Table III. Disulfide and Sulfhydryl Concentrations in Native and Extruded Soy Concentrate[a]

	native soy concentrate	extruded soy concentrate
this work (140 °C extrusion)		
(1) -S-S- content, mol/mg	22.7×10^{-8}	19.6×10^{-8}
(2) -SH content, mol/mg	0.5×10^{-8}	4.1×10^{-8}
Burgess and Stanley (1976) (178 °C extrusion)		
(1) -S-S- content, mol/mg	4.5×10^{-8}	0.9×10^{-8}
(2) -SH content, mol/mg	3.3×10^{-8}	48.9×10^{-8}

[a] This work was done on a sample extruded at 140 °C. Burgess and Stanley (1976) used the extrudate formed at 178 °C. The expected value is about 20×10^{-8} mol of disulfide/mg of native protein (Wolf and Cowan, 1975).

structural role of disulfide units as was found for high-temperature extrusion.

In summary, low-temperature (≲150 °C) thermoplastic extrusion forms structured protein primarily by intermolecular disulfide bridging accompanied by changes in noncovalent bonding. Higher temperature (≳180 °C) extrusion may produce protein polymerization through formation of intermolecular peptide bonds.

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Investigations of Carotenoid Reactions on Micro-Cel C

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The reactivity of carotenoids with Micro-Cel C, a common chromatographic adsorbent, has been investigated further. α-Carotene was substantially converted to 4-hydroxy-α-carotene. Neither 4,4′-dihydroxy-α-carotene nor 3′-hydroxy-α-carotene was formed, suggesting that the reaction was limited to positions allylic to the conjugated double bond system. β-Apo-8′-carotenal also underwent hydroxylation at the allylic 4-position of the β-ring, while retinal and β-ionone did not react. Lycopene reacted to such an extent that none of it remained unchanged. Other important characteristics of the reaction were determined by using β-carotene as the substrate. The hydroxylation process was stopped when Micro-Cel C was washed with acetone or methanol prior to mixing with β-carotene. Used Micro-Cel C could be recycled after removing the acetone from the first extraction of pigments. Drying the Micro-Cel C at 100 °C for 20 h decreased the amount of isocryptoxanthin produced but increased the level of dehydro-β-carotene 200-fold. The water content of the adsorbent was shown to play a key role in the reaction. The reaction was also shown to be solvent dependent. Petroleum ether was the best solvent while ethyl ether, chloroform, and acetone were inhibitors to different degrees. Methanol and ethanol changed the course of the reactions.

Micro-Cel C has been routinely used as a liquid chromatographic adsorbent in the separation and analysis of carotenoids, particularly the xanthophylls. It was found that β-carotene underwent substantial (65%) hydroxylation at the position 4 carbon upon exposure to this adsorbent when in the presence of a nonpolar solvent such as petroleum ether (Rodriguez et al., 1976). The extent of the conversion was directly dependent on the amount of Micro-Cel C, and the highest rate of accumulation of isocryptoxanthin occurred within the first 15 min. This type of reaction was not observed in other adsorbents such as silica gel, kieselguhr, Celite, alumina, HyfloSupercel, and MgO.

It is of interest, therefore, to determine what other carotenoids react with this adsorbent and the extent to which this reaction takes place.

EXPERIMENTAL SECTION

Apparatus. A high-performance liquid chromatograph (HPLC; Waters Associates, Milford, MA) equipped with a μ-porasil silica column and varible-wavelength detector

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was employed to aid in the analysis of the reaction products. Mass spectra were obtained by using a type AEJ MS 12 mass spectrometer, ionization by electron impact (ionizing voltage 70 eV; ion source temperature 180–200 °C), and use of a direct insertion probe.

Materials. Crystalline β-carotene and β-apo-8′-carotenal were provided by F. Hoffman-La Roche and Co. (Nutley, NJ). *trans*-Retinal and β-ionone were purchased from Eastman Kodak Co. (Rochester, NY) and Aldrich Chemical Co. (Metuchen, NJ), respectively.

α-Carotene was isolated from carrots. The carrot carotenoids were extracted with acetone, transferred to petroleum ether (PE), washed (H_2O) free of acetone, and saponified overnight at room temperature with 10% KOH in methanol. The alkali was removed by thorough washing, and the PE solution was dried over sodium sulfate and concentrated in a rotary evaporator. Separation of the pigments was accomplished on a MgO–HyfloSupercel (1:2) column developed with 2% and 5% ether in PE and 2% and 5% acetone in PE. The α-carotene band was removed from the column and extracted with acetone, transferred to PE, and purified on the same column until only the single α-carotene band remained.

