TRIPLET CARBONYL SPECIES GENERATED FROM 3-HYDROXYMETHYL-3,4,4-TRIMETHYL-1,2-DIOXETANE (HTMD) INDUCE ON *CIS/TRANS* ISOMERIZATION OF CAROTENOIDS

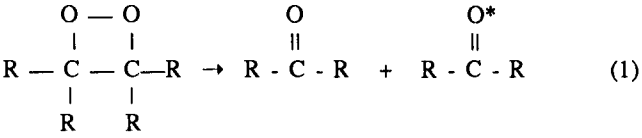
Helmut Sies*, Michael Hanusch, Wilhelm Stahl and Alfred R. Sundquist
Institut für Physiologische Chemie I - Universität Düsseldorf - Moorenstraße 5 - W-4000 Düsseldorf - Germany

The dioxetane, 3-hydroxymethyl-3,4,4-trimethyl-1,2-dioxetane (HTMD), was used to generate electronically excited ketones, and the interaction of these reactive species with several geometrical isomers of carotenoids was studied. A significant *trans*-to-*cis* isomerization was found with ß-carotene, lycopene and canthaxanthin when the respective all-trans isomers were incubated with HTMD, *cis* isomers accounting for 20-50% of products formed. The isomers formed from *all-trans*-ß-carotene were identified as 9-*cis*-, 13-*cis*- and 15-*cis*-ß-carotene by cochromatography of cis isomer standards and by UV/VIS-spectroscopy. The isomerization rate was even greater when 15-*cis*-ß-carotene was used as the starting isomer in the incubation with HTMD. The isomer patterns generated from lycopene and ß-carotene are generally similar to those reported recently for various human tissues.

Keywords: ß-carotene; cis/trans isomerization; singlet oxygen; 1,2-dioxetane; triplet ketone.

INTRODUCTION

Biologically occurring prooxidants can influence the isomeric composition of carotenoids. Triplet excited carbonyls,[1] detectable by their characteristic light emission in the visible spectral region, can be generated enzymatically with peroxidases[2-4] and lipoxygenases,[5] and they likely contribute to the low-level photoemission accompanying lipid peroxidation.[6] A proposed route to the enzymatic formation of triplet carbonyls is via a 1,2-dioxetane intermediate.[7] 1,2-Dioxetanes decompose thermally to yield ground state and excited ketones[8] (Reaction 1)



(1)

While both the singlet excited and triplet excited states are produced, with alkyl substituted 1,2-dioxetanes, the production of the triplet state exceeds that of the singlet by >100-fold.[9] Both the *all-trans* and 13-*cis* isomers of retinal undergo triplet ketone-sensitized *cis/trans* isomerization to a mixture of *all-trans*-, 13-*cis*- and 9-*cis*-retinal when incubated with tetramethyl-1,2-dioxetane.[10]

Carotenoids are present in human tissue and plasma as mixtures of the *all-trans* and *cis* isomers (E/Z isomers). In the case of lycopene, cis isomers typically account for over 50% of the total lycopene content, while with ß-carotene *cis* isomers contribute 5-30% to the total.[11] Both the factors that influence the isomeric composition of carotenoids in human tissue and serum and the relative biological activities of the different geometrical isomers, are not yet fully understood. While the absorption of carotenoid isomers from the diet is expected to contribute significantly to the isomer pattern,[12,13] other factors may also be important: in vitro studies demonstrated that the quenching of singlet oxygen by ß-carotene induces *cis*-to-*trans* isomerization,[14] as followed by a decrease in absorbance at the "*cis*-peak" or *cis*-band (lambda max ca. 345 nm) of the 15-*cis* isomer;[15] and 9-*cis*-ß-carotene was found in significant amounts in human tissues but not in serum.[11] The relevance of *cis/trans* isomerization to biological activity was recently revealed in studies demonstrating separate retinoic acid receptors for the *all-trans* and 9-*cis* isomers of retinoic acid, a carotene metabolite.[16,17] Whether the biological isomerization reactions occur at the level of the carotenoid or the retinoid is currently unknown.

The work presented here has been described in detail.[18]

MATERIALS AND METHODS

Chemicals

3-Hydroxymethyl-3,4,4-trimethyl-1,2-dioxetane (HTMD) was generously provided by Prof. W. Adam, University of Würzburg, Germany. The activity of the dioxetane stock solutions was monitored periodically by determining their specific photoemission (i.e. counts s^{-1} microliter^{-1}) as described below. 3,3'-(1,4-Naphthylidene)dipropionate (NDP) and its endoperoxide ($NDPO_2$) were prepared as described by Di Mascio et al..[19] Carotenoids were kind gifts from Drs. J. Bausch and H. E. Keller, Hoffmann-La Roche (Basel, Switzerland).

