The stability of β-carotene under different laboratory conditions

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The stability of β-carotene, the carotenoid with the highest vitamin A activity, has been investigated under laboratory conditions, other than food processing or food storage. β-Carotene appears to be fairly stable over 24 h (loss < 4%) and 48 h (loss < 15%) when micellar solutions in aqueous medium of this pigment were incubated at 37° C, in the dark, in a 5% CO_2 in air atmosphere to simulate incubation conditions (used for cell culture systems). The effect of both UV light and fluorescent light on solutions of β-carotene in toluene was highly damaging (50% loss occurred after 8 h under UV light and after 24 h under daylight). Butylated hydroxytoluene (BHT) and alpha-tocopherol, two common antioxidants, were able to reduce significantly the degradation of β-carotene under light exposure. At the same concentration of BHT (1 mM), alpha-tocopherol had a much stronger relative potency to prevent the loss of the pigment (50% loss occurred after 48 h in fluorescent and 40 h in UV light in presence of alpha-tocopherol and after 23 h and 17 h, respectively, in fluorescent and UV light in presence of BHT). Increasing the rate of air-solution exchange by increasing the surface of β-carotene solution exposed to air caused an increase of the loss of the pigment of more than 3 times. Under storage conditions (−20° C, in the dark, under N_2), the rate of loss of β-carotene was 1.5%/month and 1.1%/month, respectively, in absence or in presence of 0.025% BHT.

Keywords: beta-carotene; micellar solution; beta-carotene stability

Introduction

Carotenoids are a well characterized class of compounds present in microorganisms, algae, higher plants, animals, and humans.[1,2] More than 600 carotenoids occur in nature. About 38 are precursors of vitamin A. Of these, only a few occur in sufficient concentration to play a significant role in the human diet. The most plentiful carotenoid is beta-carotene (BC) which also has the highest vitamin A activity. Multiple biologic functions besides the pro-vitamin A activity have been ascribed to this compound, for instance the activity as singlet oxygen or free-radical scavenger,[3] as a stimulant of the immune response,[4] and the action as anti-carcinogenic agent.[5] In consequence of this variety of functions and in correlation with the nontoxicity of BC, its biologic role and metabolic fate have been the subject of emphasis in recent research. Nevertheless, very little has been done to test the stability of BC under conditions other than food storage or processing. Chichester and Simpson,[6] for instance, reviewed the metabolic degradation of BC in senescent tissues as well as the effect of acid and alkali on solutions of this pigment. El Tinay and Chichester[7] studied the oxidation of BC in toluene at various temperatures. They found that the rate of BC loss indicated a zero-order reaction in the presence of excess oxygen. Carnevale et al.[8] showed that fluorescent light catalyzed the autoxidation of BC dissolved in fatty acid. A free-radical destruction of BC was shown to occur during the aerobic conversion of sulfite, a chemical widely used in foods; to sulfate, a process known to generate radicals.[9]

The purpose of the present report was to define the conditions to be used in laboratory research on BC that would prevent its degradation. In order to do that the following was done: a) BC stability was examined under conditions usually used in an experimental setting to investigate its metabolic or functional relevance both in vitro and in cell culture systems; b) the effect of some antioxidants in preventing this degradation was determined; and c) its storage stability under controlled conditions with regard to the type of solvent, light, temperature, and atmosphere was studied.

Materials and methods

Chemicals

[^{14}C]-beta-carotene (BC) was a gift from Hoffmann-LaRoche, Nutley, NJ, USA. Unlabeled BC was purchased from Fluka Biochemica, Buchs, Switzerland. Alpha-tocopherol, butylated hydroxytoluene (BHT) and taurodeoxycholic acid (TDCA) were from Sigma Co., St. Louis, MO, USA. All the organic solvents were Fischer-brand high-pressure liquid chromatography (HPLC) grade. The HPLC column was an Ultrasphere ODS, 5

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μm, 4.6 × 150 mm, from Beckman, Fullerton, CA. Separation was performed on a Beckman/Altex HPLC system. Cytoscint, the scintillation liquid, was from ICN, Irvine, CA, USA.