Lycopene was isolated from tomatoes by the general method for plant tissue extraction (Davies, 1976; Raymundo et al., 1967) and purified by column chromatography (MgO–HyfloSupercel, 1:2 w/w) developed with 20% acetone in PE (chromatographed 2 times to ensure maximum purity).

Due to their instability, β-carotene and β-apo-8′-carotenal were purified prior to use by preparative thin-layer chromatography (TLC, Analtech, 1000 μm of silica gel G) developed with 10% acetone in PE or as described previously (Rodriguez et al., 1976).

The *trans*-retinal and β-ionone required no further purification (purity was checked prior to use by TLC; Eastman Chromatogram Sheet 13179; 10% (v/v) acetone in PE).

Exposure to Micro-Cel C. A measured amount of the carotenoid dissolved in PE was added to a specified amount of Micro-Cel C. Enough PE then was added to cover the adsorbent. The mixture was then shaken. The reactants were allowed to stand at room temperature under subdued light for 30 min, after which time they were extracted with acetone, transferred to PE by partioning with water, and dried over sodium sulfate. The products were concentrated with the use of a rotary evaporator (30 °C) and isolated by preparative TLC [Analtech 1000 or 250 μm of silica gel G developed with 10% (v/v) acetone in PE] or, for α-carotene, by column chromatography (MgO–HyfloSupercel, eluted with 10–14% acetone in PE). Experiments involving other solvents (ether, acetone, chloroform, ethanol, methanol) were carried out in the same manner with 100 mL of the solvent. The pigments were extracted and separated by column chromatography as previously described, except that 25% acetone in petroleum ether was used as a developing solvent.

In the experiments on the effect of drying, three 20-g portions of Micro-Cel C were treated as follows: (1) dried directly; (2) exposed to β-carotene for 30 min, extracted with acetone to remove the pigments, and then dried; (3) washed with acetone prior to drying. Drying was done in an oven at 100 °C for 20 h.

For some reactions, 10% (weight) distilled water was added to previously oven dried Micro-Cel C (130 °C for at least 20 h). The wetted Micro-Cel C was placed in a tightly stoppered flask and was equilibrated with occasional shaking for at least 30 min. After this time, the carotenoid in PE was added and the mixture was treated as previously described.

Table I. Conversion of α-Carotene on Micro-Cel C[a]

	amount of carotenoid, μg	
	10 g of Micro-Cel C	20 g of Micro-Cel C
original amount of α-carotene	1433	1433
after exposure to Micro-Cel C		
α-carotene	851	486
5,8-epoxy-α-carotene	41	72
4-oxo-α-carotene	7	5
4-hydroxy-α-carotene	478	645

[a] α-Carotene was mixed and allowed to stand for 30 min with the specified amount of Micro-Cel C and enough petroleum ether to cover the adsorbent. The pigments were extracted and separated on a MgO–HyfloSupercel column developed with 10–14% acetone in petroleum ether.

The reaction with *trans*-retinal was also run at elevated temperatures (40 and 68 °C) with the aid of a constant-temperature water bath.

For some experiments, Micro-Cel C was washed, prior to exposure of β-carotene, with acetone, petroleum ether, or methanol in a Büchner funnel with 250 mL of the solvent.

Recycling of Micro-Cel C. In an attempt to recycle Micro-Cel C, about 1 mg of β-carotene was exposed to 20 g of the adsorbent for 30 min, the pigments were extracted, and the Micro-Cel C was mixed with a second sample of β-carotene.

Product Identification and Quantitation. The reaction products in each experiment were identified with the aid of adsorption spectroscopy (UV–visible and IR), comparison of rention on TLC and/or HPLC, and/or chemical tests for fuunctional groups as reviewed by Davies (1976). The products were also analyzed by mass spectrometry (Liverpool and F. Leuenberger, F. Hoffmann-La Roche and Co., Ltd.).

Quantitation of the individual products was accomplished by using the extinction coefficient of the parent carotenoid (Davies, 1976) in each reaction and the absorbance at λ_{max} (Rodriguez et al., 1973, 1976).

