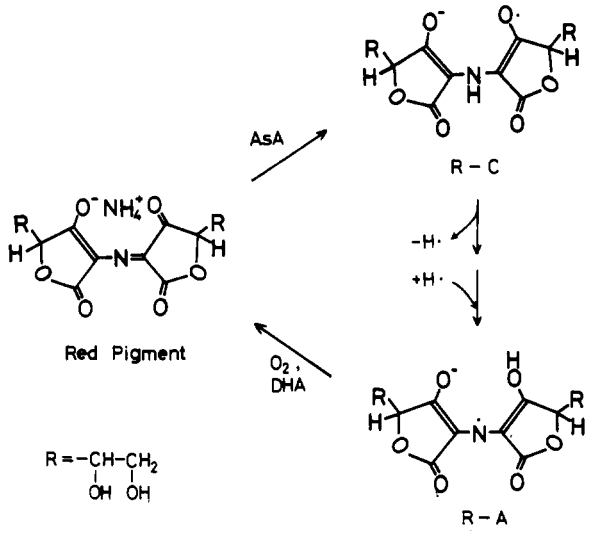

Figure 10. Possible formation mechanism of the free-radical products (Yano et al., 1974).

residue (Ranganna and Setty, 1974), from the results described above. If the structure of R-A could be coplanar, the unpaired electron on nitrogen might be able to conjugate through a long range with the carbonyl groups in DHA moieties; this could explain the abnormally high stability of R-A as a radical product. In addition it should be pointed out that the triplet spectrum of R-A is always accompanied by a minor signal in their wings as shown in Figure 4. The details of this spectrum are not yet clear; however, because of its abnormal intensity and splitting pattern, it may be due to some other product closely resembling R-A. Further investigations on the structure of R-B as well as the details of the properties and the reaction process of R-A and R-C are being undertaken. This study has revealed that the fairly stable free-radical products could easily be formed by the reaction of DHA and α-amino acids, both of which are present generally in foods and biological systems, and it is of interest in relation to a possible antioxidative action of free-radical products and to various important effects of ascorbic acid in biological systems.

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LITERATURE CITED

Euler, H. v., Hasselquist, H., *Ark. Kemi* 8, 67 (1954).
Kenyon, J., Munro, N., *J. Chem. Soc.*, 158 (1948).
Kurata, T., Fujimaki, M., Sakurai, Y., *Agric. Biol. Chem.* **37**, 1471 (1973).
Laroff, G. P., Fessenden, R. W., Schuler, R. H., *J. Am. Chem. Soc.* **94**, 9062 (1972).
Mitsuda, H., Yasumoto, K., Yokoyama, K., *Agric. Biol. Chem.* **29**, 751 (1965).
Müller-Mulat, W., *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 52 (1970).
Namiki, M., Hayashi, T., *J. Agric. Food Chem.* **23**, 487 (1975).
Namiki, M., Yano, M., Hayashi, T., *Chem. Lett.*, 125 (1974).
Orr, J. C., *Nature (London)* **205**, 1008 (1965).
Ranganna, S., Setty, L., *J. Agric. Food Chem.* **22**, 719 (1974).
Yano, M., Hayashi, T., Namiki, M., *Chem. Lett.*, 1193 (1974).
Yeferov, V. P., Froncish, W., Kharitonenkov, I. G., Kalmanson, A. E., *Biochim. Biophys. Acta* **200**, 160 (1970).

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Hydroxylation of β-Carotene on Micro-Cel C

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β-Carotene underwent substantial hydroxylation to isocryptoxanthin (4-hydroxy-β-carotene) when brought in contact with Micro-Cel C, an adsorbent long presumed to be inert. The reaction was rapid, the maximum accumulation rate of isocryptoxanthin occurring within the first 15 min. The extent of hydroxylation depended directly on the amount of Micro-Cel C; the percent yield of 1 mg of β-carotene ranged from 23% with 10 g of Micro-Cel C to 65% with 30 g of Micro-Cel C. Isozeaxanthin (4,4'-dihydroxy-β-carotene) was also formed but at markedly lower amounts. Very small quantities of mutatochrome, echinenone, 4-hydroxy-5',8'-epoxy-β-carotene, and dehydro-β-carotene were also detected. The hydroxylating property of Micro-Cel C was not observed in other adsorbents such as silica gel, kieselguhr, Celite, alumina, HyfloSupercel, and MgO.

