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The antioxidant properties of canthaxanthin and its potential effects in the poultry eggs and on embryonic development of the chick. Part 1.

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Among more than 750 known carotenoids, canthaxanthin (CX) has a special place as a carotenoid with proven antioxidant and other biologically-relevant functions. A substantial body of evidence indicates that CX possesses high antioxidant activity which has been shown in various *in vitro* model systems as well as in animal experiments *in vivo*. It seems likely that the highest protective effects of CX are seen under various stress conditions. This compound may be considered as an important element of the integrated antioxidant system of various tissues in the body, including chicken embryo development. A possibility of the recycling of vitamin E by carotenoids, including CX, is of interest for further investigation. Taken together, the data analysed in the paper clearly indicated that CX could provide benefits for animals, including in eggs and embryos as well as for chickens during early postnatal development. In particular, CX is well absorbed from the diet and effectively transferred to the egg yolk and developing embryo. It possesses high antioxidant activity and participates in building an effective antioxidant system of the body.

Keywords: canthaxanthin; carotenoids; chicken; egg; antioxidant

Introduction

Carotenoids are the most numerous and widespread group of pigments in nature and have had a long and interesting history. Studies on these pigments were started at the beginning of the 19th century, when the crystalline yellow pigment carotene was first isolated in 1831 by Wackenroder from carrots, and the yellow pigments of autumn leaves were named as xanthophylls by Berzelius in 1837 (Tee, 1992; Karnaukhov, 1990). A hundred years later, the number of characterised naturally occurring carotenoids rose from 15 in 1933 to about 80 in 1948 and increased sharply to about 300 over the next 20

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years (Ong and Tee, 1992). Today, the carotenoid family is known to include over 750 pigments (Maoka, 2009).

In nature, carotenoids are responsible for a variety of bright colours in senescent leaves, flowers (narcissus, marigold), fruits (pineapple, citrus fruits, paprika), vegetables (carrots, tomatoes), insects (ladybird), bird plumage (flamingo, cock of rock, ibis, canary) and marine animals (crustaceans, salmon) (Pfander, 1992). These pigments provide different colours from light yellow to dark red and, when complexed with proteins, can produce green and blue colorations (Ong and Tee, 1992). Carotenoids are exclusively responsible for egg yolk colour and are thought to play specific roles in avian embryonic development (Surai, 2002).

Chemical structure and properties

All carotenoids may be derived from the acylic $C_{40}H_{56}$ structure having a long central chain of conjugated double bonds (Pfander, 1992) and they can be described by the general formula $C_{40}H_{56}O_n$. Where n is 0-6 hydrocarbons (n=0) these compounds are called carotenes, whereas oxygenated carotenoids (n=1-6) are termed xanthophylls (Castenmiller and West, 1998). The basic structure of canthaxanthin (CX) is shown in *Figure 1*. Carotenoids are based upon the same C40 isoprenoid skeleton, which is modified by cyclisation, addition, elimination, rearrangement and substitution (Rice-Evans *et al.*, 1997).

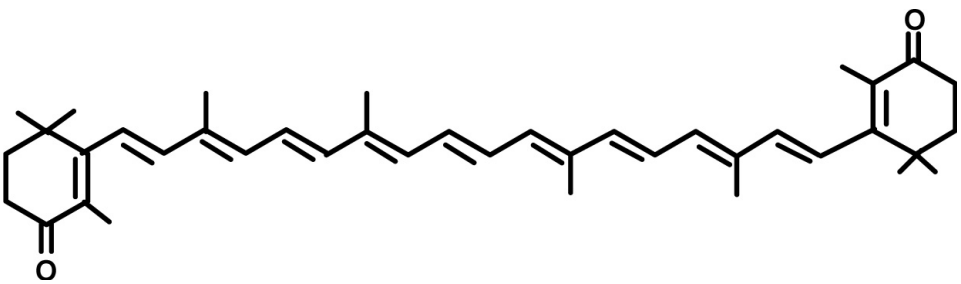

Figure 1 Chemical structure of Canthaxanthin .