HPLC

The extracts from each experiment (see below) were analyzed by HPLC in the following way: after evaporating to dryness under a stream of N_2 the samples were reconstituted in a mixture of toluene:methanol (1:3) and injected into the HPLC column. The optimized HPLC analysis was performed at a flow rate of 1.5 mL/min. using a linear gradient elution system from 100% solvent A (methanol plus 0.5% ammonium acetate) to 80% solvent A plus 20% toluene, developed in 12 min. The retention time of BC was 12 min. The effluent was monitored at 452 nm.

BC stability under simulated incubation conditions

Two different micellar solutions of BC were prepared. The first was by a modification of the method of Bertram et al[10] by rapidly mixing 20 μL of a 2 mmol/L solution of BC in THF:DMSO (tetrahydrofuran:dimethylsulfoxide) (1:1) plus 5 μL of a 0.5 mmol/L [^{14}C]-BC solution in THF:DMSO (1:1) into 5 mL of 90% Iscove's Modified Dulbecco's Medium with 10% bovine fetal serum and stirring it for 30 min. The second was prepared according to a modified procedure by Westergard and Dietschy.[11] Crystalline BC was dissolved in THF to provide a final concentration of 8 mmol/L. This solution (400 μL) was evaporated to dryness under N_2 and the resulting precipitated crystals were resuspended in 50 μL of THF and added to 55 mL of 40 mmol/L in 40 mmol/L Tris pH 7.4. The mixture was gently stirred overnight in the dark at room temperature, then diluted 1:1 with the same buffer without TCDA and sonicated for 15 min using a Branasonic 220 (Branson Sonic Power Co., Danbury, CT, USA) sonicator bath. The BC recovered in micellar solution was 60%. Both types of micellar solution were incubated at 37° C in petri dishes (60 mm diameter) in the dark, in presence of a 5% CO_2 in air atmosphere. At each time point one volume of ethanol plus 0.025% BHT was added to aliquots of each solution and, after saturating with NaCl, extracted twice with six volumes of hexane. The samples were then evaporated to dryness and reconstituted either in toluene:methanol (1:3), when analyzed by HPLC, or in benzene when BC concentration was determined by spectrophotometer. When [^{14}C]-BC was used, each fraction from the HPLC elution was collected, mixed with scintillation liquid, and counted by a liquid scintillation counter.

BC exposure to light

Crystalline BC was dissolved in a solution of toluene with or without 0.025% BHT or alpha-tocopherol to provide a final concentration of 4 μmol/L. This solution (50 mL) was put into a 200 mL beaker to produce a layer 1.5 cm deep and a surface of 45.3 cm^2. Exposure was to a fluorescent cold lamp (40W) giving an intensity of 6500 lux at the surface of the sample from a distance of 10 cm. A UV lamp at 325 nm was used for UV treatment. The BC concentration was tested at different times by monitoring the absorbance at 463 nm. The experiment with a smaller air exchange was carried out under the same conditions except that a test tube with an exchange surface of 0.785 cm^2 was used.

BC stability under storage conditions

Solutions of BC in THF were prepared in presence or absence of 0.025% BHT and were kept in plastic tubes in a brown bottle, at −20° C under N_2. At different times an aliquot was taken and analyzed both by liquid chromatography and, after diluting in benzene to (to 2–3 μmol/L), by spectrophotometer at 452 nm.