Micro-Cel is Johns-Manville's registered trade name for synthetic, hydrous calcium silicates produced by the hydrothermal reaction of diatomaceous silica with hydrated lime and water (Johns-Manville Product Corp., 1969). This product has been advertised as an inert powder with a broad range of applications in the chemical, agricultural, pharmaceutical, and food industries, being used as carriers, grinding aids, conditioning agents, etc. Micro-Cel C is one of several Micro-Cel grades available for specific needs.

On the basis that Micro-Cel C is an "inert" material, it has been widely used as an adsorbent for column chromatography in carotenoid research, particularly in the separation of xanthophylls. Contrary to this long standing assumption, we wish to report that β-carotene and other carotenoids can actually undergo substantial hydroxylation and some oxidation in the presence of Micro-Cel C. The data reported here will automatically raise serious doubts on the natural occurrence of certain carotenoids hitherto reported as inherent minor constituents of microbial, animal, and plant tissues. Moreover, considering the wide applicability of Micro-Cel powders in manufactured goods,

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our results could have far reaching implications in industry.

Hydroxylation of β-carotene on Micro-Cel C will be shown to occur at the 4 position, a reaction that to date has not been easily accomplished by other means. Treatment of a carbon tetrachloride solution of β-carotene with *N*-bromosuccinimide (NBS) in the presence of glacial acetic acid yields upon hydrolysis the monohydroxy and dihydroxy β-carotene compounds (Entschel and Karrer, 1958a,b). Alternatively, addition of BF_3-etherate to β-carotene in chloroform results in a blue complex which can be subsequently hydrolyzed with water to yield isocryptoxanthin (Petracek and Zechmeister, 1956; Wallcave and Zechmeister, 1953). These two chemical processes, however, are considerably less efficient than the conversion of β-carotene on Micro-Cel C. The present reaction can therefore be of research and commercial importance.

For this study we have identified the compounds formed, determined some of the factors that can affect the degree of conversion, and tested other adsorbents for similar transformations.

EXPERIMENTAL SECTION

Purification and Crystallization of β-Carotene. Since β-carotene in the form of crystals is rather unstable, it was separated from decomposition products immediately preceding each experiment by passage through a MgO column developed with 4–5% acetone in petroleum ether. Purification on this column was repeated (two or three times) until only the single band corresponding to β-carotene remained and a single spot was seen on a silica gel sheet (Eastman Chromagram Sheet 13179) developed with 3% methanol in benzene. Crystallization was accomplished with petroleum ether and ethanol. The resulting β-carotene crystals were tested each time and found not to yield conversion products when run through a MgO–HyfloSupercel (1:2) column with 10–25% acetone in petroleum ether as solvent.

Exposure to Micro-Cel C. Crystallized β-carotene dissolved in petroleum ether was either passed through a column of Micro-Cel C with 2% acetone in petroleum ether as developing solvent or allowed to stand with Micro-Cel C in an Erlenmeyer flask. The Micro-Cel C column was run until the band of residual β-carotene had been eluted. The column was allowed to run dry and the entire adsorbent, which contained yellowish pigments diffused throughout, was extracted with acetone. The pigments were transferred to petroleum ether, dried over sodium sulfate, and separated on a MgO–HyfloSupercel (1:2) column developed with 25% acetone in petroleum ether. Pigments eluted from the column as mixtures were resolved on silica gel plates developed with 3% methanol in benzene for identification purposes. Separation of the carotenoids derived from Micro-Cel C was also tried on an alumina (activity grade III) column, with increasing concentration of acetone in petroleum ether as solvent, to ascertain that no new compounds could be formed on the MgO–HyfloSupercel column and claimed as conversion products of Micro-Cel C.

Alternatively, Micro-Cel C was mixed and allowed to stand with β-carotene for a specified time period and then extracted with acetone. The pigments were likewise transferred to petroleum ether, dried over sodium sulfate, and separated on the MgO–HyfloSupercel column. Exposure of β-carotene to other adsorbents was undertaken in the same manner.

Identification of the Conversion Products. Identification of the carotenoids was based on the absorption spectra, polarity (position on the column, TLC R_f values, co-chromatography with authentic carotenoids) and chemical reactions. Details of the identification procedure and the quantitative determination had been described elsewhere (Rodriguez et al., 1973). Since extinction coefficients for all individual carotenoids were not available and the minor oxidation products were collected as mixtures, the quantitative data were based on the extinction coefficient of β-carotene and were therefore approximations.