Molecular mechanisms of carotenoid action in animals

It is well known that carotenes are precursors of vitamin A and this function has been well defined. However, less than 10% of known carotenoids can be converted to vitamin A, and clearly-defined roles for non-provitamin carotenoids have still to be definitively established (Thurnham and Northrop-Clewes, 1999; Faulks and Southon, 2005), but evidence is emerging for several important functions. These include:

- antioxidant activities (Krinsky, 1989a; Rice-Evans *et al.*, 1997; Edge *et al.*, 1997; Moller *et al.*, 2000; Surai *et al.*, 2001; Surai, 2002);
- the promotion of cell differentiation (Zhang *et al.*, 1991; Zhang *et al.*, 1992; Rock *et al.*, 1995);
- regulation of cell proliferation (Krinsky, 1992; Bertram and Borthiewicz, 1995);
- regulation of intracellular communication via gap junctions (Sies ans Stahl, 1997; Stahl and Sies, 2005);
- regulation of cellular levels of the detoxifying enzymes (De Flora, 1999);

- enhancement of immune function (Bendich, 1991; Hughes, 1999; Moller *et al.*, 2000);
- natural colorants providing coloration to birds, reptiles, amphibians, fish and various invertebrates (Surai, 2002);
- cell membrane stabilisers in molluscs (Surai, 2002).

These functions are considered to be responsible for the various health-promoting properties of carotenoids. However, the physiological requirement for carotenoids in avian species is not yet established and more work in this field is needed to understand how carotenoids fulfil any essential nutritional function in poultry.

Antioxidant properties of carotenoids

The antioxidant potential of carotenoids was first described in 1932 (Monoghan and Schmitt, 1932). The discovery by Foote and Denny (1968) that carotenoids, such as β-carotene, lycopene, zeaxanthin, lutein and CX, could quench singlet oxygen $^1O_2$ was an important advance in understanding the effectiveness of carotenoid pigments in preventing damage within photobiological systems (Foote *et al.*, 1970). Sixteen years later, Burton and Ingold (1984) proposed the mechanism of quenching lipid radicals within biological membranes by carotenoids. In recent years an important role of carotenoids in biological systems as antioxidants has received substantial attention (Krinsky, 1989a; Edge *et al.*, 1997; Rice-Evans *et al.*, 1997; Bast *et al.*, 1998; Surai, 2002; Stahl and Sies, 2003).

The antioxidant properties of carotenoids include scavenging singlet oxygen and peroxyl radicals (Krinsky, 1989b; Terao *et al.*, 1992), sulphur radicals (Chopra *et al.*, 1993) as well as thiyl, sulphonyl and NO_2 radicals (Everett *et al.*, 1996) and provide protection for lipids against superoxide and hydroxyl radical attack (Krinsky and Deneke, 1982). The mechanism of protection of biological systems against damage due to $^1O_2$ by carotenoids includes both a physical component as well as a chemical reaction between the carotenoid and the reactive oxygen molecule (Krinsky, 1989a). The deactivation of $^1O_2$ by carotenoids results predominantly in physical quenching, a process involving transfer of reactive energy from $^1O_2$ to the carotenoids and resulting in the formation of ground state oxygen $^3O_2$ and triplet reactive carotenoid ^{3}Car* (Stahl and Sies, 1993). In real terms, this means that, instead of participating in further chemical reactions, the carotenoid returns to its ground state, dissipating its energy by interaction with the surrounding solvent. Therefore, carotenoids can actively quench singlet oxygen ($^1O_2$) and prevent lipid peroxidation caused by singlet oxygen and can intercept the propagation step of lipid peroxidation *in vitro* (Rice-Evans *et al.*, 1997).

The physical quenching reaction involves the transfer of energy from high-energy state molecules, such as $^1O_2$, to the carotenoid (CAR) with a formation of the carotenoid triplet (Bast *et al.*, 1998):

$$^1O_2 + CAR = {}^3O_2 + {}^3CAR$$

In the subsequent reaction the carotenoid dissipates its energy as heat and returns to its basic state:

$$^3CAR = CAR + heat$$

Since the carotenoids remain intact during physical quenching of $^1O_2$ or reactive

compounds, they can be reused several fold in quenching cycles. Among the various carotenoids, xanthophylls as well as carotenes have proven to be efficient quenchers of singlet oxygen interacting with reaction rates that approach diffusion control (Foote and Denny, 1968; Conn *et al.*, 1991). The efficacy of carotenoids for physical quenching is related to the number of conjugated double bonds present in the molecule which determines their lowest triplet energy level. In this way, one molecule of β-carotene is able to quench 1000 molecules of singlet oxygen before it reacts chemically and forms products (Bast *et al.*, 1998; Krinsky, 1998). Maximum protection is afforded by carotenoids which have nine or more double bonds (Krinsky, 1989a) and CX is one such effective carotenoids. When reactions between $^1O_2$ and carotenoids takes place through chemical scavenging, oxidative products of carotenoids are formed, but this is considered to be a very minor side reaction (Edge *et al.*, 1997) and the antioxidant impact of this chemical reaction is negligible.