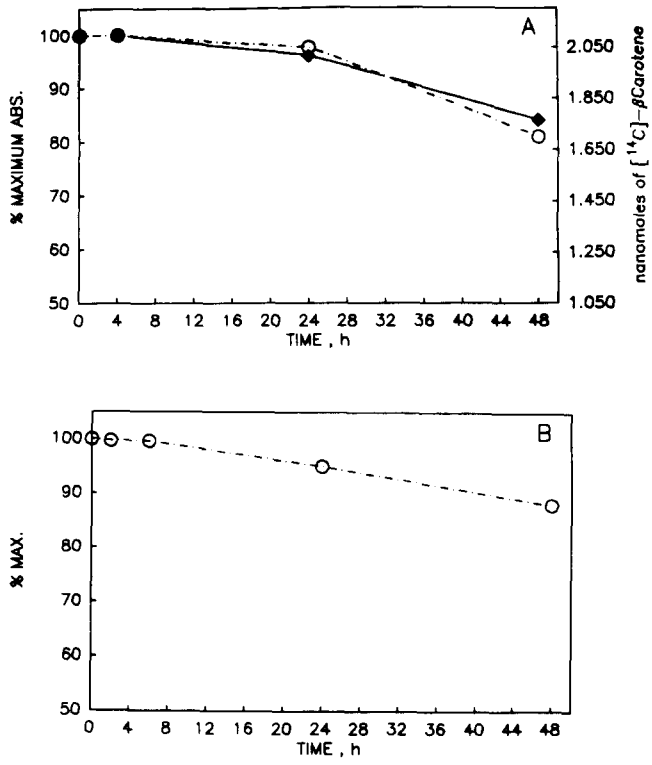

Figure 1 Rate of degradation of beta-carotene from micellar solution under simulated incubation conditions. A: a micellar solution in THF:DMSO (1:1) of unlabeled (2 mmol/L) and labeled BC (0.5 mmol/L) were mixed with cell culture medium, incubated at 37° C in the dark, in 5% CO_2, 95% air atmosphere. An aliquot of each sample at different times was extracted and analyzed both for the unlabeled BC content by measuring the absorbance of 452 nm (open circles) and for the labeled one (solid diamonds) by a monitoring system described in Materials and methods. B: micellar solutions of beta-carotene (17 μmol/L) in taurodeoxycholic acid were incubated and analyzed as described above. Each value is the mean of at least two determinations which differed by no more than 5% of the mean.

Results and discussion

Stability of BC under simulated incubation conditions

This study was carried out to test the stability of two different micellar BC solutions at different times, under dark conditions at 37° C. Detection limits of less than 1 picomole were obtained by the use of HPLC together with radioactive BC. Furthermore, as a double check, a spectrophotometric monitoring system was used. As shown in *Figure 1* the BC appeared to be fairly stable over 24 hours of incubation (loss <4%) with no significant differences between the two different methods used to prepare micellar solutions. Even after 48 hr of incubation, the loss of the pigment did not exceed 10% and 15%, respectively, for the TCDA and the THF micellar solutions. No known putative metabolic products were detected in the breakdown

products of BC. To have shown that BC is quite stable under conditions of relatively high temperature and for a long time is relevant in relation to the widespread use of cell-free and cell culture systems, which require similar incubation conditions, for the study of BC metabolism (uptake and cleavage) and for the investigation of a direct anti-carcinogenic action of this pigment.

Light degradation and antioxidant effect

To explore the factors that could cause BC degradation, the effect of different kinds of light exposure was investigated. As shown in *Figures 2 and 3*, the exposure of BC both to UV and fluorescent light, concomitant with experimental conditions that allowed a good atmospheric oxygen exchange, was highly damaging. The loss of the pigment was complete after 48 hr, resulting in a total bleaching under both conditions, where UV light was three times more effective (50% loss occurred after 8 hr) than fluorescent light (50% loss after 24 hr). In this respect the present results are in accord with those previously obtained for BC autoxidation catalyzed by fluorescent light by Carnevale et al.[8] Nevertheless, it is not easy to compare the rate of BC loss since the composition of lipids used as solvent system by these authors highly affected this

