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Diversity of Biological Functions of Carotenoids

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Abstract

Of 600 carotenoids from natural sources that have been characterized, fewer than 10% serve as precursors of vitamin A. Many dietary carotenoids, both with and without provitamin A activity are found in the blood and tissues of humans. beta-Carotene, the most nutritionally active carotenoid, comprises 15-30% of total serum carotenoids. Vitamin A is formed primarily by the oxygen-dependent central cleavage of beta-carotene and other provitamin A carotenoids. Several carotenoids show enhancement of the immune response, inhibition of mutagenesis, reduction of induced nuclear damage, and protection from various neoplastic events in cells, tissues, and whole animals. Carotenoids also protect against photo-induced tissue damage. Some carotenoids, including beta-carotene, quench highly reactive singlet oxygen under certain conditions and can block free radical-mediated reactions. In epidemiological studies, the intake of carotenoid-rich fruits and vegetables has been correlated with protection from some forms of cancer, particularly lung cancer. Similarly, serum beta-carotene levels have been associated with a decreased chance of developing lung cancer. Carotenoids are natural pigments that are also responsible for the bright colors of various fruits and vegetables.

Introduction

Carotenoids are natural pigments which are synthesized by plants and are responsible for the bright colors of various fruits and vegetables. Carotenoids are the most ubiquitos and widespread pigments which are characteristic for organisms of all taxa.

There are relationships between structure, the chemical and physical properties and the varied biological functions of carotenoids. The conjugated polyene chromophore determines not only the light absorption properties, and hence color, but also the photochemical properties of the molecule and consequent light-harvesting and photoprotective action. The polyene chain is also the feature mainly responsible for the chemical reactivity of carotenoids toward oxidizing agents and free radicals, and hence for any antioxidant role. In vivo, carotenoids are found in precise locations and orientations in subcellular structures, and their chemical and physical properties are strongly influenced by other molecules in their vicinity, especially membrane proteins and lipids. In turn, the carotenoids influence the properties of these subcellular structures. Structural features such as size, shape, and polarity are essential determinants of the ability of a carotenoid to fit correctly into its molecular environment to

allow it to function. Ability of carotenoids in modifying structure, properties, and stability of cell membranes, and thus affecting molecular processes associated with these membranes, may be an important aspect of their possible beneficial effects on human health.[2]

Of 600 carotenoids from natural sources that have been characterized, fewer than 10% serve as precursors of vitamin A. Many dietary carotenoids, both with and without provitamin A activity, are found in the blood and tissues of humans. beta-Carotene, the most nutritionally active carotenoid, comprises 15-30% of total serum carotenoids. Vitamin A is formed primarily by the oxygen-dependent central cleavage of beta-carotene and other provitamin A carotenoids. Several carotenoids show enhancement of the immune response, inhibition of mutagenesis, reduction of induced nuclear damage, and protection from various neoplastic events in cells, tissues, and whole animals. Carotenoids also protect against photo-induced tissue damage. Some carotenoids, including beta-carotene, quench highly reactive singlet oxygen under certain conditions and can block free radical-mediated reactions. In epidemiological studies, the intake of carotenoid-rich fruits and vegetables has been correlated with protection from some forms of cancer, particularly lung cancer. Similarly, serum beta-carotene levels have been associated with a decreased chance of developing lung cancer. It must be stressed, however, that these epidemiological associations do not show cause and effect. In this regard, long-term intervention trials with beta-carotene supplements are in progress. Whatever the results of these trials, carotenoids clearly show biological actions in animals distinct from their function as precursors of vitamin A.[1]

Table 1. Some of the biological functions of carotenoids.[24]

