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Formation of Free-Radical Products by the Reaction of Dehydroascorbic Acid with Amino Acids

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Short time heating of a mixture of dehydroascorbic acid with amino acid in water or ethanol gives fairly stable radical products. The ESR spectrum obtained in ethanol was composed of two kinds of spectra, a triplet (spectrum A) and a quintet–doublet (spectrum B), while that obtained in the aqueous system was composed of spectrum A and another multiplet (spectrum C). The radical products were separately detected on TLC, where that of spectrum A (R-A) was in a blue spot and that of spectrum C (R-C) was in the vicinity of the red pigment. Based on the chemical and spectral properties of these radical products, the structures and mechanism of formation of them are discussed in relation to those of the red pigment.

The browning reaction of dehydroascorbic acid (DHA) with amino acid is well known as it develops a wine red color and causes a deterioration in the quality of some foods. There have been a number of studies on this color development; recently the structure and the mechanism of formation of the red pigment have been proposed (Kurata et al., 1973; Ranganna and Setty, 1974). Additionally, there have been some studies on the development of free-radical products in the reaction of amino compounds with carbonyl compounds relating to ascorbic acid, for example, the stable radicals in melanoidin as the browning reaction products from glucose and glycine (Mitsuda et al., 1965), the unstable free radicals in the reaction of ninhydrin with amino compounds (Orr, 1965; Yeferov et al., 1970), and a radical intermediate in a redox reaction between ascorbic acid and dehydroascorbic acid (Laroff et al., 1972).

Recently, the authors have found the development of free radicals in various amino–carbonyl reactions. Sugar–amino acid or amine systems gave unstable free-radical products in an early stage of the reaction (Namiki and Hayashi, 1975). On the other hand, the reactions of DHA with various amino acids or amines have been found to provide fairly stable free radicals of a different type (Namiki et al., 1974; Yano et al., 1974); this paper is concerned with the details of the formation and isolation of the radical products from DHA and amino acids, and moreover with some speculation on their structure and formation mechanism, in relation to those of the red pigment.

MATERIALS AND METHODS

DHA was prepared from L-ascorbic acid (AsA) using *p*-benzoquinone as an oxidizing reagent (Euler and Hasselquist, 1954; Müller-Mulat, 1970). Barium 2,3-diketogulonate was prepared from DHA (Kenyon and Munro, 1948), and the free acid was obtained by desalting with Dowex 50-W. AsA, amino acids, and other reagents used were guaranteed grade.

The reactions of DHA with amino acids were done in Pyrex test tubes using distilled water or purified 95% ethanol as a medium, and the ESR spectrum was measured in a quartz tube with a JES-ME-1X ESR spectrometer. The splitting constant and g value of the ESR spectrum were determined by means of potassium peroxylamine disulfonate as a standard. Since the ESR signal is recorded as the first derivative, the concentrations of free-radical products were measured for convenience as the intensity relative to the standard, polycrystalline Mn^{2+}. Thin-layer chromatography (TLC) was performed on microcrystalline cellulose (Avicel) with ethyl acetate–pyridine–water (7–10:4:3) as solvent. Ninhydrin and 2,4-dinitrophenylhydrazine were used for visualization of colorless substances. The densitometry was performed with a Shimazu dual-wavelength TLC scanner CS-900. Uv and visible spectra of solutions were measured with a Hitachi EPS-3T spectrometer.

RESULTS AND DISCUSSION

Development of ESR Spectra of the Reaction Mixtures of DHA and Amino Acids. A mixture of DHA and α-Ala (each 1 M) in 95% ethanol was heated in a boiling water bath. The reaction mixture turned pink immediately, then wine red, and gradually brown red with further heating. Simultaneously, the characteristic ESR signal developed and increased rapidly at an early stage of the reaction (Figure 1a); prolonged heating caused little change in the shape of the spectrum.