RESULTS AND DISCUSSION

Reaction of α-Carotene. The major product formed on exposure of α-Carotene to Micro-Cel C was identified as 4-hydroxy-α-carotene by its visible absorption spectrum (λ_{max} at 421, 442, and 473 nm in PE) and by its positive reaction to acetylation with acetic anhydride and dehydration with acidic chloroform. Also detected as minor products were 5,8-epoxy-α-carotene and 4-oxo-α-carotene, but these compounds could be the result of spontaneous oxidation of α-carotene and 4-hydroxy-α-carotene and were not necessarily attributable directly to Micro-Cel C.

It was shown in a previous study (Rodriguez et al., 1976) that the allylic 4-position of β-carotene was efficiently hydroxylated on exposure of β-carotene to Micro-Cel C. In the same manner, marked hydroxylation of α-carotene to 4-hydroxy-α-carotene is now reported to occur on exposure of α-carotene to Micro-Cel C (Table I). Unlike β-carotene, which was also hydroxylated to isozeaxanthin (4,4′-dihydroxy-β-carotene) to a lesser extent, α-carotene was not converted to the dihydroxy derivation nor was it converted to 3′-hydroxy-α-carotene. This suggested that the isolated allylic position of the α-ionone ring of α-carotene was not subject to hydroxylation by Micro-Cel C. Thus, the Micro-Cel C mediated hydroxylation of caro-

Table II. Products from the Reaction of β-Apo-8′-carotenal (0.33 mg, $E_{1cm}^{1\%}$ = 2640) with "Fresh" Micro-Cel C (10 g)

compound	mg	% of recovered[a] product
a, β-apo-8′-carotenal	0.018	20
b, (5,6-epoxy-β-apo-8′-carotenal)	trace	
c, 5,8-epoxy-β-apo-8′-carotenal	0.014	15
d, unidentified	0.019	21
e, unidentified mixture	0.006	6
f, 4-hydroxy-β-apo-8′-carotenal	0.035	38

[a] 28% recovery.

tenoids appeared limited to positions allylic to the conjugated double bond system.

Reaction of β-Apo-8′-carotenal. Exposure of β-apo-8′-carotenal to Micro-Cel C gave, as expected, 4-hydroxy-β-apo-8′-carotenal as the major product. Other products included the 5,6- and 5,8-epoxide derivatives and other as yet unidentified compounds (Table II and Figure 1).

β-Apo-8′-carotenal has the same number of double bonds in conjugation as α-carotene but only one ring and therefore, only one likely position for hydroxylation. The 4-hydroxy derivative was identified by its visible absorption spectrum (λ_{max} at 426, 447, and 476 nm on PE) and by its mass spectrum. The molecular ion, $M^{+}\cdot$, was found at m/z 432 (33%, $C_{30}H_{40}O_2$). A major fragment ion at m/z 414 (100%, M − H_2O) with a metastable, m*, at m/z 397, a very strong loss of water, is indicative of an allylic, C-4 hydroxyl group on the β-ring. The presence of the hydroxyl function was also confirmed by its visible absorption spectrum after dehydration (λ_{max} 450 → 455 nm) and IR absorption a broad band at 3340 cm^{-1}. Also, increased retention time on HPLC (Figure 1) demonstrates an increase in polarity in relation to the parent β-apo-8′-carotenal.

Identification of the 5,8–epoxy derivative was achieved through its visible absorption spectrum (λ_{max} 405, 427.5, and 454 nm). The molecular ion, $M^{+}\cdot$, at m/z 432 (100%, $C_{30}H_{40}O_2$) and characteristic fragment ions at m/z 417 (4%, M − CH_3) with mt at m/z 402.5, m/z 403 (1.5%, M − CHO) with m* at 376, m/z 352 (19%, M − 80) with m* at 287, m/z 205 (45%), and m/z 165 (33%) support this structure. The ions at m/z 205 and 165 and the M − 80 fragmentation are characteristic of epoxy-β-ring carotenoids (both 5,6 and 5,8 derivatives), the absorption spectrum suggesting the 5,8 isomer.