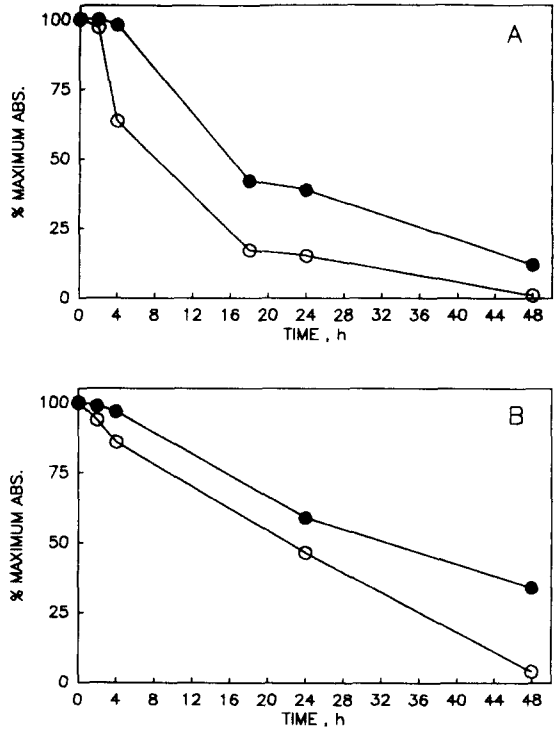

Figure 2 Rate of degradation of a toluene solution of beta-carotene exposed to different light sources in presence (open circles) or in absence (solid circles) of butylated hydroxytoluene. Solutions of BC (4 μmol/L) were put in a 200 mL beaker and exposed to ultraviolet light (*Figure 2A*) or to fluorescent light (*Figure 2B*) for different times. At each time point an aliquot was taken and the BC content was determined by spectrophotometer at 463 nm. Each data point is the mean of two determinations which differed by no more than 5% of the mean.

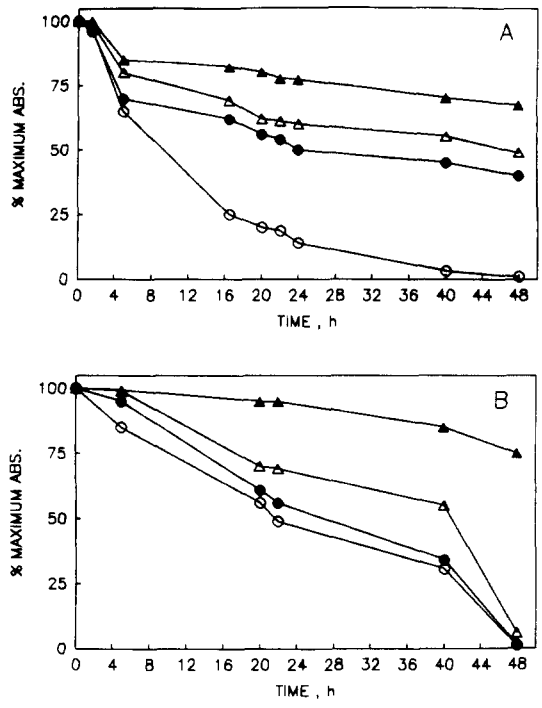

Figure 3 Effect of different concentrations of alpha-tocopherol on BC degradation by air-light exposure. Solutions of BC (4 μmol/L) in toluene were exposed to UV light (*Figure 3A*) or to fluorescent light (*Figure 3B*) in presence of different concentrations of alpha-tocopherol: control, no alpha-tocopherol (open circles); 0.1 mmol/L alpha-tocopherol (solid circles); 1 mmol/L alpha-tocopherol (open triangles); 10 mmol/L alpha-tocopherol (solid triangles). The BC content was determined by measuring the absorbance at 463 nm. Each value is the mean of two determinations which differed by no more than 4% of the mean.

rate (50% loss occurred after 15 hr in laurate solution, while no loss was observed in oleate solution), showing that a protective effect had been introduced with greater efficiency as unsaturation was increased.

Having established the degradation rate induced by light and air exposure on BC, the protective effect of two common antioxidants, BHT and alpha-tocopherol, was further investigated. BHT, at the usual concentration used in biological assays (1 mmol/L), accounted for a loss reduction that ranged from more than 210% (50% loss occurs after 17 hrs compared to 8 hrs in absence of antioxidant) to more than 140% (50% loss after 33 hrs compared to 23 hrs in absence of antioxidant), respectively on UV light and fluorescent light exposure. The effect of the most potent natural antioxidant, alpha-tocopherol, is shown in *Figure 3*. There was a concentration dependent effect under both conditions (UV and fluorescent light). At the same concentration of BHT (1 mmol/L) the 50% BC loss was equal to 48 hr and 40 hr, respectively of UV and fluorescent light exposure, showing that alpha-tocopherol has stronger relative potency to prevent BC degradation than BHT in our assay conditions. The protective effect of alpha-tocopherol and BHT was also shown by Peiser and Young[9] in an experimental system in which BC destruction was induced by free radicals formed from transformation of

sulfite to sulfate. This might suggest that even in the present incubation, BC degradation could be by a free radical-mediated process, promoted by light absorption either by BC or by the toluene used as a solvent. Nevertheless, since cis/trans isomerization has been suggested to occur in the autoxidation process of carotenoids,[12,13] further investigation will be required to assess the relevance of this interconversion in reducing the maximum absorbance used as monitoring signal of BC degradation in the present assay.