Measurement of low-level photoemission

This was done with a single-photon counting system with a photomultiplier (EMI-Gencom 9658 A, Plainview, NY, USA) cooled to -22°C.[20] Measurements were made at 37°C in n-hexane. An aerated solvent was employed to quench triplet excited ketone and thus selectively measure the fluorescence from singlet excited ketone.

Incubations

Incubations of carotenoids with HTMD were conducted in chloroform at 37°C in a water bath. Spectrophotometric assays were done in a dual-beam spectrophotometer (Beckman Instruments model 25, San Ramon, CA) at 37°C. The change in absorbance of the sample (0.05 mM carotenoid with 2 mM HTMD) over the reference (0.05 mM carotenoid without HTMD) was measured at 350 nm. The loss of HTMD was followed by assaying the incubation mixture for photoemission, and it was found to be < 20% after 1 h of incubation. Controls with the decomposition products of HTMD, acetone and hydroxy-acetone, did not yield carotenoid reaction products.

Iodine-catalyzed photoisomerizations were conducted in n-hexane with 0.04 mM I_2 and 0.1 mM ß-carotene as described previously.[15,21] Incubations with $NDPO_2$ were done in chloroform:ethanol (1:1) with 10 mM endoperoxide and 0.1 mM ß-carotene; control incubations were done with the parent compound NDP.

HPLC analysis

Routine reversed-phase HPLC was conducted with a Hitachi pump (model 665A12) and variable wavelength detectors (models L4200 and 665A) on a LiChrospher RP-18 column (endcapped, 5 µm, 4x250 mm), from Merck (Darmstadt, Germany). The eluent for the separation of ß-carotene and lycopene was composed of acetonitrile: methanol: dichloromethane: water (7:7:2:0.16) and for canthaxanthin: acetonitrile: methanol:toluene: water (7:7:1:0.8). Spectral analysis of carotenoid isomers was done with a diode array detector (Beckman model 168).

RESULTS

ß-Carotene: The *all-trans* isomer of ß-carotene was incubated with HTMD in chloroform and monitored continuously at 350 nm (the absorbance maximum of the "*cis*-band" of ß-carotene in chloroform) to detect *trans*-to-*cis* isomerization. The absorbance increased during the first 60 min of the incubation relative to a control without HTMD and then remained constant; the half-maximal change in absorbance was achieved 16 min after the addition of HTMD (Fig. 1, upper panel). To study the reaction products, aliquots were removed from similar incubations and analyzed by HPLC. Detection at 450 nm (Fig. 2A) revealed an HTMD-dependent decrease in *all-trans*-ß-carotene and distinct increases in two peaks eluting at 21 and 22 min. The peak at 21 min eluted together with authentic 9-*cis*-ß-carotene added to the sample, while the 22 min peak coeluted both with 13-*cis*- and 15-*cis*-ß-carotene standards in separate spiking experiments. Absorption spectra were made using on-line diode array detection, and a comparison of the intensity of the *cis*-bands in these spectra indicate that both *cis* isomers are present. Peak identifications were later confirmed using a new chromatographic system which better resolves the three cis isomers.[22] In these experiments, the relative amounts of 9-*cis*-, 13-*cis*- and 15-*cis*-ß-carotene formed from *all-trans*-ß-carotene after incubation with HTMD as described above was found to be 1:1:0.3.

The increases in the *cis* isomer peaks after 60 min of incubation accounted for 20% of the HTMD-dependent loss of *all-trans*-ß-carotene (Table 1). Other peaks detectable at 340 nm (Fig. 2B), assigned as ß-carotene oxidation products, make up the balance of loss of the *all-trans* isomer and were not further investigated here.

Incubation of the 15-*cis* isomer of ß-carotene with HTMD resulted in a decrease in absorbance at the *cis*-band which reached the half-maximal change after 9 min and ceased to decrease after 50 min (Fig 1, lower panel). An HTMD-dependent increase in the *all-trans*- and 9-*cis*-ß-carotene peaks was observed (Fig. 3A) and accounted for over 70% of the 15-*cis*-ß-carotene lost after 60 min of incubation (Table 1). Decomposition of the 15-*cis*-ß-carotene also occurred, and a similar pattern of oxidation products was seen in the incubations with 15-*cis*- and *all-trans*-ß-carotene (compare Figs. 2B and 3B).