Carotenoids are able to react with a range of free radicals (R*) and in this case three possible mechanisms are considered:

- electron transfer with a formation of carotenoid radical cation:

$$R^* + CAR = R^- + CAR^{*+}$$

- addition reaction with the formation of a carotenoid-adduct radical which can react with another radical to form a non-radical product:

$$ROO^* + CAR = ROO\text{-}CAR^*$$
$$ROO\text{-}CAR^* + ROO^* = ROO\text{-}CAR\text{-}ROO$$

- hydrogen abstraction with a formation of the neutral carotenoid radical:

$$R^* + CAR(H) = RH + CAR^*$$

In accordance with widely accepted views, the addition reaction and/or hydrogen abstraction are the more probable reactions that occur between free radicals and carotenoids (Kennedy and Liebler, 1991; 1992).

The relative reduction potentials of a variety of carotenoids have been established by monitoring the reaction of carotenoid radical anion (CAR1(*-)) with another carotenoid (CAR2) in hexane and benzene (Edge *et al.*, 2007). This work illustrated that the presence of a carbonyl group causes the reducing ability to decrease. Indeed, the radical cations are strong oxidising agents and the authors have shown that the radical anions are very strong reducing agents.

The chemical reactions between radical species and carotenoids should result in certain products, which include epoxy, hydroxy and carbonyl derivatives of the original molecules. When CX was reacted with peroxyl radicals generated by thermolysis of 2,2‘-azobis(2,4-dimethylvaleronitrile) (AMVN) in benzene (Yamauchi and Kato, 1998), the peroxyl radical addition occurred during the AMVN-initiated peroxidation of methyl linoleate. In contrast to the action of other antioxidants such as vitamin E, where reactions with free radicals involves electron or hydrogen transfer, the above products appear to be formed by radical addition to the carotenoid molecule (Canfield *et al.*, 1992).

Evaluation of the antioxidant activity of carotenoids

The antioxidant activity of carotenoids *in vitro* has been characterised by using different methodological approaches (Soffers *et al.*, 1999) including their ability to scavenge various radicals in solutions, their relative rate of oxidation by a range of free radicals, and their capacity to inhibit lipid peroxidation in multilamelar liposomes.

The comparative mechanisms and relative rates of nitrogen dioxide, thiyl and sulphonyl radical scavenging by such carotenoids as lycopene, lutein, zeaxanthin, astaxanthin and CX have been determined by pulse radiolysis (Mortensen *et al.*, 1997). Under experimental conditions, all carotenoids react with the NO_2^* radical via electron transfer to generate the carotenoid radical cation. In marked contrast, the glutathione and 2-mercaptoethanol thiyl radicals react via a radical addition process to generate carotenoid-thiyl radical adducts. The sulphonyl radical undergoes both radical addition, and electron abstraction. The mechanism and rate of scavenging is strongly dependent on the nature of the oxidising radical species, but much less dependent on the carotenoid structure.

The peroxyl trapping activity of carotenoids was shown to be in the following order: astaxanthin= CX >>β-carotene = zeaxanthin (Terao, 1989), astaxanthin > CX > β-carotene > zeaxanthin (Jorgensen and Skibsted, 1993). In contrast, when the interaction with the stable radical cation (ABTS+) was used to evaluate the antioxidant activity of carotenes and xanthophylls, the ranking was as follows: lycopene >> β-carotene = β-cryptoxanthin > lutein = zeaxanthin = α-carotene>echinenone>>astaxanthin= CX (Rice-Evans *et al.*, 1997) which was similar to that reported by Miller *et al.* (1996).