The same reaction in an aqueous system gave similar changes in the development of color and ESR signal, although both proceeded far more quickly than those in

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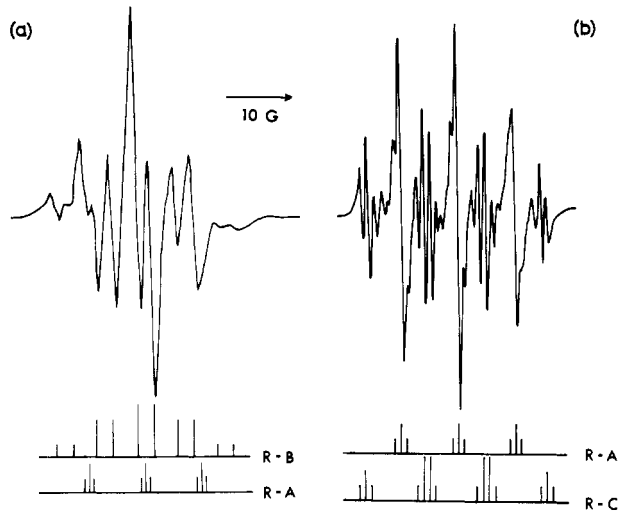

Figure 1. ESR spectra of the free-radical products formed by the reaction of DHA with α-Ala: (a) in EtOH, (b) in water (Namiki et al., 1974).

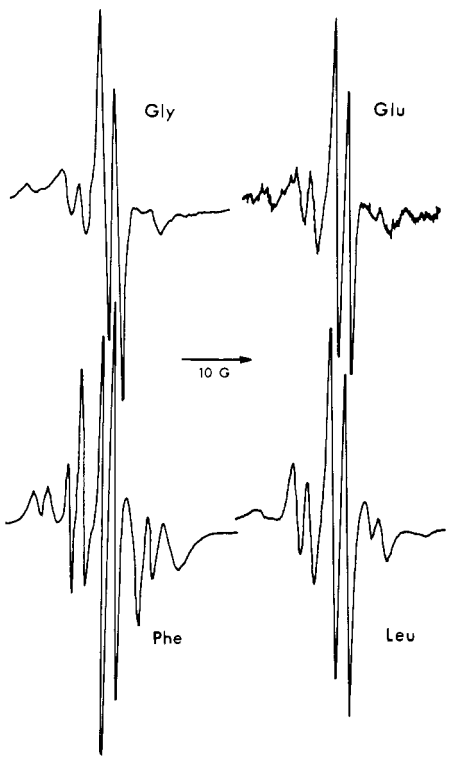

Figure 2. ESR spectra of the free-radical products formed by the reaction of DHA with various α-amino acids in EtOH.

ethanol, and the ESR spectrum showed somewhat different features as illustrated in Figure 1b. The ESR spectra in each solvent were found to be a mixture of several signals, that is, the spectrum in the ethanol system is assumed to be composed mainly of a triplet signal (spectrum A) and a quintet–doublet signal (spectrum B), while that in the aqueous system is composed of spectrum A and another multiplet signal (spectrum C) as shown by the stick lines in each figure; the radical products corresponding to them are termed R-A, R-B, and R-C, respectively.

Effect of Structure of the Materials on the Formation of Radical Products. Figure 2 shows the spectra observed in the reactions of DHA with the α-amino acids Gly, Phe, Leu, and Glu, in the ethanol system; here it should be noted that these spectra are essentially the same.

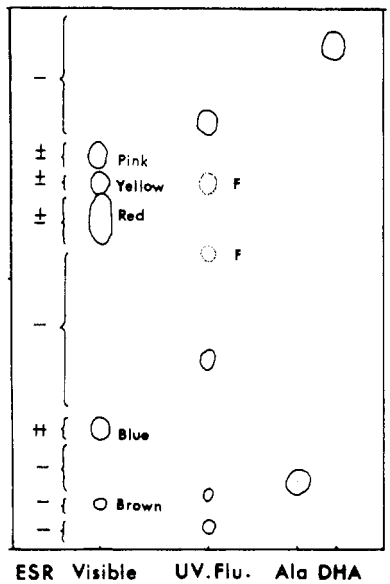

Figure 3. Thin-layer chromatogram of the reaction product of DHA with α-Ala in EtOH; Avicel plate, ethyl acetate-pyridine-water (7:4:3).