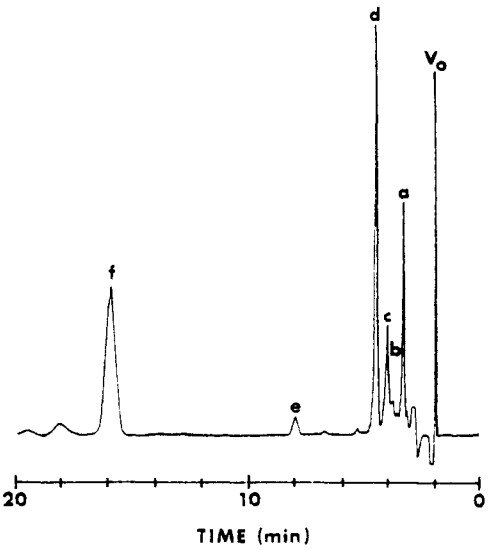

Figure 1. HPLC separation of the products from the reaction of β-apo-8′-carotenal with Micro-Cel C (10% H_2O added). The solvent system used was 7.5% acetone (v/v) in hexane with a flow rate of 1.5 mL/min, detector at λ = 450 nm (see Table II for the key).

Identification of compound b (Table II) as the 5,6-epoxide remains only tenative as we were unable to obtain sufficient pure compound for analysis by MS. The absorption spectrum (λ_{max} at 403, 424, and 451 nm) of this compound is nearly identical with that of the 5,8-epoxide, which suggests isomerization to the more stable 5,8 derivative (known to occur readily under acidic conditions) during isolation. Another possibility is that the Micro-Cel C aided in the formation of cis double bonds in the conjugated system of the 5,8-epoxide. This may account for the observed 4 nm shift in λ^{max} and its slightly shorter retention on HPLC.

Compound d (Table II) has also eluded positive identification at the present time. This compound showed positive response to sodium borohydride reduction (λ_{max} at 433, 444 nm $\xrightarrow{[H]}$ 379, 399, 424 nm $\xrightarrow{[O]}$ 426, 444 nm), indicating retention of the terminal carbonyl. There was a slight increase in polarity observed on HPLC over the parent molecule. The mass spectra data suggests a molecular ion, $M^{+}\cdot$, at m/z 448 (100%, $C_{31}H_{44}O_2$ or possibly $C_{30}H_{40}O_3$). The diagnostic fragments are m/z 430 (28%, M − H_2O), indicative of a hydroxyl group, and m/z 416 (M − 32) with m* at m/z 386. The MS data suggests that the

Table III. Conversion of β-Carotene on Recycled[a] and Dried[b] Micro-Cel C

	amount of carotenoid, μg				
	fresh Micro-Cel C	used Micro-Cel C	dried Micro-Cel C	dried used Micro-Cel C	dried acetone-washed Micro-Cel C
original amount of β-carotene	1048	1300	1594	1594	1594
after exposure to Micro-Cel C					
β-carotene	36	1121	359	128	289
mutatochrome	61	8	64	43	75
echinenone	trace		trace	trace	trace
isocryptoxanthin	490	6	277	353	233
dehydro-β-carotene	trace		266	229	219
isozeaxanthin,[c] 4-hydroxy-5′,8′-epoxy-β-carotene	92	2	34	40	25

[a] β-Carotene was mixed with 20 g of Micro-Cel C and allowed to stand for 30 min under petroleum ether (first column). After extraction of the pigments, the Micro-Cel C was mixed with a second sample of β-carotene (second column). [b] Twenty grams of Micro-Cel C was either dried directly, treated as in footnote a prior to drying, or washed with acetone prior to drying. Drying was at 100 °C for 20 h in an oven. [c] Mixture of isozeaxanthin and 4-hydroxy-5′,8′-epoxy-β-carotene.

Table IV. Products from the Reaction of β-Apo-8′-carotenal (0.33 mg) with Oven-Dried Micro-Cel (9 g) plus 10% Water (1 mL)

compound	mg	% of recovered[a] product
a, β-apo-8′-carotenal	0.071	37
b, 5,6-epoxy-β-apo-8′-carotenal	trace	
c, 5,8-epoxy-β-apo-8′-carotenal	0.009	5
d, unidentified	0.068	36
e, unidentified mixture	0.002	1
f, 4-hydroxy-β-apo-8′-carotenal	0.040	21

[a] 58% recovery.

Table V. Products from the Reaction of Lycopene (5 mg, $E_{1cm}^{1\%}$ = 3450) with Oven-Dried Micro-Cel C (150 g) plus 10% Water (17 mL)[a]

fraction	λ_{max}, nm (petroleum ether)	mg	%	compound
1	440, 457, 485	0.012		unidentified
2	464	0.010		unidentified
3	424, 450, 478	0.090		unidentified
4	*428, 453, 484*	*0.249*	*22.8*	*lycopene 5,6-epoxide*
5	439, 467, 495	0.027		unidentified
6	*455, 478, 513*	*0.136*	*12.5*	*6′-apolycopenal*
7	428, 449, 475	0.064		unidentified
8	440, 450, 465	0.014		unidentified
9	427, 447, 466	0.014		unidentified
10	429, 453, 481	0.051		unidentified
11	*430, 454, 483*	*0.357*	*32.7*	*lycopene-5,6-diol*
12	408, 432, 458	0.067		unidentified

[a] The products were separated by TLC (silica gel G) and the numbers correspond to the relative position on the chromatogram, 1 being the fastest moving component and 12 being the slowest.

aldehyde had undergone acetal formation; however, spectral data conflict, making identification of this compound elusive at the present time.