Several studies have looked at carotenoids, mainly *β*-carotene and CX, as inhibitors of LDL oxidation (Carpenter *et al.*, 1997). The order of carotenoid oxidation in LDL exposed to Cu^{2+} was as follows: lycopene > β-cryptoxanthin > lutein/zeaxanthin > α and β-carotene (Esterbauer *et al.*, 1992). Similarly, as a result of carotenoid interaction with the ABTS+ radical cation, the order of carotenoid oxidation was lycopene > β-carotene > lutein > CX = astaxanthin (Rice-Evans *et al.*, 1997). It has been shown that CX can protect liposomes against Cu+-initiated lipid peroxidation (Rengel *et al.*, 2000). The ability of xanthophylls (CX, zeaxanthin, and astaxanthin) as chain-breaking antioxidants was investigated in peroxyl radical-mediated peroxidation of phosphatidylcholine (PC) liposomes under atmospheric conditions using lipid-soluble and water-soluble radical generators (Lim *et al.*, 1992). These xanthophylls retarded the chain propagation reaction of phosphatidylcholine hydroperoxides (PC-OOH) formation, although their activities to trap chain-carrying peroxyl radical were much less than that of α-tocopherol. When peroxidation was initiated by a water soluble initiator (AAPH) the order of antioxidant activity changed: astaxanthin=zeaxanthin>>β-carotene>CX (Lim *et al.*, 1992).

When an electrochemical method of antioxidant activity evaluation was applied to pure compounds, the order of antioxidant activity was as follows: lycopene > β-carotene > zeaxanthin > α-carotene > β-cryptoxanthin > lutein > α-tocopherol > astaxanthin > CX (Buratti *et al.*, 2001). Methylene blue plus visible light in the presence of oxygen, induced lipid peroxidation in rat liver microsomes, as assessed by the formation of thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides and the loss of membrane-bound enzymes (Kamat and Devasagayam, 1996). Protective effects were observed with natural antioxidants such as CX, β-carotene, lipoic acid, glutathione, α-tocopherol and, to a lesser extent, ascorbic acid.

In a detailed study, the ability of carotenoids to protect egg-yolk phosphatidylcholine lipids against oxidation by peroxyl radicals generated from azo-initiators was evaluated

(Woodall *et al.*, 1997). In a homogeneous organic solution, β-ring carotenoids showed a correlation between a protective effect and the rate of carotenoid destruction. The reactivity and protective ability of the 4,4'-diketocarotenoids, astaxanthin and CX was less than β-carotene and zeaxanthin. It is necessary to underline that yellow xanthophylls and β-carotene have the highest rates of oxidation, with the ketocarotenoids and violaxanthin degrading at lower rates (Perez-Galvez and Minguez-Mosquera, 2001).

The ability of astaxanthin and CX as chain-breaking antioxidants was studied in Cu^{2+}-initiated peroxidation of phosphatidyl choline large unilamellar vesicles (Rengel *et al.*, 2000). Both carotenoids increased the lag period that preceeds the maximum rate of lipid peroxidation, although astaxanthin showed stronger activity. Differential scanning calorimetry assays demonstrated that, when incorporated, xanthophylls interact with the lipid matrix, becoming interspersed among the phospholipid molecules. It is interesting that, in HL 60 cells, lutein and CX scavenged phenoxyl radical faster than β-carotene or lycopene (Tyurin *et al.*, 1997).

Using a model system based on brain homogenate oxidation, antioxidant activities of lutein, zeaxanthin, CX, lycopene, β-cryptoxanthin and β-carotene were clearly shown (Surai *et al.*, 1995). At concentrations, comparable with those in tissues of chickens fed on diets low (1-5 ppm) in CX supplementation, CX (10 ppm in the incubation medium) showed antioxidant activities in terms of prevention of MDA formation, comparable to vitamin E. The ability of several dietary carotenoids to quench singlet oxygen in a model membrane system (unilamellar DPPC liposomes) has been investigated. Singlet oxygen was generated in both the aqueous and the lipid phase, with quenching by a particular carotenoid independent of the site of generation. Lycopene and *β*-carotene exhibit the fastest singlet oxygen quenching rate constants with lutein the least efficient. The other carotenoids, astaxanthin and CX, were intermediate (Cantrell *et al.*, 2003).

By using cell-free systems, it was found that carotenoids could scavenge superoxide anion generated by xanthine/xanthine oxidase system. Their ability to scavenge the superoxide anion decreased in the order of CX > bixin > lutein > *β*-carotene (Zhao *et al.*, 1998). CX also showed a scavenging effect for the superoxide anion generated from irradiation of riboflavin. Bleaching of *β*-carotene, bixin and CX by peroxynitrite resulted in the increasing absorption between 290 and 365 nm and the diminishing absorption between 400 and 500 nm.