The development of the ESR signal could also be observed when similar reactions were done with β-amino acids, alkylamines, and ammonia, although the spectral patterns were different from those of α-amino acids; the details will be reported elsewhere.

On the other hand, there remained the possibility that the free-radical products could be caused by the contamination of compounds other than DHA such as AsA, *p*-benzoquinone, and 2,3-diketogulonic acid, which is a main decomposition product of DHA. The reactions of α-Ala with these compounds gave no measurable ESR signal, indicating that free-radical products are formed by the reaction of DHA with amino acids.

Isolation of the Radical Products by TLC. The reaction mixture of DHA with α-Ala in the ethanol system heated in a boiling water bath for several minutes was subjected to TLC. A typical chromatogram is shown in Figure 3. Among the several spots that appeared, a most definite one was the red pigment (Kurata et al., 1973), below which a blue spot of unknown nature was observed. Each colored band on preparative TLC was scraped off and was subjected to ESR spectrometry without treatment. Interestingly, an intense ESR signal could be detected on the scraping of the blue band and a weak one on that of the band neighboring the red pigment. The water extracts of each scraping gave the hyperfine ESR spectra (Figures 4 and 5), which were found to correspond well to those termed as spectra A and C, respectively. A similar TLC treatment was made on the reaction mixture employed in the aqueous system and, in this case, R-C was also detected on the scraping of the band neighboring the red pigment. However, neither the blue band nor R-A was detected on TLC, perhaps because of its instability in the aqueous system.

Effects of Various Reaction Conditions on the Formation of Radical Products. The effects of oxygen, pH, and temperature on the formation of free radicals were each examined by the reaction of DHA with α-Ala in the aqueous system, and the relative intensities of R-A and R-C were measured at the modulation width of 0.5 G. In experiments to examine the process of formation of the reaction products and the effect thereon of ascorbic acid in ethanol medium, the intensities of the signals of radical products in the original solution or of the isolated one on

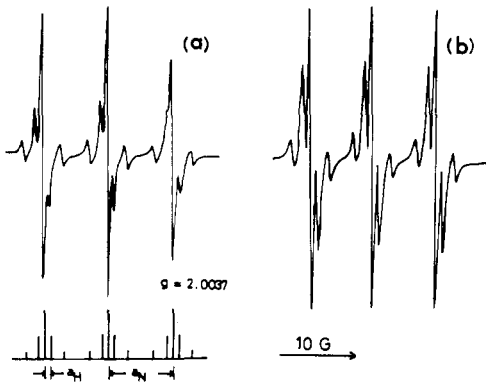

Figure 4. ESR spectrum of the extract of the blue spot on TLC of the reaction mixture of DHA with α-Ala in EtOH: (a) water extract, a_N = 8.45 G (t), a_H = 0.81 G (d), a_H = 0.81 G (d); (b) spectrum synthesized from the hyperfine pattern indicated by the stick line in (a), assuming the ratio of R-A and unknown accompanying radical species in the wings of R-A is 5000:1500 (Yano et al., 1974).

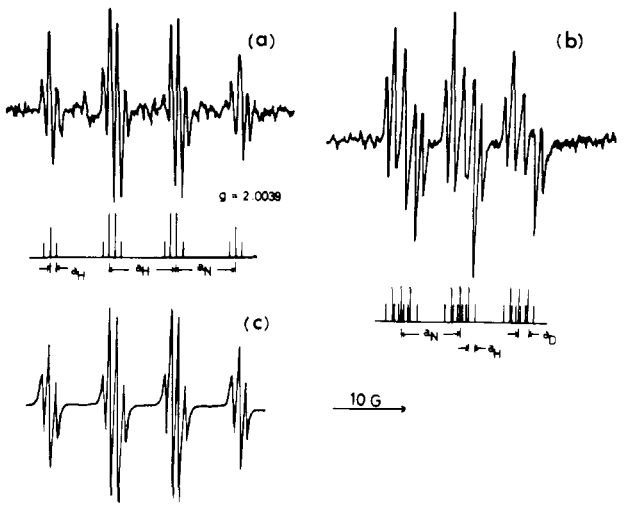

Figure 5. ESR spectrum of the extract in the vicinity of the red spot on TLC of the reaction mixture of DHA with α-Ala in EtOH: (a) water extract, a_{N-H} = 9.18 G (d), a_N = 8.27 G (t), a_H = 0.92 G (d), a_H = 0.92 G (d); (b) D_2O extract, a_{N-D} = 1.41 G (t); (c) spectrum synthesized from the hyperfine pattern indicated by the stick line in (a) (Yano et al., 1974).