The ß-carotene isomer pattern obtained with HTMD was compared with those arising from iodine-catalyzed photoisomerization.[15] Starting with *all-trans*-ß-carotene it gives chiefly the 9-*cis* (40% of products) and 13-*cis* (40% of products) isomers.[21] Analyzed with the present HPLC conditions,

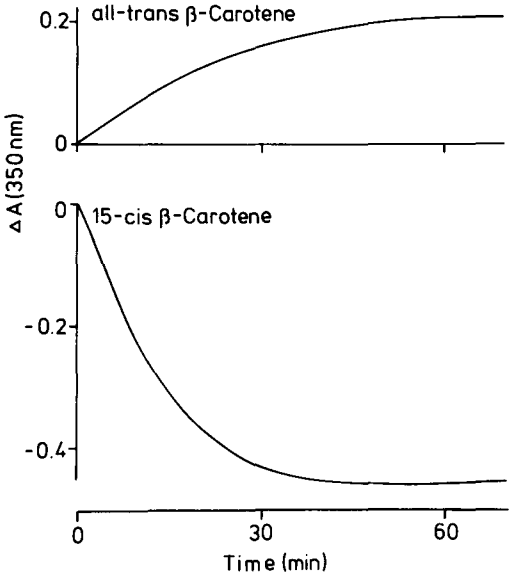


Figure 1. Reaction of 3-hydroxymethyl-3,4,4-trimethyl-1,2-dioxetane (HTMD) with β-carotene monitored as a change in absorbance at the cis*-band of β-carotene.*
All-trans- or 15-cis-β-carotene (0.050 mM) was incubated with HTMD (added to 2 mM at t= 0 min) in chloroform at 37°C in a dual-beam spectrophotometer. Absorbance at 350 nm was recorded against a reference sample without HTMD. (From Ref.[18])

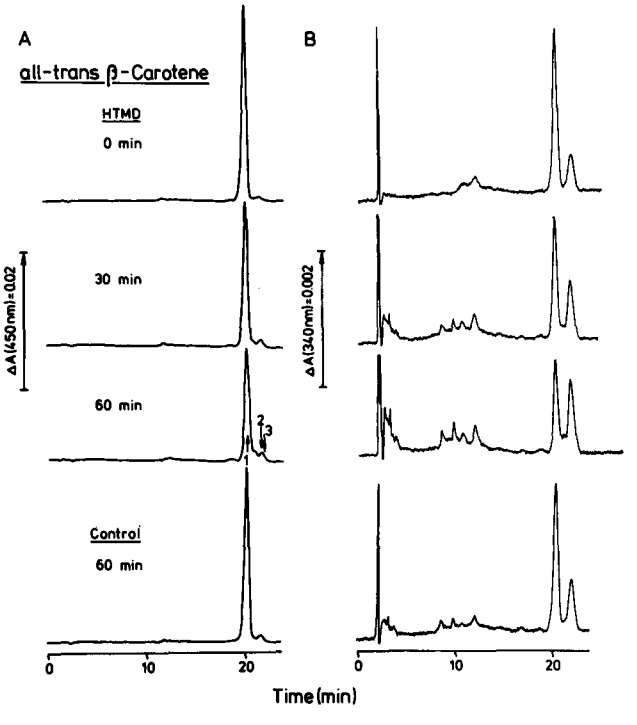


Figure 2. Reversed-phase HPLC analysis of products formed upon incubation of all-trans-β-carotene with HTMD.
All-trans-β-carotene was incubated with HTMD as in Fig. 1. Aliquots were removed and chromatographed with detection at (A) 450 nm and (B) 340 nm, the absorbance maximum of the cis-band in the eluant. The control was without HTMD. Authentic 9-cis-, 13-cis- and 15-cis-β-carotene eluted at 21, 22 and 22 min, respectively. (From Ref.[18])

Table 1. Reaction of carotenoids with 3-hydroxymethyl-3,4,4-trimethyl-1,2-dioxetane (HTMD).

The carotenoids (0.10 mM) were incubated for 60 min with 2 mM HTMD in N_2-saturated chloroform at 37°C, after which the incubation mixtures were analyzed by HPLC with detection at 450 nm (see Figs. 2A and 3A). Values were calculated from isomer peak heights and were corrected for product formation observed in control assays without HTMD (see Materials and Methods).