Electron transfer to the radical cations of *β*-carotene, zeaxanthin, CX, and astaxanthin from each of the three acid/base forms of the diphenolic isoflavonoid daidzein and its C-glycoside puerarin, as studied by laser flash photolysis in homogeneous methanol/chloroform (1/9) solution, was found to depend on the carotenoid structures involved and, more significantly, on the deprotonation degree of the isoflavonoids (Han *et al.*, 2010). Electron transfer from isoflavonoids to the carotenoid radical cation, as formed during oxidative stress, was faster for astaxanthin and CX than for the other carotenoids, which may relate to astaxanthin and CX more effective antioxidative properties and in agreement with their higher electron accepting index.

The antioxidant activity of *β*-carotene and oxygenated carotenoids lutein, CX, and astaxanthin was investigated during spontaneous and peroxyl-radical-induced cholesterol oxidation (Palozza *et al.*, 2008). Cholesterol oxidation, measured by the generation of 7-keto-cholesterol (7-KC), was evaluated in a heterogeneous solution with cholesterol, AAPH, carotenoids solubilised in tetrahydrofuran in water, and in a homogeneous solution of chlorobenzene, with AIBN as a pro-oxidant. The formation of 7-KC was dependent on temperature and on cholesterol and pro-oxidant concentrations. All the carotenoids tested, exhibited significant antioxidant activity by inhibiting spontaneous, AAPH- and AIBN-induced formation of 7-KC, although the overall order of efficacy of these compounds was astaxanthin > CX > lutein = *β*-carotene.

It is necessary to mention that different model systems have been employed to test antioxidant properties of carotenoids including cultured hepatocytes, normal and tumour thymocytes, kidney fibroblasts, embryonic hippocampal cultures, embryo fibroblast ovary cells, primary cultures of chicken embryo fibroblasts, leukaemia HL-60 cells, monocyte-macrophages, cultured Ito cells, LDL in different systems, *Salmonella typhimurium*, pigmented yeast *Rhodotorula mucilaginosa* and liver microcosms. In most cases, β-carotene was tested in the model systems as an antioxidant. But other carotenoids have been studied as well, including lutein, lycopene, α-carotene, CX, astaxanthin, zeaxanthin (Surai, 2002). In the model systems, different stress factors were used including CCl_4, tret-butyl hydroperoxide, UV light, ethanol, paraquat, T-2 toxin, aflatoxin B1, H_2O_2, adriamycin, peroxynitrous acid and $CuSO_4$. In many cases the accumulation of TBARS was used to monitor lipid peroxidation. In addition, lipid hydroperoxide formation, cholesteryl ester hydroperoxide formation, sister chromatid exchanges and DNA breaking were monitored. Under the conditions of the model systems the carotenoids confirmed their antioxidative protective effects preventing lipid peroxidation, decreasing cytotoxicity or decreasing DNA breaking. Thus, *in vitro* experiments using different modelling systems clearly showed an antioxidative protective effect for various carotenoids and the ranking of their antioxidant activity depends on the model system used. In general, CX was shown to be quite effective as an antioxidant (Surai, 2002; Palozza *et al.*, 2008), as measured in terms of prevention TBARS formation, lipid hydroperoxide formation, cholesteryl ester hydroperoxide formation, sister chromatid exchanges and DNA breaking.

Antioxidant action of carotenoids in vivo

In vivo experiments devoted to examining the antioxidant properties of carotenoids have been previously described (Palozza and Krinsky, 1992; Moller *et al.*, 2000; Surai, 2002). In other trials, various carotenoids were included in the diet of chickens, mice, rats, guinea pigs, fish, and humans. In some cases, diets were deficient in antioxidants (vitamin E and Se), whilst in others, diets were sufficient in major antioxidants. In many situations various stress factors were applied. This included injection with CCl_4, tumour development or inoculation with tumour cells. In addition, exposure to UV or X-ray radiation, dietary inclusion of oxidised oils, a zinc deficient diet, a diet with iron overload or challenged with *Aeromonas salmonicida* were used (Surai, 2002).

