Carotenoids in Avian Nutrition and Embryonic Development. 2. Antioxidant Properties and Discrimination in Embryonic Tissues

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Introduction

In the first part of the review (Surai et al., 2001) we described features of carotenoid assimilation from the feed by poultry and analysed carotenoid concentrations in plasma and egg yolk depending on their concentration in the feed. In spite of a long history our knowledge on molecular mechanisms of carotenoid action in human and animals are still in the initial stage. One of the important suggestion which has attracted a lot of attention in the recent years is an antioxidant role of carotenoids in biological systems. If proven this could have a great implication for the understanding of the role of carotenoids in the avian egg yolk and embryonic development. Therefore in this part of the review we will characterise antioxidant properties of carotenoids in vitro and in vivo and describe carotenoid profile of the embryonic tissues in relation to antioxidant protection in the developing chicken embryo.

Antioxidant properties of carotenoids

In recent years it has become obvious that carotenoids play an important role in biological systems as antioxidants (Krinsky, 1989; Edge et al., 1997; Rice-Evans et al., 1997; Bast et al., 1998). Carotenoids can actively quench singlet oxygen ($^1O_2$) and prevent lipid peroxidation caused by singlet oxygen and they can intercept the propagation step of lipid peroxidation in vitro (Rice-Evans et al., 1997). The mechanism of protection of biological system against damage due to $^1O_2$ by carotenoids includes both physical component as well as a chemical reaction between a carotenoid and the excited oxygen molecule (Krinsky, 1989).

The physical quenching reaction involves the transfer of the 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):

$$^1O_2 + \text{CAR} \rightarrow ^3O_2 + ^3\text{CAR}$$

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

$$^3\text{CAR} \rightarrow \text{CAR} + \text{heat}$$

In this way, one molecule of $\beta$-carotene is able to quench 1000 molecules of singlet oxygen before it reacts chemically and form products (Bast et al., 1998; Krinsky,
Maximum protection is afforded by carotenoids which have 9 or more double bonds (Krinsky, 1989).

When the reaction between $^1$O$_2$ and carotenoids takes place through chemical scavenging, oxidative products of carotenoids are formed, but this reaction is considered as 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:
  $$R^* + \text{CAR} \rightarrow R^- + \text{CAR}^*$$

- addition reaction with a formation of a carotenoid-adduct radical which can react with another radical to form a nonradical product:
  $$\text{ROO}^* + \text{CAR} \rightarrow \text{ROO}^- \text{CAR}$$
  $$\text{ROO}^- \text{CAR}^* + \text{ROO}^* \rightarrow \text{ROO}^- \text{CAR} + \text{ROO}$$

- hydrogen abstraction with a formation of the neutral carotenoid radical:
  $$R^* + \text{CAR(H)} \rightarrow RH + \text{CAR}^*$$

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

The chemical reactions between radical species and carotenoids should result in certain products. The carotenoid oxidation products include epoxy, hydroxy and carbonyl derivatives of the original molecules. The major $\beta$-carotene oxidation products of free radical reactions are carbonyl products. One of the most important products of chemical scavenging of singlet oxygen by $\beta$-carotene is $\beta$-carotene-5, 8-endoperoxide (Stratton et al., 1993). A number of other products of carotenoid reaction with excited oxygen molecule or free radicals can be formed as well (Handelman et al., 1991; Yamauchi et al., 1993). For example, products formed by the reaction of $\beta$-carotene with alkoxyl, and alkylperoxyl free radicals generated by thermolysis of azobis (2, 4-dimethylvaleronitrile) (AMVN) in benzene were determined and characterised (Liebler and McClure, 1996). It was also shown that peroxy radicals generated in hexane by thermolysis of AMVN at 37°C oxidized $\beta$-carotene to 5, 6-epoxy-beta, beta-carotene and to 15, 15'-epoxy-beta, beta-carotene in addition to several unidentified polar products (Kennedy and Liebler, 1991). In contrast to the action of other antioxidants such as vitamin E, where reaction 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). Therefore $\beta$-carotene is an efficient scavenger of peroxy radicals and the efficiency of such reactions is increased at low oxygen tensions (Burton and Ingold, 1984; Kennedy and Liebler, 1992). The similarity in its antioxidant effects at 15 and 160 torr O$_2$ suggests that $\beta$-carotene could provide antioxidant protection to any tissue within the normal physiologic range of pO$_2$ (Kennedy and Liebler, 1992).