Structure			Polyene chain		
Properties	electronic			Mechanic	stereo-specific
	ability for close-range energy transfer	π-electron cloud mobility		Rigidity	
		light absorption	high reducing potential (easily oxidized molecule)		
Functions	transfer of absorbed light energy to chlorophyll (all photosynthetics)	photo-synthetic antenna pigments (all photosynthetics) light reception in vision (retinal in all animals)	antioxidant defense via elimination of free radicals (bacteria, plants, animals)	Stabilization of membrane fluidity (bacteria, mycoplasms, fungi, mollusca)	animal (retinoic acid) and plant (abscisic acid) hormones
	triplet chlorophyll quenching (all photosynthetics)	coloration -species/sex/social status -masking (animals) -attractive or repellent (animals, plants)	defense against NO_2 nitrosative action (plants)	Stabilization and defense of caroteno-proteins in skeleton structures (crustaceans, mollusca, echinoderms)	
	singlet oxygen quenching (O_2-photosynthetics)	chromophor in bacteriorhodopsin photosynthesis (*Halobacterium)* light-protective screening (shown in microalgae) selective light filtering in retina (insects, birds) metabolic activation via heating (lake planktonic crustaceans)		Defense of reserve proteins in molluscan and crustaceans eggs against proteases	

Mechanical functions

The natural pigments carotenoids were first emerged in archaebacteria. Their function in the oldest Earth organisms was that of lipids reinforcing bacterial cell membrane. Carotenoidic molecules have an extremely rigid backbone due to the linear chain of 10-11 conjugates double bonds – the length corresponding to the thickness of the hydrophobic zone of the membrane that they penetrate.[19] Their polyene structure is very hard to bend or twist.

Carotenoids decrease the fluidity of membrane, so their amounts control the stability of that parameter, affecting all membrane functions. The membrane reinforcing function of carotenoids is retained in mycoplasms, some fungi and animals. This is the main reason for the colouration of flesh of many mollusca and ascidians (yellow-to-red colour). [11, 12, 21, 25]

It is known that carotenoids and proteins form complexes (carotenoproteins) which are highly resistant to environmental action due to carotenoidic and protein parts.[26] In molluscan and crustacean eggs, the major reserve of proteins are carotenoproteins, which are not affected by proteases present in cytoplasm, because carotenoids defend the protein core from digestion.

Subczynski W.K. et al. [23] have discovered, using a spin-label study, the effects of polar carotenoids on dimyristoylphosphatidylcholine membranes. Spin labeling methods were used to study the structure and dynamic properties of dimyristoylphosphatidylcholine (DMPC) membranes as a function of temperature and the mole fraction of polar carotenoids. The results in fluid phase membranes were as follows: (i) Dihydroxycarotenoids, zeaxanthin and violaxanthin, increase order, decrease motional freedom and decrease the flexibility gradient of alkyl chains of lipids. The activation energy of rotational diffusion of the 16-doxylstearic acid spin label was about 35% less in the presence of 10 mol% of zeaxanthin. (ii) Carotenoids increase the mobility of the polar headgroups of DMPC and increase water accessibility in that region of membrane.(iii) Rigid and highly anisotropic molecules dissolved in the DMPC membrane exhibit a bigger order of motion in the presence of polar carotenoids . Carotenoids decrease the rate of reorientational motion of cholestane spin label (CSL) and do not influence the rate of androstane spin label (ASL), probably due to the lack of the isooctyl side chain. The abrupt changes of spin label motion observed at the main phase transition of the DMPC bilayer are broadened and disappear at the presence of 10 mol% of carotenoids. In gel phase membranes, polar carotenoids increase motional freedom of most of the spin labels employed showing a regulatory effect of carotenoids on membrane fluidity. Their results support the hypothesis that carotenoids regulate the membrane fluidity in Procaryota as cholesterol does in Eucaryota. A model is proposed by these researchers to explain these results in which intercalation of the rigid rod-like polar carotenoid molecules into the membrane enhances extended trans-conformation of the alkyl chains, decreases free space in the bilayer center, separate the phosphatidylcholine headgroups and decreases interaction between them. [19]

Photosynthetic and antioxidant functions

Carotenoids function as accessory light-harvesting pigments in all plants due the polyene chain of 9-11 double bonds which absorb light in the gap of chlorophyll absorption (420-500nm). [22] The unique arrangement of molecular energy levels provided by polyene makes carotenoids the only natural compounds capable of close-range excitation energy transfer:

- from the carotenoid excited state (S1) to chlorophyll S1 in the light-harvesting complex, this is how light energy caught by carotenoid is channeled to photosynthetic reactions;
- from the triplet state of chlorophyll (a highly unstable state produced in photosynthesis and resulting in destruction of that molecule) to triplet carotenoid;

- from the singlet state of oxygen to carotenoid triplet state.