When a Se and vitamin E deficient chicken diet was supplemented with CX (0.5 g CX/kg diet), liver homogenates exhibited significantly ($P=0.02$) decreased formation of thiobarbituric acid-reactive substances over time in ferrous ion-induced peroxidation conditions (Mayne and Parker, 1989). It is worth mentioning the protective effect of CX observed in an experiment where chickens were fed diets containing full-fat flaxseed supplemented with mixed tocopherols and CX (Ajuyah *et al.*, 1993). Cooked meat from both the tocopherol and CX-supplemented chickens was more stable during refrigerated storage. The best stability of meat lipids was obtained with the combination of the two compounds. Tocopherol is known to act as a hydrogen donor; however, canthaxanthin can stop peroxy free radical chain propagation by trapping the radical in its conjugated polyene system (Ruiz *et al.*, 1999). When vitamin E and CX were both included in the diet of fish, a strong antioxidant effect was shown. This was attributed to the concept of antioxidant interaction. Indeed, these two compounds use different mechanisms to control lipid oxidation. α-Tocopherol is a hydrogen donor and can donate the hydrogen from its C-6 carbon while CX captures the peroxyl free radical in its conjugated polyene system. It is interesting to mention that in the liver of chickens fed on CX-enriched diet vitamin E

concentrations significantly increased (Mayne and Parker, 1989). Furthermore, antioxidant interactions play an important role in antioxidant defences. For example, SOD activity was increased in plasma newly hatched chicks obtained from CX-enriched eggs (Zhang *et al.*, 2011)

When carotenoid antioxidant activities were measured, a range of state of the art techniques of assessing oxidative stress and lipid peroxidation were employed. In most of the cases the accumulation of TBARS was used as a test for lipid peroxidation. In other cases phospholipid hydroperoxide accumulation, lysis of erythrocytes, pentane production, levels of antioxidant vitamins in tissues, plasma antioxidant capacity, activities of antioxidant enzymes, resistance of LDL to oxidative stress, oxidative damage to lipids, lipoproteins and DNA, the frequency of micronuclei of polychromatic erythrocytes and the mitotic index of bone marrow cells, photosensitivity, survival rate after irradiation or survival of lymphoma-bearing animals were used as the end points in experiments (Surai, 2002). In most of *in vivo* experiments β-carotene was tested. But other carotenoids showed positive effects as well including lutein, CX, citranaxanthin and carotenoic acid, zeaxanthin and CX, ethyl-β-apo-8^{I}-carotenoate, CX, astaxanthin, lutein and lycopene (Surai, 2002).

The protective effect of carotenoid dietary supplementation varied but was sufficient to conclude that the carotenoids express their antioxidant properties *in vivo*. It is necessary to underline that the efficiency of antioxidant protection afforded by carotenoids depends on their accumulation in the experimental tissues. On the other hand, an interaction of carotenoids with other antioxidants could be considered as an additional factor regulating the efficiency of antioxidant defence in the tissue.

Taken together, the results clearly show that carotenoids express their antioxidant properties not only *in vitro* but *in vivo* as well. The efficiency of the antioxidant defence provided by carotenoids depends on many factors including stress conditions, method of oxidative stress detection, concentrations of carotenoid used, model system employed, oxygen tension and interaction with other antioxidants (Rock, 1997; Rice-Evans *et al.*, 1997; Edge *et al.*, 1997). It has been suggested that depending on the redox potential of the carotenoid molecules and oxygen tension, carotenoid concentration and interactions with other antioxidants these pigments could show antioxidant or pro-oxidant properties (Palozza, 1998). However, it is necessary to underline that in physiological conditions all those factors are usually favourable for antioxidant activity of carotenoids. Therefore, carotenoids, such as CX, are efficient quenchers of singlet oxygen and are also effective scavengers of free radicals. Indeed, in biological systems carotenoids could be considered as an integral part of the antioxidant systems operating inside the membranes. Furthermore, recent results demonstrate a very strong modifying effect of CX with respect to the dynamic and structural properties of lipid membranes (Sujak *et al.*, 2005). However, the precise details of the processes associated with the quenching of radical species by carotenoids, as well as inhibition of the propagation of chain reactions, remain to be elucidated.

Conclusions

Among more than 750 known carotenoids, CX has a special place as a carotenoid with proven antioxidant and other biologically-relevant functions. A great body of evidence indicates that CX possesses high antioxidant activity that was shown in various *in vitro* model systems as well as in animal experiments *in vivo*. It seems likely that the highest protective effects of CX would be seen under various stress conditions and this compound could be considered as an important element of the integrated antioxidant

system of various tissues in the body, including chicken embryo development. A possibility of the recycling of vitamin E by various carotenoids, including CX, is of great importance for further investigation. Taking together, the aforementioned results clearly indicate that CX could provide a great deal of benefits for animals, including chicken eggs, embryos and chickens during early postnatal development.

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