If carotenoids take part in antioxidant reactions in vivo the oxidative products of such reactions should be detected in tissues. Unfortunately, the sensitivity of tech-
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Techniques used has been insufficient to detect such products. For example, under different experimental conditions, Iannone et al. (1998) were not able to detect any radical species deriving from carotenoids but provided direct evidence that carotenoids quench peroxyl radicals. However potentially biologically active oxidation products of lycopene have been identified in human plasma (Sies and Stahl, 1998). Recently it has been shown that lutein, zeaxanthin, 3'-epilutein, and 3-hydroxy-beta,epsilon-caroten-3'-one in human retina may be interconverted through a series of oxidation-reduction reactions (Khachik et al., 1997). The presence of the direct oxidation product of lutein and 3'-epilutein in human retina is a direct confirmation of antioxidant action of these carotenoids. Oxidation products of carotenoids in human plasma were also identified (Khachik et al., 1992). Among the carotenoid metabolites in human milk and serum were two oxidation products of lycopene and four of lutein/zeaxanthin (Khachik et al., 1997 a). In chicken plasma and tissues (Tyczkowski et al., 1986) and egg yolk (Schaeffer et al., 1988) 3'-oxolutein was detected and characterised.

Evaluation of the antioxidant activity of carotenoids

Antioxidant activity of the carotenoids in vitro is characterised by using different methodological approaches (Soffers et al., 1999): their ability to scavenge various radicals in solutions; their relative rate of oxidation by a range of free radicals; their capacity to inhibit lipid peroxidation in multilamellar liposomes.

The comparative mechanisms and relative rates of nitrogen dioxide, thyl and sulphonyl radical scavenging by such carotenoids as lycopene, lutein, zeaxanthin, astaxanthin and canthaxanthin have been determined by pulse radiolysis (Mortensen et al., 1997). In the experiment all the carotenoids react with the NO₂⁻ radical via electron transfer to generate the carotenoid radical cation. In marked contrast, the glutathione and 2-mercaptoethanol thyl radicals react via a radical addition process to generate carotenoid-thyl 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 order: astaxanthin = canthaxanthin > β-carotene = zeaxanthin (Terao, 1989), astaxanthin > canthaxanthin > β-carotene > zeaxanthin (Jorgensen and Skibsted, 1993). In contrast, when interaction with the stable radical cation (ABTS⁺) was used to evaluate antioxidant activity of carotenes and xanthophylls the ranking was as follows: lycopene > β-carotene = β-cryptoxanthin > lutein = zeaxanthin = α-carotene > echinenone > astaxanthin = canthaxanthin (Rice-Evans et al., 1997) which was similar to that reported by Miller et al. (1996).

The order of carotenoid oxidation in LDL exposed to Cu²⁺ 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 > canthaxanthin = astaxanthin (Rice-Evans et al., 1997). Bleaching of carotenoid mixtures mediated by
NaOCl, addition of azo-bis-isobutyronitril, and the photoirradiation of carotenoid mixtures by natural sunlight lead to the following sequence of breakdown rates: lycopene > beta-carotene > zeaxanthin > lutein (Siems et al., 1999). Therefore the rate of oxidation of lutein and zeaxanthin is moderate compared to other carotenoids studied.

When phosphatidyl choline (PC)-containing liposomes were incubated with AMVN in the presence of alpha-, beta-carotene, lutein and zeaxanthin, they limited phosphatidyl choline hydroperoxide (PCOOH) formation by 77%, 68%, 85% and 82%, respectively, while alpha-tocopherol elicited 90% reduction. Alpha-Carotene significantly suppressed thiobarbituric acid-reactive substances (TBARS) formation by 78% whilst beta-carotene, lutein, zeaxanthin elicited 60%, 91% and 80% reductions respectively (Farombi and Britton, 1999). These results confirmed the antioxidant activity ranking of carotenoids which was shown earlier: astaxanthin > zeaxanthin > anthaxanthin >> beta-carotene (Lim et al., 1992). In contrast, when peroxidation was initiated by water soluble initiator (AAPH) the order of antioxidant activity changed: astaxanthin = zeaxanthin > beta-carotene > canthaxanthin (Lim et al., 1992).