RESULTS AND DISCUSSION

Conversion Products of β-Carotene. β-Carotene, when passed through a column of Micro-Cel C or when allowed to stand in contact with Micro-Cel C, produced a number of hydroxy, epoxy, and keto carotenoids. Six products were detected whether the pigments were separated on a MgO–HyfloSupercel column or an alumina column. The first product had the absorbance (λ_{max} at 404, 425, and 450 nm in petroleum ether) and other properties of mutatochrome. It adsorbed just above echinenone on the silica gel sheets. The absorbance was unaffected by the addition of dilute HCl to an ethanolic solution of the pigment, but chromatograms of the pigment (yellow) turned intense blue on exposure to HCl gas. Product 2, a keto carotenoid, co-chromatographed with authentic echinenone and exhibited an asymmetric peak with the maximum at 453 nm and a shoulder at 474 nm in petroleum ether. Reduction of the pigment with $NaBH_4$ changed the color from orange to yellow, increased the polarity, and transformed the single peak to three peaks (λ_{max} at 424, 447, and 475 nm). The reduced compound was identified as isocryptoxanthin. The third compound, the major product, was identified as isocryptoxanthin by its absorbance (λ_{max} at 424, 447 and 475 nm in petroleum ether), co-chromatography with authentic isocryptoxanthin while being separable from cryptoxanthin, and positive reaction to acetylation, methylation, and dehydration. The fourth product showed maximum absorptions at 437, 463, and 490 nm in petroleum ether, indicating an extended chromophore, and ran at the solvent front on the silica gel sheets developed with 3% methanol in benzene, showing that it was devoid of substituents. These properties are consistent with a dehydro-β-carotene compound. Product 5, which absorbed maximally at 404, 425, and 450 nm, appeared to be 4-hydroxy-5′,8′-epoxy-β-carotene. The presence and location of the hydroxy group were ascertained by the pigment's positive response to acetylation, methylation, and dehydration. The furanoid character was shown by its absorbance and by exposure of the chromatograms to HCl gas which changed the yellow color to blue. Addition of dilute HCl to the pigment in ethanol did not alter the absorbance. The sixth product was identified as isozeaxanthin (λ_{max} at 425, 447, and 474 nm in petroleum ether). It co-chromatographed with authentic isozeaxanthin but not with zeaxanthin and lutein, and reacted positively to acetylation (producing two acetylated compounds), methylation, and dehydration.

Conversion on Column. The presence of conversion products was first noted on Micro-Cel C columns. The products, however, did not appear as distinct bands but were diffused throughout the zone travelled by β-carotene. In the analysis of complex, natural mixtures, they are likely to be masked by the bands of major carotenoid constituents and thus go undetected. This could explain why the activity of this adsorbent remained unreported for a long time.

Table I shows the amounts of products formed from β-carotene on two sizes of Micro-Cel C columns. The sum total of the unreacted substrate and the reaction products was not expected to account for the total input of β-

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

	Amount of carotenoid, μg		
	1st small column (2.5 × 20 cm)[b]	2d small column (2.5 × 20 cm)[b]	Large column (5 × 25 cm)[b]
Original amount of β-carotene	2916	1591[c]	5584
After passing through Micro-Cel C column:			
β-Carotene	1591	819	2454
Mutatochrome[d] / Echinenone[d]	Trace	24	38
Isocryptoxanthin	559	537	1625
Dehydro-β-carotene[e] / Isozeaxanthin[e] / 4-OH-5′,8′-epoxy-β-carotene[e]	36	69	117
% yield of isocryptoxanthin	19	34	29

[a] The columns were run under 12-in. vacuum with 2% acetone in petroleum ether as solvent. Running time was 20 min for the first column and 30 min for the second and third columns. The carotenoids were subsequently separated on a MgO–HyfloSupercel column developed with 25% acetone in petroleum ether. [b] Measurements given refer to actual dimensions of the adsorbent, not the glass column. [c] β-Carotene recovered from the first column was run through the second column. [d] Mixture in which mutatochrome was predominant. [e] Mixture in which 4-hydroxy-5′,8′-epoxy-β-carotene and isozeaxanthin were predominant.

carotene for at least three reasons: (1) use of the β-carotene extinction coefficient for all carotenoids tended to underestimate the quantity of the products; (2) recovery from the adsorbents of carotenoids, especially the more polar products, is always incomplete; and (3) breakdown of the rather unstable carotenoids to smaller fragments could occur during the reaction, the separation, and the extraction.