***trans*-Retinal and β-Ionone.** When *trans*-retinal and β-ionone were each exposed to Micro-Cel C, no reaction was observed. Even at elevated temperature (40 and 68 °C) only what is apparently a cis isomer of retinal could be detected (HPLC, 7.5% v/v, acetone in hexane, 1.5 mL/min). No diagnostic tests were done on this compound as isolation by TLC was difficult. Although unlikely, it is acknowledged that this compound could acutally be the 3,4-dehydro derivative. None of the 4-hydroxy derivative was found, which strongly suggests that the reactivity of carotenoids on Micro-Cel C to yield hydroxylated compounds is dependent upon the length of the polyene chain, the six conjugated double bonds of retinal being insufficient for reaction.

Effect of Drying. Drying Micro-Cel C at 100 °C for 20 h significantly changed the proportion of the products (Table III). The amount of isocryptoxanthin was reduced while the level of dehydro-β-carotene increased markedly. Only trace amounts of dehydro-β-carotene were detected when undried Micro-Cel C was used; the amount increased to over 200 μg on drying.

We have found that Micro-Cel C placed in an oven at 130 °C for 24 h exhibits a weight loss of about 7%. Reactivity is restored when 10% water (w/w) is added back to the oven-dried absorbent after a 30-min equilibration time (Table IV). At 20% water, reactivity is considerably diminished.

Reaction of Lycopene. Lycopene reacted to near completion when exposed to Micro-Cel C (10% H_2O) for 30 min. More than 11 bands could be seen on the TLC plate (Analtech, 1000 μm of silica gel G, developed with 7% acetone in PE). The major components were analyzed by absorption spectroscopy and mass spectrometry. The results can be seen in Table V.

The identification of compound **6** as 6′-apolycopenal was confirmed by comparison of its mass spectrum with that of a semisynthetic sample. The molecular ion, $M^{+\cdot}$, at m/z 442 (40%, $C_{32}H_{42}O$) with key fragment ions at m/z 373 (2%, M − 69) with m* at m/z 314.5 confirming a lycopene end group, m/z 336 (12%, M − 106) indicating an acyclic structure, m/z 350 (0.5%, M − 92), and a base peak at m/z 69 (100%).

Although not confirmed, the evidence strongly suggests that compound **4** is lycopene 5,6-epoxide. Its absorption spectrum λ_{max} is indicative of a 10 conjugated double bond chromophore. Mass spectral analysis gave the molecular ion, $M^{+\cdot}$, at m/z 552 (50%, $C_{40}H_{56}O$) and key fragment ions at m/z 460 (2.5%) and m/z 446 (35%). The ratio of the relative intensities of these two fragment ions (approximately 1:15) is consistent with an acyclic structure. This in turn is supported by the existence of the base peak at m/z 69 (100%). Other diagnostic fragment ions were found at m/z 483 (2%, M − 69) and m/z 467 (1%, M − 69 − O). On the basis of this data, identification as the 5,6-epoxide is reasonable, though not proved.

Tentative identification of compound **11** as lycopene-5,6-diol was based upon its absorption (λ_{max}, 10 conjugated double bonds) and mass spectra. The molecular ion, $M^{+\cdot}$, at m/z 570 (100%, $C_{40}H_{58}O_2$) and key fragment ions at m/z 552 (100%, M − H_2O), m/z 534 (100%, M − $2H_2O$), m/z 478 (28%), m/z 464 (78%), m/z 460 (22%, 478 − H_2O), m/z 446 (63%, 464 − H_2O), m/z 442 (15%, 478 − $2H_2O$), and m/z 428 (34%, 464 − $2H_2O$) are consistent with the