TLC (R-A) were measured at 5 G. The amounts of the red pigment and the blue substance were estimated by measuring the density of their spots on the TLC at 520 and 635 nm using a dual-wavelength scanner.

Effect of Oxygen. Oxygen is often known to play an important role in the formation and/or stability of free-radical products. In order to examine these effects of oxygen on the DHA–amino acid system, the reaction of DHA and α-Ala in the aqueous system was done in an open tube and in a closed one which had previously been evacuated at 10^{-4} mmHg in a frozen state. Consequently, there was no significant difference between them in the shape and amplitude of the ESR spectrum. The reaction products were equally stable whether kept in air or in vacuo. This seems to indicate that oxygen does not influence the formation of the radical products or their stability as long as they are kept in the reaction mixture. However, as is noted later, the isolated radical product is unstable especially in the presence of air.

Effect of pH. The reactions of DHA and α-Ala, each 1.5 M in the aqueous system, were done at 80 °C and at

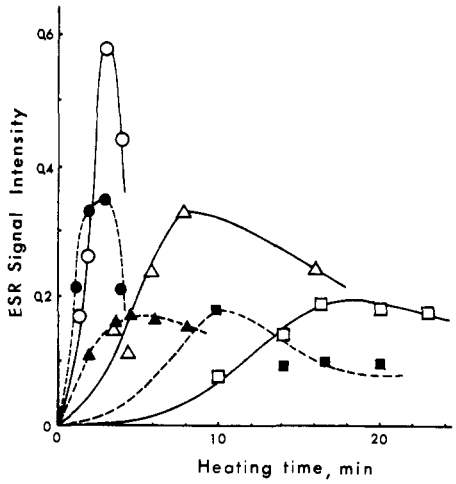

Figure 6. Effect of temperature on the development of the free-radical products, DHA and α-Ala in water (each 1.2–1.3 M), heated in a water bath at 70, 85, or 95 °C: 95 °C, R-A (-○), R-C (---●); 85 °C, R-A (-△), R-C (---▲); 70 °C, R-A (-□), R-C (---■).

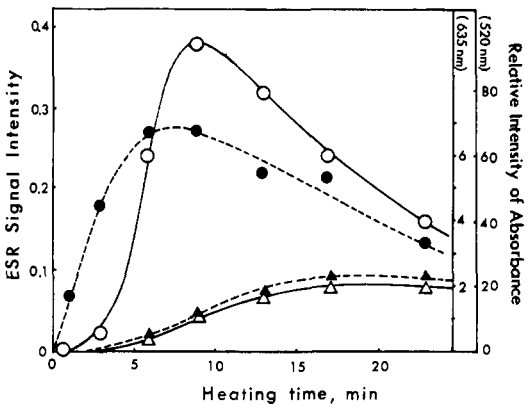

Figure 7. Formation of products by the reaction of DHA with α-Ala in EtOH (each 1 M), heated in a boiling water bath: free radicals in the reaction mixture (-○); R-A (-△); red pigment (---●); blue substance (---▲).

constant pH values by using a pH stat. The results indicate that the development of the radical products A and C was greatly affected by pH during the reaction, that is, the time to reach the maximum concentration was about 20 min at pH 2.3 while it was less than 8 min at pH 4.8, and the maximum intensity increased with increasing pH above 2 and especially above 4, although the development of the radical products was hardly observed in the reactions controlled at pH 6 or above. Thus, the formation of radical products is maximum at around pH 4.8.