Conversion of β-carotene to isocryptoxanthin, the major reaction, was substantial and directly dependent on the size of the column or the amount of Micro-Cel C. Passing the unreacted β-carotene recovered from one column through another column continued the conversion process but at a higher efficiency. While the initial amount of β-carotene of the first column was about twice that of the second column, the isocryptoxanthin yields were nearly equal. Therefore, a substrate-to-adsorbent ratio above that of the second column should provide saturation of the active sites. The running time was 10 min longer for the second column but, as it will be shown later, the time difference was apparently not a critical factor after the first 15 min.

The amount of isozeaxanthin produced was markedly lower than isocryptoxanthin. Hence the allylic position of the second β-ionone ring appeared to be less susceptible to hydroxylation.

The reactions which apparently occurred on exposure of β-carotene to Micro-Cel C are summarized in Figure 1. The limited oxidation to echinenone and the limited epoxidation reactions may occur spontaneously and may not be attributed directly or solely to Micro-Cel C. The noticeably higher amount of 4-hydroxy-5′,8′-epoxy-β-carotene and the slightly higher level of echinenone in the bigger column might only be due to the production of more isocryptoxanthin from which these compounds could be derived, and not necessarily to the larger amount of Micro-Cel C.

Conversion of β-Carotene as a Function of Time and Amount of Micro-Cel C. Batch-type experiments confirmed the dependence of the yield of isocryptoxanthin on the mass of adsorbent (Table II). The percent yield increased from 23% with 10 g of Micro-Cel C to 40% with 20 g of Micro-Cel C and 65% with 30 g of Micro-Cel C.

The time course study indicated that the greatest rate of isocryptoxanthin formation occurred within the first 15 min. Extending the duration of contact between β-carotene and Micro-Cel C to 30 min and 1 h increased the amount of isocryptoxanthin moderately. A distinct increase in the other products was observed after 1 h.

Conversion on Other Adsorbents. Table III demonstrates that the efficient hydroxylation of β-carotene to isocryptoxanthin is unique to Micro-Cel C. Although isocryptoxanthin was detected on silica gel, kieselguhr, and Celite, the amount found was negligible. Isocryptoxanthin was not detected on HyfloSupercel, MgO, and alumina.

Table III agrees with previous observations that the recovery of carotenoids from different adsorbents varies with the adsorbent used and the polarity of the carotenoid.

Table II. Conversion of β-Carotene as a Function of the Amount of Micro-Cel C[a] and Time of Exposure[b]

	Amount of carotenoid, μg					
	10 g of Micro-Cel	20 g of Micro-Cel	30 g of Micro-Cel	15 min	30 min	1 h
Original amount of β-carotene	1093	1093	1093	1077	1077	1077
After exposure to Micro-Cel C:						
β-Carotene	531	59	25	510	418	262
Mutatochrome[c] / Echinenone[c]	20	28	34	17	24	30
Isocryptoxanthin	246	437	714	234	304	314
Dehydro-β-carotene[d] / Isozeaxanthin[d] / 4-OH-5′,8′-epoxy-β-carotene[d]	35	43	52	43	45	69
% yield of isocryptoxanthin	23	40	65	22	28	29

[a] Mixtures consisting of an equal amount of β-carotene, the specified amount of Micro-Cel C, and enough petroleum ether to cover were allowed to stand for 15 min. The carotenoids were then extracted and separated on a MgO–HyfloSupercel column with 25% acetone in petroleum ether as solvent. The values listed are the averages of two trials. [b] Mixtures consisting of 1077 μg of β-carotene, 10 g of Micro-Cel C, and 50 ml of petroleum ether were allowed to stand for the specified time period. The carotenoids were then extracted and separated as described above. [c] Mixture in which mutatochrome was predominant. [d] Mixture in which the epoxide and isozeaxanthin were predominant.