By employing a newly developed fluorometric assay Naguib (2000) examined three categories of carotenoids: the hydrocarbon carotenoids lycopene, alpha-carotene, and beta-carotene; the hydroxy carotenoid lutein; and the alpha-hydroxy-ketocarotenoid astaxanthin. The results showed that the relative peroxyl radical scavenging activities of Trolox (water-soluble vitamin E analogue), astaxanthin, alpha-tocopherol, lycopene, beta-carotene, lutein, and alpha-carotene in octane/butyronitrile (9:1, v/v) were determined to be 1.0, 1.0, 1.3, 0.5, 0.4, 0.3, and 0.2, respectively. In dioleoylphosphatidyl choline liposomal suspension in Tris-HCl buffer the relative reactivities of astaxanthin, beta-carotene, alpha-tocopherol, and lutein were found to be 1.00, 0.9, 0.6, and 0.6, respectively (Naguib, 2000).

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 homogeneous organic solution, beta-ring carotenoids showed a correlation between protective effect and rate of carotenoid destruction. The reactivity and protective ability of the 4, 4'-diketocarotenoids, astaxanthin and canthaxanthin was less than beta-carotene and zeaxanthin.

It is well recognised that the structure of a particular carotenoid has a significant effect on its location and orientation within phospholipid bilayers (Britton, 1995). In particular hydrocarbons, including beta-carotene and lycopene, are located entirely within the hydrophobic membrane core and xanthophylls such as lutein and zeaxanthin have membrane-spanning orientation and can trap radicals across most of the bilayer width (Rice-Evans et al., 1997). Therefore carotenoids incorporated into ordered membrane systems (egg yolk PC liposomes) displayed different protective efficacies. Zeaxanthin and beta-cryptoxanthin with polar end groups located near the hydrophobic-hydrophilic interface were more effective than beta-carotene or lycopene against oxidation initiated in the aqueous and lipid phases. Thus the hydrocarbons lycopene and beta-carotene which are located in the hydrophobic inner core of the bilayer, would not be
in a position to readily intercept free-radicals entering the membrane from the aqueous phase. Therefore it has been concluded that the chemical reactivity of a carotenoid is not the only factor that determines its ability to protect membranes against oxidation. The position and orientation of the carotenoid in the bilayer is also of importance (Woodall et al., 1997).

The ability of astaxanthin and canthaxanthin 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 precedes 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.

Both lutein and zeaxanthin were found to slightly modify the mechanical properties of the liposomes. Linear dichroism analysis of the mean orientation of the dipole transition moment of the xanthophylls incorporated to the lipid multibilayers revealed essentially different orientation of zeaxanthin and lutein in the membranes. In this respect, the differences in the protective efficacy of lutein and zeaxanthin in lipid membranes were attributed to a different organisation of zeaxanthin-lipid and lutein-lipid membranes (Sujak et al., 1999). It is interesting that, in HL 60 cells, lutein and canthaxanthin scavenged phenoxyl radical faster than \(\beta\)-carotene or lycopene (Tyurin et al., 1997). Since carotenoids are essential constituents of the antioxidant defences in cells and biological fluids, their depletion through the reaction with phenoxyl radicals formed from endogenous, nutritional and environmental phenolics, as well as phenolic drugs, may be an important factor in the development of oxidative stress (Tyurin et al., 1997).

In another study, the antioxidant activity of carotenoids in multilamellar liposomes assayed by inhibition of formation of TBARS was in the ranking: lycopene > \(\alpha\)-tocopherol > \(\alpha\)-carotene > \(\beta\)-cryptoxanthin > zeaxanthin = \(\beta\)-carotene > lutein (Stahl et al., 1998). In that experiment, mixtures of carotenoids were more effective than the single compounds and the synergistic effect was most pronounced when lycopene or lutein was present. It has been suggested that the superior protection of mixtures may be related to specific positioning of different carotenoids in membranes.