In photosynthetic reactions of all photosynthetics take place the two latter processes, absolutely vital for protection of reaction centre (RC) from photodamage. Carotenoids return from triplet to ground state just dissipate the excessive energy as heat, possible due to the very close range interactions inside the pigment-protein complex (RC or LHC-light harvesting chlorophyll).[24]

It is known that only about 50 of total of 600 carotenoids have provitamin A activity. Despite being one of the first vitamins to be discovered, the full range of biological activities for vitamin A remains to be defined. Within the body, vitamin A can be found as *retinol, retinal* and *retinoic acid*. Because all of these forms are toxic at high concentrations, they are bound to proteins in the extracellular fluids and inside cells. Vitamin A is stored primarily as long chain fatty esters and as provitamin carotenoids in the liver, kidney and adipose tissue. The antioxidant activity of vitamin A and carotenoids is conferred by the hydrophobic chain of polyene units that can quench singlet oxygen, neutralize thioyl radicals and combine with and stabilize peroxyl radicals. In general, the longer the polyene chain, the greater the peroxyl radical stabilizing ability. Because of their structures, vitamin A and carotenoids can autoxidize when O2 tension increases, and thus are most effective antioxidants at low oxygen tensions that are typical of physiological levels found in tissues.[18]

These compounds can theoretically participate in a biological antioxidant network. Under physiological conditions, vitamin A esters are transported and stored in a lipid matrix that contains other antioxidants, and retinol and its active metabolites are largely bound in clefts of specific retinoid-binding proteins. Thus, vitamin A seems to be protected in vivo by other antioxidants and proteins rather than protecting other molecules. Carotenoids are largely distributed in lipoproteins, membranes, and the lipid phases of intracellular structures, usually together with vitamin E. Carotenoids can interact with other antioxidants in vitro, but whether they play similar significant roles in vivo is not clear.[16]

Much effort has been expended in evaluating the relative antioxidant potency of carotenoid pigments in both in vitro and in vivo experiments. It is quite clear that in vitro, carotenoids can inhibit the propagation of radical-initiated lipid peroxidation, and thus fulfill the definition of antioxidants.

When it comes to in vivo systems, it has been much more difficult to obtain solid experimental evidence that carotenoids are acting directly as biological antioxidants. In fact, under nonphysiological circumstances, carotenoids may act as prooxidants.

These results can be modified by altering the oxidant stress, the cellular or subcellular system, the type of animal, and environmental conditions, such as oxygen tension. Results of this type raise the question as to whether it is still appropriate to group the carotenoids with such antioxidant vitamins as vitamin E and vitamin C. Thus, the biological properties of the carotenoids may be much more related to the products of the interaction of carotenoids with oxidant stress, that is, such breakdown products as apocarotenoids and retinoids.[13]

Singlet molecular oxygen (1O2) has been shown to be generated in biological systems and is capable of damaging proteins, lipids and DNA. The ability of some biological antioxidants to quench 1O2 was studied by using singlet oxygen generated by the thermodissociation of the endoperoxide of 3,3'-(1,4-naphthylidene) dipropionate (NDPO2). The carotenoid lycopene was the most efficient 1O2 quencher (kq + kr = 31 x 10(9) M-1 s-1). The singlet oxygen quenching ability decreased in the following order: *lycopene, gamma-carotene, astaxanthin, canthaxanthin, alpha-carotene, beta-carotene, bixin, zeaxanthin, lutein, bilirubin, biliverdin, tocopherols* and *thiols*. However, the compounds with low quenching rate constants occur at higher levels in biological tissues. Thus, carotenoids may contribute almost equally to the protection of tissues against the deleterious effects of 1O2. The quenching abilities of carotenoids were mainly due to physical quenching.[9]

Carotenoids as antioxidants have also been studied for their ability to prevent chronic disease.