Table III. Conversion of β-Carotene on Other Adsorbents[a]

	Amount of carotenoid, μg					
	Silica gel	Kieselguhr	Celite	HyfloSupercel	MgO	Alumina
Original amount of β-carotene	1564	1300	1300	1828	1828	1524
After exposure to adsorbent:						
β-Carotene	1099	880	1048	1808	1468	982
Mutatochrome	6	9	11			
Isocryptoxanthin	6	11	10			
Mixture with epoxides predominating (R_f values lower than that of isocryptoxanthin)	11	3	3			
Mixture with epoxides predominating (R_f values equal or higher than that of isocryptoxanthin)				5[b]	16[b]	4[b]

[a] The specified amount of β-carotene was mixed and allowed to stand for 30 min with 20 g of the adsorbent and enough petroleum ether to cover. The carotenoids were extracted and separated on a MgO–HyfloSupercel column developed with 25% acetone in petroleum ether. [b] The small amounts of conversion products on these adsorbents did not form distinct bands on the MgO–HyfloSupercel column and were therefore extracted as a whole after elution of β-carotene.

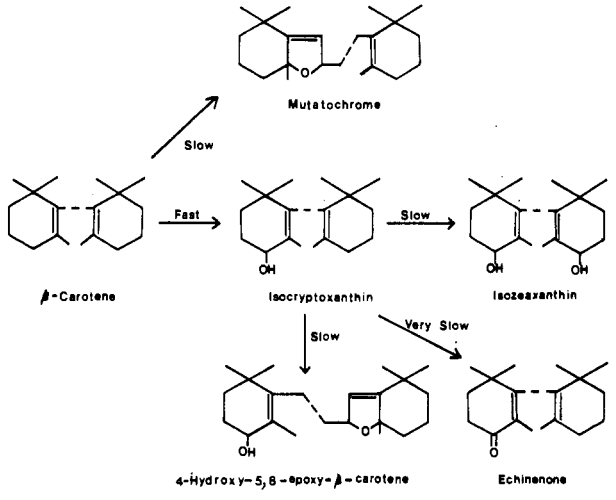

Figure 1. Proposed reactions of β-carotene on Micro-Cel C.

At the present time, the mechanism of the hydroxylation reaction on Micro-Cel C has not been elucidated. The presence of trace amounts of dehydro-β-carotene may indicate that the Micro-Cel C reaction follows the same route as the NBS–acetic acid and the BF_3 methods mentioned above, both of which have been reported to proceed through dehydro-β-carotene as an intermediate (Entschel and Karrer, 1958a,b; Petracek and Zechmeister, 1956; Wallcave and Zechmeister, 1953). Catalytic dehydrogenation of β-carotene can conceivably occur on the surface of Micro-Cel C as a step prior to hydroxylation. Dehydro-β-carotene is known to be a dehydration product of isocryptoxanthin under acidic conditions but this possibility is not compatible with the alkaline pH of Micro-Cel C.

While investigators in this area are encouraged to carefully evaluate analytical and biosynthetic research in which Micro-Cel C has been employed, they are cautioned not to automatically invalidate such studies. High amounts of pure β-carotene were used in the present study; thus, isocryptoxanthin and other conversion products were easier to detect and determine quantitatively. When actual samples are analyzed, the conversion products can pass unrecognized due to their lower quantities and their diffused distribution on the Micro-Cel C column. On the other hand, the natural occurrence of low levels of hydroxylated carotenoids and the other carotenoids reported here and the absolute values of these carotenoids may be of doubtful validity. Our laboratories have therefore withdrawn papers accepted for publication dealing with the isolation of small amounts of isocryptoxanthin, isozeaxanthin and echinenone from *Phycomyces blakesleeanus* and *Rhodotorula aurantiaca*.

α-Carotene and lycopene were also observed to undergo similar transformations on Micro-Cel C. Thus, the effect of Micro-Cel C on carotenoids is a general reaction not just limited to β-carotene. Studies on other carotenoids and on the mechanism of hydroxylation will be reported separately.

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LITERATURE CITED

Entschel, R., Karrer, P., *Helv. Chim. Acta* **41**, 402 (1958a).
Entschel, R., Karrer, P., *Helv. Chim. Acta* **41**, 983 (1958b).
Johns-Manville Product Corp., "Celite and Micro-Cel Functional Fillers", Johns-Manville Research and Development Center, Denver, Colo., 1969.
Petracek, F. J., Zechmeister, L., *J. Am. Chem. Soc.* **78**, 3188 (1956).
Rodriguez, D. B., Simpson, K. L., Chichester, C. O., *Int. J. Biochem.* **4**, 213 (1973).
Wallcave, L., Zechmeister, L., *J. Am. Chem. Soc.* **75**, 4495 (1953).

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