Interestingly, there is a marked difference in the disappearance curves for BC under UV and fluorescent illumination. The BC concentration, in the absence of antioxidant, decreased in a hyperbolic fashion when exposed to UV light and linearly under fluorescent illumination. Thus UV light appears to operate by a first-order kinetic model, while the action of fluorescent light is best described as zero order. Speculation about this difference in terms of mechanism requires further investigation, although the direct absorption of fluorescent light by BC (λ = 450) may account for such a difference.

The stability of BC exposed to fluorescent light in relation to different rates of air-solution exchange was also studied in order to investigate the effect of oxygen. The rate of gas-liquid exchange is affected by the size of the surface of the solution exposed to air. An experiment was carried out comparing the degradation rate of BC solution in a test tube (exchange surface of 0.785 cm^2) and in a 200 mL beaker (exchange surface of 45.3 cm^2). As shown in *Figure 4* the 50% loss occurred after 72 hr in the case of the solution in the test tube and after 23 hr for the beaker.

BC stability under storage conditions

To test the stability under conditions of prolonged storage, BC in THF solution was kept at −20° C, avoiding any illumination by using a double brown container (two brown bottles, one inside the other), in a nitrogen atmosphere. I analyzed the concentration at different times both by liquid chromatographic and spectrophotometric systems. The rate of BC loss ranged from 1.1% per month in presence of BHT to 1.5% per month in absence of antioxidant during the 4 month experiment as shown in *Figure 5*. This clearly strengthens the importance of controlling air, light exposure, and temperature to reduce any degradative processes. An antioxidant like BHT can effectively improve the storage life of BC even when the degradative factors are minimized.

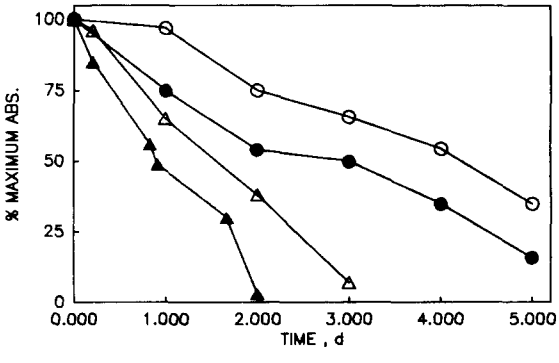

Figure 4 Rate of degradation of a toluene solution of beta-carotene exposed to fluorescent light under conditions of varying air-exchange. Solutions of BC (4 μmol/L) in toluene in presence (solid symbols) or in absence (open symbols) of 0.025% BHT were put into a test tube (exchange surface 0.785 cm^2–open and filled circle) or a 200 mL beaker (exchange surface 45.3 cm^2–open and filled triangle) and exposed to fluorescent light. At each time point the BC content was analyzed as described in *Figure 3*. The values are the mean of two determinations which differed by no more than 6% of the mean.

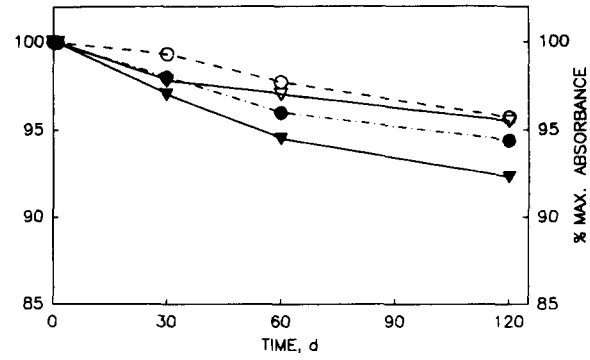

Figure 5 Stability of beta-carotene under storage condition. Solutions of BC in THF in presence (solid symbols) or in absence (open symbols) of 0.025% BHT were kept at −20° C in a double brown bottle, in the dark, and under N_2. At each time point an aliquot was taken and analyzed for BC content both by a liquid chromatographic (circles) and spectrophotometric (triangles) monotoring system. Percent of maximum peak area on HPLC was calculated on the basis of the nmol of BC (5 nmol) detected by a liquid chromatographic system. Each data point is the mean of two determinations which differed by no more than 4% of the mean.