		Composition of incubation mixture after 1 h (%)		
			Reaction products	
Carotenoid	Relative rate[a]	Starting isomer	Isomers[b]	Other
ß-Carotene,				
all-trans-	1.0	66	7	27
15-*cis*-	1.8	56	31	13
all-trans-Lycopene	1.1	61	20	19
all-trans-Canthaxanthin	0.5	71	14	15

[a]Based on initial reaction rates calculated from spectrophotometric assays such as those shown in Figure 1.
[b]The ε_{450} value of the all-trans isomer was used to calculate levels of resolved *cis* isomer.

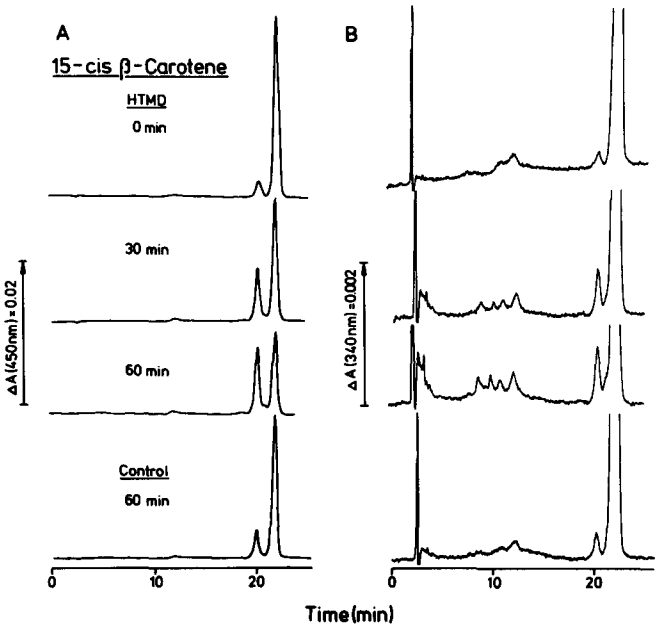


Figure 3. Reversed-phase HPLC analysis of products formed upon incubation of 15-cis-ß-carotene with HTMD.
15-cis-ß-carotene was incubated with HTMD as in Fig. 1. Aliquots were removed and chromatographed with detection at (A) 450 nm and (B) 340 nm. The control was without HTMD. Authentic all-trans-, 9-cis- and 13-cis-ß-carotene eluted at 20, 21 and 22 min, respectively. (From Ref.[18])

the iodine-catalyzed photoisomerization of *all-trans*-ß-carotene generated the same two peaks of *cis* isomers observed with HTMD and identified as 9-*cis*-ß-carotene and 13-*cis*-and 15-cis-ß-carotene. Photophysically-generated singlet oxygen was reported to have no detectable effect on *all-trans*-ß-carotene but to sensitize the isomerization of 15-*cis*-ß-carotene to the *all-trans* isomer in high yield (ca. 90%).[14] Incubation of *all-trans*-ß-carotene with $NDPO_2$, an efficient chemical source of singlet oxygen,[23-25] resulted in isomerization, and the isomer pattern was similar to that observed with HTMD (Fig. 2A). Both singlet oxygen-induced isomerization and iodine-catalyzed photoisomerization of 15-*cis*-ß-carotene yielded the same isomer pattern as with *all-trans*-ß-carotene.

Additional carotenoids: Lycopene and canthaxanthin also underwent an HTMD-dependent loss and *trans*-to-*cis* isomerization. While both the rate and extent of loss seen with *all-trans*-lycopene was similar to that of *all-trans*-ß-carotene, the yield of *cis* isomers was considerably higher with lycopene after the same incubation period (Table 1). The lycopene *cis* isomers eluted as two peaks and a shoulder immediately following the *all-trans* isomer. Diode array spectra revealed a *cis* absorption band at 360 nm which was more intense for the isomers eluting later, suggesting that as with ß-carotene both central and terminal *cis* isomers are formed.

The *all-trans* isomer of canthaxanthin, an oxycarotenoid, reacted most slowly and to the least extent of the carotenoids tested. Canthaxanthin *cis* isomers eluting as two peaks after the *all-trans* isomer accounted for 50% of the products after 60 min of incubation (Table 1). Spectra from both peaks were characterized by absorption bands at 365 nm, with the *cis*-band of the second being the more intense. Oxidation products of lycopene and canthaxanthin eluting before the geometrical isomers were also detected (data not shown).