Thus, in vitro experiment with different model systems clearly showed an antioxidative protective effect of various carotenoids and the ranking of their antioxidant activity depends on the model system used. In general lutein and zeaxanthin, two major carotenoids of avian egg yolk and tissues, were shown to be quite effective in antioxidant defence.

**Antioxidant action of carotenoids in vivo**

In vivo experiments devoted to the antioxidant properties of carotenoids have been previously described (Palozza and Krinsky, 1992a; Moller et al., 2000). In brief, it is possible to summarise this activity as follows.

Inclusion of various carotenoids in different doses into the diet of chickens, mice,
rats, guinea pigs, fish, and human was used. In some cases, diets were deficient in antioxidants (Prohazka, 1966; Mayne and Parker, 1987; 1989; North and Tappel, 1997); in other cases diets were sufficient in other antioxidants (Surai and Speake, 1998; Woodall et al., 1996). In many cases, stress factors were applied including injection with CCl4 (Iyama et al., 1996), tumour development (Chew et al., 1999; Basu et al., 2000) or inoculation with tumor cells (Park et al., 1998), exposure to UV (Someya et al., 1994; Lee et al., 2000) or X-ray radiation (Kazanskaya et al., 1994; Forssberg et al., 1959; Ben-Amotz et al., 1996; Umegaki et al., 1994; El-Habit et al., 2000), dietary inclusion of oxidised oils (Levin et al., 1997; Nakano et al., 1995; 1999), a zinc deficient diet (Kraus et al., 1997) or a diet with iron overload (Whittaker et al., 1996), or challenge with *Aeromonas salmonicida* (Christiansen et al., 1995).

In humans, patients with cystic fibrosis (Lepage et al., 1996; Winklhofer-Roob et al., 1995; 1996), erythropoietic protoporphyria (Mathews-Roth, 1986; 1986a), elderly women (Maydani et al., 1994) or patients on carotenoid deficient diet (Dixon et al., 1994; Mobarhan et al., 1990) as well as heavy smokers (Allard et al., 1994) were investigated. In some experiments healthy human subjects were also used (Rao and Agarwal, 1999; 1998; Riso et al., 1999). In most of the cases the accumulation of TBARS were used as a test for lipid peroxidation. In other cases phospholipid hydroperoxide accumulation (Nakagawa et al., 1996), lysis of erythrocytes (Miki, 1991; Niki, 1991), pentane production (Kunert and Tappel, 1983; Allard et al., 1994), levels of antioxidant vitamins in tissues (Christiansen et al., 1995), plasma antioxidant capacity (Meydani et al., 1994), activities of antioxidant enzymes (El-Habit et al., 2000), resistance of LDL to oxidative stress (Winklhofer-Roob et al., 1995), oxidative damage to lipids, lipoproteins and DNA (Rao and Agarwal, 1999; Riso et al., 1999), the frequency of micronuclei of polychromatic erythrocytes and the mitotic index of bone marrow cells (El-Habit et al., 2000), photosensitivity (Mathews-Roth, 1986), survival rate after irradiation (Forssberg et al., 1959) or survival of lymphoma-bearing animals (Basu et al., 2000) were used as the end points in experiments.

In most cases β-carotene was tested. But other carotenoids showed positive effects as well including lutein, canthaxanthin, citranaxanthin and carotenoic acid (Surai and Speake, 1998), zeaxanthin and canthaxanthin (Woodall et al., 1996), ethyl-β-apo-8-carotenoate (Prohaszka, 1966), canthaxanthin (Mayne and Parker, 1987, 1989), astaxanthin (Chew et al., 1999; Miki, 1991; Niki, 1991; Nakano et al., 1995, 1999; Christiansen et al., 1995), lutein (Park et al., 1998) and lycopene (Forssberg et al., 1959; Rao and Agarwal, 1998, 1999, Riso et al., 1999).

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.