Table VI. Conversion of β-Carotene in Different Solvents[a]

	amount of carotenoid, μg					
	petroleum ether	ethyl ether	acetone	chloroform	ethanol	methanol
original amount of β-carotene	1594	1620	1300	1100	1100	1620
after exposure to Micro-Cel C						
β-carotene	425	1452	1204	803	852	1292
mutatochrome	72	trace	6	trace		
echinenone	trace	trace		trace		
isocryptoxanthin	575	32		133	trace	trace
dehydro-β-carotene	trace					
isozeaxanthin	25			trace		
4-hydroxy-5′,8′-epoxy-β-carotene	12			24		
unidentified					18	30

[a] Mixtures consisting of the specified amount of β-carotene, 20 g of Micro-Cel C, and 100 mL of the solvent were allowed to stand for 30 min. The pigments were extracted with acetone, transferred to petroleum ether, and separated on a MgO-HyfloSupercel column.

proposed structure. The very strong losses of one and two molecules of water from $M^{+\cdot}$, m/z 478, and m/z 464 would be expected from the 5,6-diol since the hydroxyl group at C-6 is allylic to the conjugated system and water would very readily be eliminated, thus causing the hydroxyl at C-5 to be allylic, which, in turn, would readily undergo elimination. The complete absence of the M − 69 fragmentation ion by cleavage of the 3,4 or 3′,4′ bonds is, however, surprising. Therefore, identification of this compound remains tentative.

An interesting point of note is that the three compounds that constitute the majority of product (compounds **4**, **6**, and 11; 68% of total) can be depicted as derivatives of each other according to the reaction scheme lycopene → lycopene 5,6-epoxide → lycopene-5,6-diol → 6′-apolycopenal + [6-methyl-5-hepten-2-one].

This is also the first reported molecular cleavage on Micro-Cel C. Cyronak et al. (1978) reported cleavage of the 13,14- and 11,12-monoepoxide derivative of canthaxanthin on magnesia. Siliceous adsorbents were noted to alter the epoxide groups of neoxanthin and violaxanthin by Strain et al. (1967), who demonstrated conversion of the 5,6-epoxides to the 5,8- epoxides but reported no cleavage products. It should be noted that isomerization of the 5,6- to the 5,8-epoxide is known to occur readily under acid conditions.

Recycling Micro-Cel C. Micro-Cel C could only effectively be used as hydroxylating agent for carotenoids if recycling of the used adsorbent is possible. Table III shows that virtually no conversion took place on Micro-Cel C that had been used in a previous reaction. This suggested at first glance that the hydroxylating agent or a necessary reagent in Micro-Cel C was consumed by the first exposure of β-carotene. In succeeding experiments, however, it became clear that the conversion was stopped by virtue of the acetone used to extract the carotenoids produced during the first exposure.

Micro-Cel C, which had been washed with acetone, failed to carry out the hydroxylation reaction. Two interpretations could be given for this observation: (1) acetone washed off a necessary reagent from Micro-Cel C and (2) acetone inactivated the hydroxylating agent or the reactive sites on the adsorbent.

Inactivation of Micro-Cel C was not limited to acetone. Washing Micro-Cel C with methanol produced the same effect.

Solvent Effect. Of the solvents tested, PE proved to be the best solvent for the conversion (Table VI). Acetone completely inhibited the hydroxylation reaction. Although isocryptoxanthin was formed when the reaction was carried out in chloroform or ether, the amounts formed were relatively small. Methanol or ethanol not only prevented the hydroxylation of β-carotene but also changed the course of the reaction to yield another as yet unidentified product.

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Registry No. Micro-Cel C, 1344-95-2; α-carotene, 7488-99-5; β-carotene, 7235-40-7; β-apo-8′-cartenal, 1107-26-2; lycopene, 502-65-8; ethyl ether, 60-29-7; chloroform, 67-66-3; acetone, 67-64-1; ethanol, 64-17-5; methanol, 67-56-1; 5,8-epoxy-α-carotene, 88314-76-5; 4-oxo-α-carotene, 3297-23-2; 4-hydroxy-α-carotene, 61825-48-7; 5,8-epoxy-β-apo-8′-carotenal, 41548-57-6; 4-hydroxy-β-apo-8′-carotenal, 88253-14-9; mutatochrome, 515-06-0; isocryptoxanthin, 472-62-8; isozeaxanthin, 29065-03-0; 4-hydroxy-5′,8′-epoxy-β-carotene, 59173-49-8; lycopene 5,6-epoxide, 51599-10-1; 6′-apolycopenal, 22255-36-3; lycopene-5,6-diol, 66803-17-6.

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