoids, specifically β-carotene, have been reported. Wallcave and Zechmeister (1953) prepared isocryptoxanthin from 3,4-dehydro-β-carotene via a boron trifluoride–etherate complex at a yield of 34%. By changing the solvent system to chloroform, Petracek and Zechmeister (1956) were able to prepare the isocryptoxanthin (32% yield) directly from β-carotene. They did, however, propose that the BF_3 first acted to form dehydro-β-carotene and that the BF_3–etherate complex did not actually form with β-carotene but rather with dehydro-β-carotene, as described earlier by Wallcave and Zechmeister (1953). Isocryptoxanthin was also the product obtained from the hydrolysis of the blue complex that formed when 3,4-dehydro-β-carotene was treated with BF_3–etherate.

Entschel and Karrer (1958a,b) reported on the reactions of β-carotene with *N*-bromosuccinimide (NBS). They prepared ketones, ethers, and esters of β-carotene with allylic substitution on one or both of the rings. The esters produced were subsequently saponified to the corresponding alcohols. The yield of isocryptoxanthin was about 24%.

The reaction scheme for the Micro-Cel C hydroxylations may be closely related to that proposed by Petracek and Zechmeister (1956), with specific sites on the Micro-Cel C functioning as Lewis acids in a manner similar to the BF_3. The decrease in production of isocryptoxanthin with the simultaneous increase in 3,4-dehydro-β-carotene observed (Ritacco et al., 1984) when oven-dried Micro-Cel

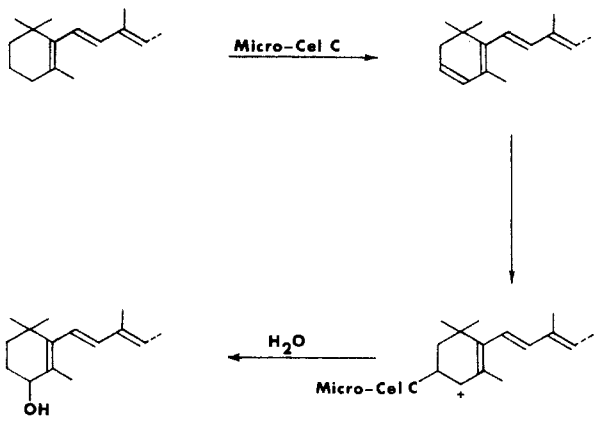

Figure 2. Suggested mechanism for the hydroxylation of carotenoids on Micro-Cel C. Possible Lewis acid sites on the surface of the adsorbent may function in a similar manner to the BF_3 reaction described by Petracek and Zechmeister (1956).

C was used, coupled with the positive reaction of 3,4-dehydro-β-apo-8′-carotenal, suggests a mechanism such as depicted in Figure 2. Lewis acid sites on Micro-Cel C are conceivable in light of the conversion of the 5,6-epoxide group of violaxanthin to the 5,8 isomer reported by Strain et al. (1967).

Registry No. Micro-Cel C, 1344-95-2; β-carotene, 7235-40-7; β-apo-8′-carotenal, 1107-26-2; β-apo-10′-carotenal, 640-49-3; β-apo-12′-carotenal, 1638-05-7; water, 7732-18-5; oxygen, 7782-44-7; nitrogen, 7727-37-9; methanol, 67-56-1; silica, 7631-86-9; 3,4-dehydro-β-apo-8′-carotenal, 74683-01-5; methanesulfonyl chloride, 124-63-0; trimethylamine, 75-50-3; 4-hydroxy-β-apo-8′-carotenal, 88253-14-9; 5,8-epoxy-β-apo-8′-carotenal, 41548-57-6; 3,4-dehydro-β-carotene, 864-87-9; echinenone, 432-68-8.

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Comparison of a High-Performance Liquid Chromatographic and Microbiological Method for the Determination of Niacin in Foods

Pieter J. van Niekerk,* Salomien C. C. Smit, Emmerentia S. P. Strydom, and Gurli Armbruster

A high-performance liquid chromatographic (HPLC) method has been developed for the determination of niacin in foods. Samples are extracted in alkaline medium. The niacin is subjected to chromatography on both reverse-phase and anion-exchange columns to ensure adequate separation from interfering substances. Automatic column switching is used to transfer the niacin fraction from the reverse-phase to the anion-exchange column, and the niacin is detected by its absorption at 254 nm. This method was compared with a microbiological method. The results obtained by both methods agreed reasonably well.

A number of HPLC methods for the determination of niacin in foods have been published, and a review of the available methods is given by van Niekerk (1982). With the available single-column methods (using UV detection) we could not obtain chromatograms where the niacin peak was adequately separated from interfering peaks. A HPLC method, using the principle of column switching (Snyder and Kirkland, 1979) to improve separation, has, therefore, been developed by us.

EXPERIMENTAL PROCEDURES

Apparatus. The HPLC equipment consisted of a Varian 5000 pump with three proportioning valves, a Valco inlet valve, a Model 1203 UV monitor from Laboratory Data Control with a 254-nm filter and a 3390 A reporting integrator from Hewlett-Packard. A motorized Valco six-port valve, controlled by the microprocessor of the pump, was used for column switching. A schematic diagram of the column configuration is shown in Figure 1. Stainless steel columns (15 × 0.46 cm) were slurry packed with Nucleosil 5 C_{18} for reverse-phase chromatography or Nucleosil 5 SB (Machery-Nagel) for anion-exchange chromatography.

A Metrohm potentiograph E 436 was used for titrations in the microbiological method.

Reagents. Mobile phase A was prepared by mixing 5.7 mL of glacial acetic acid with 800 mL of water, adjusting the pH to 3.0 with sodium hydroxide, and diluting to 1 L with water. Mobile phase B was prepared by mixing 5.7 mL of glacial acetic acid with 800 mL of water, adjusting to pH 3.0 with sodium hydroxide, diluting to 1 L with water and finally mixing this solution with methanol in a ratio of 5:95. Mobile phace C was prepared by mixing 22.8 mL of acetic acid with 800 mL of water, adjusting the pH to 3.0 with sodium hydroxide, and diluting to 1 L with water.

National Food Research Institute, Council for Scientific and Industrial Research, Pretoria, 0001, Republic of South Africa.

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