Effect of Temperature. As shown in Figure 6 the concentration and rate of development of the radical products were considerably affected by temperature during the reaction. In the case of R-A, the time to reach a maximum radical concentration was less than 3 min at 95 °C, while it was over 15 min at 70 °C with a marked decrease in the maximum concentration. Here, it should be noted that, at 70 °C, the development of R-C not only preceded that of R-A but also showed a maximum peak at an earlier stage; its decrease appeared to be correlated to the increase in R-A.

The Course of the Formation of the Radical Products. Figure 7 shows the course of the formation of the main reaction products of DHA with α-Ala in the ethanol system, namely, the blue substance, the red pigment, total radical amount in the reaction mixture, and isolated R-A. It is evident that the red pigment formed

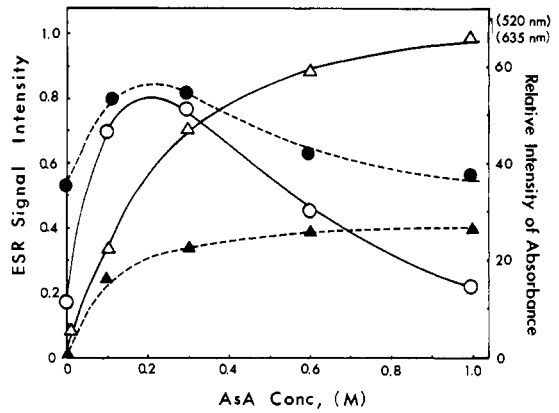

Figure 8. Effect of ascorbic acid on the formation of the radical products by the reaction of DHA with α-Ala in EtOH (each 1 M), each heated for 6 min in a boiling water bath. Refer to Figure 7 for explanation of symbols.

initially and its concentration reached a maximum at about 6 min. After that the formation of the blue substance occurred parallel with that of R-A, indicating that the formation of the red pigment precedes that of the blue substance or R-A. On the other hand, the concentration of total radical products in the reaction mixture increased markedly after 5 min to its maximum and then fell after 10 min with prolonged heating. Although the relative intensities of the ESR signals of the reaction mixture and R-A could not be directly compared with each other, there seems to be a significant difference between their changes especially at an earlier stage, which might be due mostly to the formation and decrease of R-B in the reaction mixture which is labile and could not be detected on TLC.

Effect of Ascorbic Acid on the Formation of Radical Products. It was revealed that the reaction of DHA with α-Ala in the presence of ascorbic acid gave rise to an apparent increase in the concentration of the blue substance as well as R-A as shown in Figure 8; namely, in the presence of 0.5 M ascorbic acid they increased to 8–10 times those in the absence of ascorbic acid. As the addition of ascorbic acid to the reaction products had no effect on the stability of either R-A or the blue substance, their apparent increases may be caused by the participation of ascorbic acid not in the protection of the products against oxidation but in their formation process. In addition, the similarity of behavior of the blue substance and R-A indicated their identity.

Chemical and Spectral Properties of the Isolated Radical Products. The water extract of the blue band, which showed the ESR signal of type A, colored blue and gave the absorption spectrum having maxima at 376 and 630 nm (Figure 9). When the extract was allowed to stand in air the blue color turned red along with a disappearance in the maximum at 630 nm and an increase of the peaks at 376 and 510 nm. On the other hand, the ESR signal of R-A decreased rapidly and disappeared almost completely after several hours in air at room temperature, although it remained considerably longer in nitrogen. The addition of a slight amount of *p*-benzoquinone to the water extract of the blue substance similarly caused the color to change to red instantaneously, resulting in the disappearance of the blue spot and the development of the red pigment on the TLC along with a complete disappearance of the ESR signal. From these results, the blue substance, or R-A, is assumed to be oxidized to the red pigment by oxygen or other weak oxidizing agents such as *p*-benzoquinone. There seemed to be some possibility that the oxidative formation of the red pigment from the water

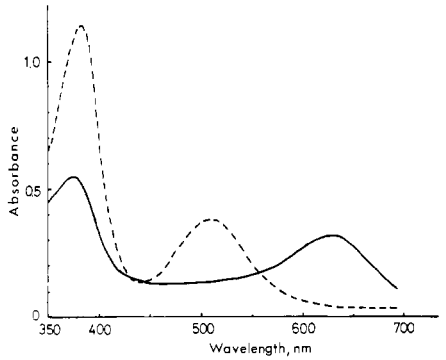

Figure 9. Visible spectra of the blue substance (—) and the red pigment (- - -), in distilled water solution.