Various natural carotenoids were proven to have anticarcinogenic activity. Epidemiological investigations have shown that cancer risk is inversely related to the consumption of green and yellow vegetables and fruits. Since beta-carotene is present in abundance in these vegetables and fruits, it has been investigated extensively as possible cancer preventive agent. However, various carotenoids which co-exist with beta-carotene in vegetables and fruits also have anti-carcinogenic activity. And some of them, such as alpha-carotene, showed higher potency than beta-carotene to suppress experimental carcinogenesis. Thus, we have carried out more extensive studies on cancer preventive activities of natural carotenoids in foods; i.e., lutein, lycopene, zeaxanthin and beta-cryptoxanthin. Analysis of the action mechanism of these natural carotenoids is now in progress, and some interesting results have already obtained; for example, beta-cryptoxanthin was suggested to stimulate the expression of RB gene, an anti-oncogene, and p73 gene, which is known as one of the p53-related genes. Based on these results, multi-carotenoids (mixture of natural carotenoids) seems to be of interest to evaluate its usefulness for practice in human cancer prevention.[14]

Epidemiologic studies have shown an inverse relationship between presence of various cancers and dietary carotenoids or blood carotenoid levels. However, three out of four intervention trials using high dose beta-carotene supplements did not show protective effects against cancer or cardiovascular disease. Rather, the high risk population (smokers and asbestos workers) in these intervention trials showed an increase in cancer and angina cases. It appears that carotenoids (including beta-carotene) can promote health when taken at dietary levels, but may have adverse effects when taken in high dose by subjects who smoke or who have been exposed to asbestos.

It will be the task of ongoing and future studies to define the populations that can benefit from carotenoids and to define the proper doses, lengths of treatment, and whether mixtures, rather than single carotenoids (e.g. beta-carotene) are more advantageous.[17]

Recent evidence has shown vitamin A, carotenoids and provitamin A carotenoids can be effective antioxidants for inhibiting the development of heart disease. Although there is considerable discrepancy in the results from studies of humans regarding this relationship, carefully controlled experimental studies continue to indicate that these compounds are effective for mitigating and defending against many forms of cardiovascular disease. More work, especially concerning the relevance of how tissue concentrations, rather than plasma levels, relate to the progression of tissue damage in heart disease is required.[18]

Despite a large number of studies demonstrating protection by carotenoids, the characteristics that render a given carotenoid effective and the relative efficiency of the individual carotenoids are not known. Moreover, dose-response and pharmacokinetic relationships remain virtually unexplored. Research to uncover mechanisms of protection by carotenoids is, for technical reasons, painfully slow. Epidemiological studies reveal associations but not cause and effect. To explore cause and effect, intervention trials are underway, hampered by the paucity of data regarding optimal choice of carotenoid, dosage, and regimen. The in vitro test systems that would provide this information are not available because the molecular sites relevant to the chemopreventive action of carotenoids are obscure. Each of these problems has a solution, but not a simple one. Until these are resolved, blanket recommendations regarding supplementation will remain problematic.[8]

Vision

Carotenoids represent the only natural source where from men and and animals can synthetize vitamin A, which have an important biochemical role in human and animal organisms. The oxidized half of several careotenoids, i.e. the retinals, are receptor molecules in eyes of all animals. Retinals, complexed with the protein opsin, isomerize upon absorbing light quants, causing change of conformation of the whole rhodopsin, thus launching the further cascade of reactions lead to nerve excitation. This process very much resembles the light reception in ancient bacteriorhodopsin photosynthesis. Retinal, complexed with three different types of opsin, can absorb light of different wavelenghts – this is the base for colour vision in mammals. [24]

Communicative coloration

Light-absorption within visible range is used for for the most spectacular function of carotenoids – communicative species-specific coloration of plants and animals. Coloration of plants in many cases are due to antocyans, in animals primarly by melanins, but carotenoids have roles in really bright coloration of animals and plants. There are: [24]

- species and sex specific coloration for the animals of the same species to recognize each other;
- sex/social status coloration (some corals change their color pattern due carotenoids, as a sign of changed sex and social status);
- masking coloration (chameleon);
- attractive coloration (flowers and fruits);
- warning coloration (coral aspid);
- pigmentation of green microalga *Haematococcus pluvialis*, which accumulates large amounts of carotenoids astaxanthin in cytoplasm under high light. This pigmentation is for the screening of chloroplasts from excessive light;
- carotenoids serve for rising body temperature by absorption of solar radiation and dissipation of the energy as heat (planktonic copepodes in temperate lakes).[3]