DISCUSSION

An HTMD-dependent depletion of starting isomer was observed with each of the carotenoids tested (Table 1). With the *all-trans* isomers of ß-carotene, lycopene and canthaxanthin two types of products were detected by HPLC analysis. *Cis* isomers, which eluted later than the *all-trans* isomer accounted for between 20 and 50% of the loss of the *all-trans* isomer. The ß-carotene isomers were identified as 15-*cis*-, 13-*cis*- and 9-*cis*-ß-carotene by coelution experiments with authentic isomers and by their absorption spectra.

In addition to *cis*/*trans* isomerization, HTMD-dependent decompostion of the carotenoids was seen. Products eluting earlier than the geometrical isomers and having insignificant visible-range absorbances formed from each carotenoid (Figs. 2A and 3A) and were assigned as oxidation products; they were not characterized further in the present study.

The products formed by HTMD appear to be independent of the carotenoid isomer used at the start of the incubation, as *all-trans*- and 15-*cis*-ß-carotene yielded corresponding isomers and the same pattern of oxidation products (Figs. 2 and 3). A similar finding was reported for the dioxetane-dependent

isomerization of retinal; incubation of either *all-trans*- or 13-*cis*-retinal with tetramethyl-1,2-dioxetane yielded the identical mixture of the *all-trans*, 13-*cis* and 9-*cis* isomers.[10] The more efficient HTMD-dependent isomerization seen with 15-*cis*-ß-carotene (Table 1) was also reported for the singlet oxygen-sensitized,[14] triplet anthracene-sensitized[26] and triplet chlorophyll a-sensitized[27] isomerization of ß-carotene. Whether the observed isomer products form directly from *all-trans*- and 15-*cis*-ß-carotene, or if an intermediate isomer is involved, is not known. However, it has been reported that the triplet-sensitized isomerization of 15-*cis*-ß-carotene to other cis isomers occurs via the *all-trans* isomer.[26,27]

The observed HTMD-mediated isomerization of carotenoids may involve the triplet excited ketone produced by 1,2-dioxetanes (Reaction 1). Isomerization of carotenoids can occur via the lowest carotenoid excited triplet,[26,28] and the triplet energy of aliphatic ketones is suffienctly high to excite ground state carotenoid to its triplet. Accordingly, the ß-carotene isomer pattern arising from incubations with singlet oxygen (data not shown), which is quenched by carotenoids to yield the carotenoid triplet,[29] and HTMD (Figs. 2A and 3A) are similar. The effectiveness of the triplet excited hydroxyacetone and acetone which form from HTMD at mediating isomerization appear to be different, however; tetramethyl-1,2-dioxetane, which decomposes only to acetone but yields a steady-state concentration of triplet ketone comparable to that of HTMD,[30] did not induce isomerization of either *all-trans*- or 15-*cis*-ß-carotene when incubated under the conditions described for HTMD (data not shown), indicating that the structure of the excited ketone may play a role as well.

Singlet oxygen-sensitized *trans*-to-*cis* isomerization of ß-carotene was seen using the thermodissociable endoperoxide of 3,3'-(1,4-naphthylidene)dipropionate as singlet oxygen source. This finding contrasts with that of an earlier study with singlet oxygen, in which isomerization of *all-trans*-ß-carotene was not detected using a spectrophotometric assay for the formation of *cis* isomer,[14] but this may reflect the more sensitive method of analysis used in the present study. The sensitized isomerization of *all-trans*-ß-carotene detected by HPLC had been previously reported.[26]

The pattern of carotenoid isomers arising upon incubation with HTMD is comparable to those found in human tissues. For example, in four tissues found to contain significant amounts of carotenoids, lycopene was resolved by the HPLC protocol used in the present study into the *all-trans* isomer (50% of the total) followed by 3 peaks of *cis* isomers.[11] While the isomeric composition of dietary carotenoids largely influences the isomer patterns observed in tissues, the contribution of further *cis/trans* isomerization reactions within the tissues becomes a topic of interest.

ACKNOWLEDGEMENTS

Support for this study came from the National Foundation for Cancer Research, Bethesda, the Ministerium für Wissenschaft und Forschung, Nordrhein-Westfalen, and the Bundesministerium für Forschung and Technologie, Bonn. A.R.S. was an Alexander-von-Humboldt-Stiftung Stipendiat.