extract of the blue band may be caused by the contamination of scorbamic acid (SCA), the possible precursor to the red pigment, since SCA was found to appear near the blue substance on the TLC. However, a comparison of authentic SCA with the reaction products in the ethanol system on TLC by the ninhydrin test demonstrated no contamination of SCA in the blue substance and eliminated the above hypothesis. It may be concluded, therefore, that R-A is a reduced compound of the red pigment.

The ESR spectrum of R-A resolved into the 8.45 G triplet and the 0.81 G triplet responsible for a nitrogen and two equivalent protons, respectively, as shown by the stick diagram in Figure 4. Since the deuterium substitution caused no significant change in spectrum A, it is apparent that no proton is attached to the nitrogen nucleus. Similar reactions done in ethanol with amino acids such as Gly, Leu, Phe, Glu, and Lys showed essentially the same ESR spectrum as α-Ala as described above and provided a blue spot of the same R_f value on TLC from which R-A could be extracted in each case. This indicates that R-A does not contain the amino acid residue.

On the other hand, as for the other radical product, R-C, which was detected very weakly on the spot in the vicinity of the red pigment, the ESR spectrum is resolved into the 9.18 G doublet, 8.27 G triplet, and 0.92 G triplet responsible for a proton, a nitrogen, and two equivalent protons, respectively. This was supported by the simulated pattern shown in Figure 5. With the examination of R-C in D_2O the spectrum was significantly changed (Figure 5), namely, the 9.18 G proton was replaced by the 9.18 G/6.514 = 1.41 G deuterium splitting. Thus, it is concluded that the 9.18 G proton should be attached to the 8.27 G nitrogen nucleus. The reaction of DHA with Gly gave a spectrum identical with that seen in the DHA and α-Ala system, which was composed of R-A and R-C. Thus, R-C is also seen not to contain the amino acid residue. The addition of an appropriate amount of 5% ascorbic acid solution to the water extract of the red band gave a remarkable increase in spectrum C, which could hardly be detected originally. Thus, it is considered that R-C is produced by the reduction of the red pigment. In addition, when the water extract of R-C was allowed to stand at room temperature, the spectrum was found to change remarkably, namely, that of R-C faded out and spectrum A appeared and thereafter increased. This may suggest the possibility of the conversion of R-C to R-A.

Figure 10 shows the possible structures and formation mechanism of R-A and R-C. The structure of the red pigment, 2,2′-nitrilodi-2(2′)-deoxy-L-ascorbic acid monoammonium salt (Kurata et al., 1973), is also illustrated, which may be more closely correlated to the structures of R-A and R-C than that proposed to have the amino acid

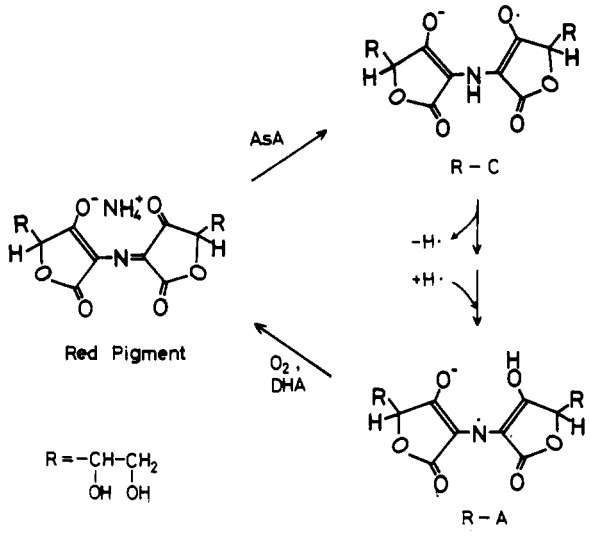

Figure 10. Possible formation mechanism of the free-radical products (Yano et al., 1974).

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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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.

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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,