Stereospecific functions

Carotenoids can be attacked enzymatically at almost every position in the molecule. On the other hand, under dark nonoxidative conditions, they can be very stable. Thus, the precise chemical and biological environment is of crucial importance in determining whether, and how, they are transformed. Three important biologically active derivatives of carotenoids, vitamin A, trisporic acid and abscisic acid, all of which serve as hormones in appropriate cells, are, or can be, formed from precursor carotenoids. Highly active forms of all three hormones are 9-cis isomers. In all cases, the products are involved both in light-induced reactions as well as in cellular differentiation, often related to sexual maturation. Each of these hormones is formed by an initial dioxygenase attack on the central conjugated chain of carotenoids followed by a series of specific reactions. Indeed, when various carotenoids of different structure are studied in humans, each shows a characteristic, if not unique, set of metabolites. Thus, generalizations about carotenoid metabolism must be constrained by precise metabolic information about given compounds. In dealing with the manifold biological activities of carotenoids, it is useful to categorize them as functions, actions or associations. In the hope of gaining greater insight into the relationship between carotenoids, health and longevity, these distinctions should be helpful. [15]

Retinoic acid (oxidized half of beta-carotene molecule) in mammals is a hormone regulating epidermal growth [20], abscisic acid (modified part of neoxanthin) is one of hrowth

hormones.[10] These functions are not based on the rigidity of carotenoid molecules or their unique electronic properties. The configuration of molecule is a key to the receptor lock.

Other functions

More recently, research interests on carotenoids have been revived, largely because of their immunomodulatory activities in humans and animals. These carotenoids enhance lymphocyte blastogenesis, increase the population of specific lymphocyte subsets, increase lymphocyte cytotoxic activity, and stimulate the production of various cytokines. In addition, carotenoids also stimulate the phagocytic and bacteria-killing ability of blood neutrophils and peritoneal macrophages. The action of these carotenoids is widely accepted to be independent of their provitamin A activity. The immunostimulatory action of carotenoids may be translated into improved health, including mammary and reproductive health in dairy cattle. More studies are needed to establish fully the beneficial effects of supplementation of different carotenoids on the health of dairy cattle. Furthermore, studies on carotenoids other than beta-carotene are needed. [4]

Zhao W et. al [27] have studied the effect of carotenoids on the respiratory burst of rat peritoneal macrophages. The effect of four carotenoids (beta-carotene, lutein, bixin and canthaxanthin) on the respiratory burst of rat peritoneal macrophages was investigated. The results obtained showed that carotenoids suppressed the luminol-dependent chemiluminescence generated from PMA-stimulated macrophages at the beginning and after 2 min of the stimulation. Canthaxanthin and bixin had higher suppressive activity than beta-carotene and lutein. The changes in absorption spectra of carotenoids showed that the absorption by carotenoids was diminished during the stimulation of macrophages by PMA and their absorption peaks were either further diminished or blue-shifted after addition of L-arginine to the system, indicating that the carotenoids were consumed and converted to new compounds during the two processes. By using cell-free systems, it was found that carotenoids could scavenge superoxide anion generated by xanthine/xanthine oxidase system. Their ability to scavenge superoxide anion decreased in the order of *canthaxanthin* > *bixin* > *lutein* > *beta-carotene*. Canthaxanthin also showed the scavenging effect on superoxide anion generated from irradiation of riboflavin. The hydroxyl radical scavenging activity of carotenoids was investigated in the reaction system of Fe^{2+} and H_2O_2. There was little difference among their activities. The reaction between carotenoids and nitric oxide led to the decreasing absorption between 400 and 540 nm and the concomitant appearance of the new absorption peaks between 330 and 395 nm. Bleaching of beta-carotene, bixin and canthaxanthin by peroxynitrite resulted in the increasing absorption between 290 and 365 nm and the diminishing absorption between 400 and 500 nm. But the increasing absorption between 280 and 490 nm was observed in bleaching of lutein by peroxynitrite. Carotenoids inhibited thiobarbituric acid-reactive substance (TBARS) formation in AAPH-induced lipid peroxidation of PC liposomes in air. The results suggest that the suppressive effect of carotenoids on the respiratory burst of macrophages may be just a way by which carotenoids in vivo protect host cells and tissues from harmful effects of oxygen metabolites overproduced by macrophages and enhance the generation of specific immune responses. [27]

Conclusion

The overview of the diversity of carotenoids' biological functions shows that the general structure of the carotenoid molecule, originally evolved for membrane-reinforcement in archaebacteria, remained practically unchanged in the great diversity of organisms and functions. Carotenoids proved to be tailor-made for many roles, some of which are of vital importance – like their protective function in photosynthetic reaction centers.