REFERENCES

1. Adam, W.; Cilento, G. In *Chemical and biological generation of electronically excited states*, Academic Press, New York, 1982.
2. Cilento, G.; *Pure. Appl. Chem.* (1984), **56**, 1179.
3. Rivas, E. I.; Paladini, A. Jr.; Cilento, G.; *Photochem. Photobiol.* (1984), **40**, 565.
4. Baader, W. J.; Bohne, C.; Cilento, G.; Dunford, H. B.; *J. Biol. Chem.* (1985), **260**, 10217.
5. Schulte-Herbrüggen, T.; Sies, H.; *Photochem. Photobiol.* (1989), **49**, 697.
6. Cadenas, E.; Ginsberg, M. E.; Rabe, U.; Sies, H.; *Biochem. J.* (1984), **223**, 755.
7. Cilento, G.; Duran, N.; Zinner, K.; Vidigal, C. C. C.; Faria Oliveira, O. M. M.; Haun, M.; Faljoni, A.; Augusto, O.; Casadei de Baptista, R.; Bechara, E. J. H.; *Photochem. Photobiol.* (1978), **28**, 445.
8. Adam, W.; Cilento, G.; *Angew. Chem. Int. Ed. Engl.* (1983), **22**, 529.
9. Adam, W., A. Beinhauer and H. Hauer In *The Handbook of Photochemistry*; Scaiano, J. C., Ed.; vol. II, CRC Press, Boca Raton, (1989), pp 271.
10. Waddell, W. H.; Crouch, R.; Nakanishi, K.; Turro, N. J.; *J. Amer. Chem. Soc.* (1976), **98**, 4189.
11. Stahl, W.; Schwarz, W.; Sundquist, A. R.; Sies, H.; *Arch. Biochem. Biophys.* (1992), **294**, 173.
12. Jensen, C. D.; Howes, T. W.; Spiller, G. A.; Pattison, T. S. Whittam, J. H.; Scala, J.; *Nutr. Rep. Inter.* (1987), **35**, 413.
13. Erdman, J. *Clin. Nutr.* (1988), **7**, 101.
14. Foote, C. S.; Chang, Y. C.; Denny, R. W.; *J. Am. Chem. Soc.* (1970), **92**, 5218.
15. Zechmeister, L. In *Cis-trans Isomeric Carotenoids, Vitamin A and Arylpolyenes*, Springer Verlag, Vienna, (1962).
16. Heyman, R. A.; Mangelsdorf, D. J.; Dyck, J. A.; Stein, R. B.; Eichele, G.; Evans, R. M.; Thaller, C.; *Cell*, **8**, 397.
17. Levin, A. A.; Sturzenbecker, L. J.; Kazmer, S.; Bosakowski, T.; Huselton, C.; Allenby, G.; Speck, J.; Kratzeisen, Cl.; Rosenberger, M.; Lovey,A.; Grippo, J. F.; *Nature* (1992), **335**, 359.
18. Sundquist, A. R.; Hanusch, M.; Stahl, W.; Sies, H. *Photochem. Photobiol.* (1993), **57**, 785.
19. Di Mascio, P.; Sundquist, A. R.; Devasagayam, T. P. A.; Sies, H.; *Methods Enzymol.* (1992), **213**, 429.
20. Murphy, M. E.; Sies, H.; *Methods Enzymol.* (1990), **186**, 595.
21. Tsukida, K.; Saiki, K.; Takii, T.; Koyama, Y.; *J. Chromatog.* (1982), **245**, 359.
22. Stahl, W.; Sundquist, A. R.; Hanusch, M.; Schwarz, W.; Sies, H.; *Clin. Chem.* (1993), **39**, 810.
23. Saito, I.; Matsuura, T.; Inoue, K.; *J. Amer. Chem. Soc.* (1983), **105**, 3200.
24. Aubry, J. M. *J. Am. Chem. Soc.* (1985), **107**, 5844.
25. Di Mascio, P.; Sies, H.; *J. Am. Chem. Soc.* (1989), **111**, 2909.
26. Hashimoto, H.; Koyama, Y.; *J. Phys. Chem.* (1988), **92**, 2101.
27. Jensen, N.-H.; Nielsen, A. B.; Wilbrandt, R.; *J. Am. Chem.* (1982), **104**, 6117.
28. Truscott, T. G.; *J. Photochem. Photobiol.* (1990), B: Biol. **6**, 359.
29. Farmilo, A.; Wilkinson, F.; *Photochem. Photobiol.* (1973), **18**, 447.
30. Adam, W.; Beinhauer, A.; Hauer, H.; Saha-Möller, C.; *J. f. Prakt. Chemie* (1990), **332**, 161.

This special issue of Química Nova is dedicated to Prof. G. Cilento on the occasion of his 70th birthday and is financed by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).