Conclusion

The present work is to my knowledge the first attempt of a systematic investigation of the factors that could affect the stability of BC under common laboratory conditions generally used to investigate its biologic role and its metabolic pathway. BC remains fairly stable during 48 hr under conditions defined as "incubation conditions" (37° C, 5% CO_2, in the dark), generally used for cell-free or cell culture model systems.

BC is rapidly degraded by ultraviolet and visible light in the presence of atmospheric oxygen, the degradation rate being lower with a lower air-oxygen exchange. This points to the following laboratory working conditions to preserve BC solutions from degradation: use of a subdued light environment and a dark container with a small air-surface exchange. A significant improvement in BC stability was also obtained by the routine use of antioxidants such as the two well-known radical trapping agents, BHT and alpha-tocopherol. These conditions suggest that the light-induced degradation of this pigment could involve a chain-radical mechanism, particularly in absence of sensitizers that could produce singlet oxygen. Finally, the relative stability of BC was confirmed

when the degradative factors were strictly controlled in the presence of antioxidants, suggesting conditions to be used to prolong the storage life of BC.

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Abbreviations

BC	beta-carotene
THF	tetrahydrofuran
DMSO	dimethylsulfoxide
BHT	butylated hydroxytoluene
TDCA	taurodeoxycholate

References

1 Goodwin, T.W. (1952). *The Comparative Biochemistry of Carotenoids*. Chapman and Hall. London, UK
2 Goodwin, T.W. (1980). *The Biochemistry of Carotenoids. Vol. 1, Plants*. Chapman and Hall. London, UK
3 Burton, G.W. and Ingold, K.U. (1984). β-Carotene: an unusual type of lipid-antioxidant. *Science* **224**, 569–573
4 Bendich, A. (1988). A role for carotenoids in immunofunction. *Clin. Nutr.* **7**, 113–117
5 Krinsky, N.I. (1988). The evidence for the role of carotenoids in preventive health. *Clin. Nutr.* **7**, 107–112
6 Simpson, K.L. and Chichester, C.O. (1982). Metabolism and nutritional significance of carotenoids. *Ann. Rev. Nutr.* **1**, 351–374
7 El Tinay, A.H. and Chichester, C.O. (1979). Oxidation of β-carotene. Site of initial attack. *J. Org. Chem.* **35**, 2290–2293
8 Carnevale, J., Cole, E.R., and Crank, G. (1979). Fluorescent light catalyzed autoxidation of β-carotene. *J. Agric. Food Chem.* **27**, 462–463
9 Peiser, G.D. and Yang, S.F. (1979). Sulfite mediated destruction of β-carotene. *J. Agric. Food Chem.* **27**, 446–449
10 Bertram, J.S., Pung, A., Churley, M., Kappok IV, T.J., Wilkens, L.R., and Cooney, R.V. (1991). Diverse carotenoids protect from chemically-induced neoplastic transformation. *Carcinogenesis*. **12**, 671–678
11 Westergaard, H. and Dietschy, J.M. (1976). The mechanism whereby bile acid micells increase the rate of fatty acid and cholesterol uptake into the mucosal cells. *J. Clin. Invest.* **58**, 97–108
12 Holman, R.T. (1949). Spectrophotometric studies of the oxidation of fats. VIII. Coupled oxidation of β-carotene. *Arch. Biochem. Biophys.* **21**, 51–57
13 Budowski, P. and Bondi, A (1960). Autoxidation of carotene and vitamin A. Influence of fat and antioxidant. *Arch. Biochem. Biophys.* **89**, 66–73