In addition to their roles as precursors of retinol and retinoids, carotenoids have distinct functions of their own in animals and humans. In vitro they are antioxidants with a broad range of potencies.

The biological functions of carotenoids are not entirely known, this might be the object of the further studies of the researchers.

References

1. Bendich, A., Olson, J.A., – *FASEB J.*, **3**(1), 927-932 (1989).
2. Britton, G., – *FASEB J.*, **9**(1), 551-558 (1995).
3. Byron, E.A., – *Proc. Natl. Acad. Sci. USA*, **78**, 1765-1767 (1981).
4. Chew, B.P., – *J. Dairy Sci.*, **76**, 2804-2811 (1993).
5. Corol, D.I., Toma, N., Dorobantu, I.I., – *Progrese in Biotehnologie*, Ed. Ars Docendi, 136-162 (2001).
6. Corol, D.I., Toma, N., Dorobantu, I.I., – *Progrese in Biotehnologie*, Ed. Ars Docendi, 204-231 (2001).
7. Corol, D.I., Toma, N., Dorobantu, I.I., – *Roum. Biotechnol. Lett.*, **6**(6), 493-500 (2001).
8. Davison, A., Rousseau, E., Dunn, B., – *Can. J. Physiol. Pharmacol.*, **71**, 732-745 (1993).
9. Di Mascio, P., Devasagayam, T.P., Kaiser, S., Sies, H., – *Biochem. Soc. Trans.*, **18**, 1054-1056 (1990).
10. Goodwin, T.W., – *The Biochemistry of Carotenoids. 1. Plants*, Chapman and Hall, N.Y. (1980).
11. Hsiao, K.C., Moller, I.M., – *Physiol. Plant.*, **62**, 167-174 (1984).
12. Huang, L., Huang, A., – *Biochim. Biophys. Acta*, **352**, 361-370 (1974).
13. Krinsky, N.I., – *Ann. N.Y. Acad. Sci.*, **854**, 443-447 (1998).
14. Nishino, H., Tokuda, H., Murakoshi, M., Satomi, Y., Masuda, M., Onozuka, M., Yamaguchi, S., Takayasu, J., Tsuruta, J., Okuda, M., Khachik, F., Narisawa, T., Takasuka, N., Yano, M., – *Biofactors*, **13**, 89-94 (2000).
15. Olson, J.A., – *Ann. N.Y. Acad. Sci.*, **691**, 156-166 (1993).
16. Olson, J.A., – *J. Nutr. Sci. Vitaminol. (Tokyo)*, **39** Suppl., 57-65 (1993).
17. Paiva, S.A., Russell, R.M., – *J. Am. Coll. Nutr.*, **18**, 426-433 (1999).
18. Palace, V.P., Khaper, N., Qin, Q., Singal, P.K., – *Free Radic. Biol. Med.*, **26**, 746-761 (1999).
19. Rohmer, M., Bouvier, P., Ourisson, G., – *Proc. Natl. Acad. Sci. USA*, **76**, 847-851 (1979).
20. Ross, A.C., Ternus, M.E., – *J. American Dietic. Assoc.*, **93**, 1285-1290 (1993).
21. Rottem, S., Markowitz O., – *J. Bacteriol.*, **140**, 944-948 (1979).
22. Siefermann-Harms, D., – *Biochem. Biophys. Acta*, **811**, 325-355 (1985).
23. Subczynski, W.K., Markowska, E., Gruszecki, W.I., Sielewiesiuk, J., – *Biochem. Biophys. Acta*, **1105**, 97-108 (1992).
24. Vershinin, A., – *BioFactors*, **10**, 99-104 (1999).
25. Vershinin, A., – *Comp. Biochem. Physiol.*, **107B**, 63-72 (1996).
26. Zagalsky, P.F., Eliopulos, E.E., – *Comp. Biochem. Physiol.*, **97B**, 1-18 (1990).
27. Zhao, W., Han, Y., Zhao, B., Hirota, S., Hou, J., Xin, W., – *Biochem. Biophys. Acta*, **1381**, 77-88 (1998).