In addition, different model systems were employed to test antioxidant properties of carotenoids including cultured hepatocytes (Kim, 1995; Leal et al., 1998; Martin et
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al., 1996), normal and tumor thymocytes (Palozza et al., 1996), kidney fibroblasts (O'Connor and O'Brien, 1998), embryonic hippocampal cultures (Mitchell et al., 1999), embryo fibroblast (Zhang et al., 1991), ovary cells (Weitberg et al., 1985), primary cultures of chicken embryo fibroblasts (Lawlor and O'Brien, 1995; 1997), leukemia HL-60 cells (Hiramoto et al., 1999), monocyte-macrophages (Carpenter et al., 1997; Levy et al., 1996), cultured Ito cells (Kim et al., 1997), LDL in different systems (Dugas et al., 1999; 1998; Jialal et al., 1991; Oshima et al., 1996), Salmonella typhimurium (DeMejia et al., 1997), pigmented yeast Rhodotorula mucilaginosa (Moore et al., 1989) and liver microcosms (Nakagawa et al., 1997; Vile and Winterbourn (1988).

In most cases, β-carotene was tested in the model systems as an antioxidant. But other carotenoids were studied as well including lutein (Kim, 1995; O'Connor and O'Brien, 1998, Zhang et al., 1991; Martin et al., 1996; DeMejia et al., 1997), lycopene (Kim, 1995; Zhang et al., 1991; Leal et al., 1998), α-carotene (Kim, 1995; Zhang et al., 1991; Leal et al., 1998), canthaxanthin (Palozza et al., 1996; Zhang et al., 1991; Carpenter et al., 1997), astaxanthin (O'Connor and O'Brien, 1998; Lawlor and O'Brien, 1997; Nakagawa et al., 1997), zeaxanthin (Carpenter et al., 1997; Kim et al., 1997).

In the model systems different stress factors were used: CCl₄, tret-butyl hydroperoxide, UV light, ethanol, paraquat, T-2 toxin, aflatoxin B1, H₂O₂, adriamycin, peroxynitrous acid and CuSO₄. 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. In those conditions of the model systems the carotenoids confirmed their antioxidative protective effects preventing lipid peroxidation, decreasing cytotoxicity or decreasing DNA breaking.

There is some inconsistency in relation to the antioxidant activity of carotenoids in vivo. For example Alam and Alam (1983) were not able to demonstrate a beneficial effect of large doses of β-carotene on rats and increased peroxide levels in plasma and liver were shown. Similar data were reported by Lomnitski et al. (1991) when testes TBARS values were increased as a result of β-carotene dietary supplementation. Nevertheless these reports are minor and the effects could be explained as a result of poor carotenoid storage in the tissues (Palozza and Krinsky, 1992a). However available data currently show that the antioxidant activity of carotenoids may shift into prooxidant activity, depending on the redox potential of the carotenoid molecules as well as on the biologic environment in which they act. The prooxidant potency of these compounds is determined by several factors, including oxygen tension, carotenoid concentration, and interactions with other antioxidants (Palozza, 1998). However, the prooxidant effect is usually seen at 100% oxygen and not in physiological conditions on tissue level where oxygen tension is equivalent to 1–2% oxygen (Krinsky, 1998). Furthermore, carotenoids can express their prooxidant properties only in conditions where other antioxidants in particular vitamin E absent or very low. Thus there is convincing evidence that in physiological conditions carotenoids play an important role
as antioxidants.

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). Carotenoids are efficient quenchers of singlet oxygen and are also effective scavengers of free radicals. Therefore in biological systems carotenoids could be considered as an integral part of the antioxidant systems operating inside the membranes.

The important point is that lutein as a major carotenoid of the avian diet, and avian egg yolk and tissues has been shown to be a better antioxidant than β-carotene in quenching lipid peroxyl radicals and even more efficient in vitro than α-tocopherol (Chopra et al., 1993). Since all elements of antioxidant systems operate in concert providing a cooperative and in many cases synergistic effect the interactions between carotenoids and other antioxidants may have considerable high biological relevance.

**Carotenoid-vitamin E interactions**

In many experiments, carotenoid-vitamin E interactions have been shown. When rat liver microsomal lipids in hexane solution were exposed to the lipid-soluble radical initiator azobis-isobutyronitrile, both α-tocopherol and β-carotene acted as chain-breaking antioxidants, suppressing lipid peroxidation and producing a lag period before any peroxidation occurred (Palozza and Krinsky, 1992). In addition, β-carotene delayed the radical-initiated destruction of the endogenous α-tocopherol and γ-tocopherol in this system. It has also been shown that a combination of β-carotene and α-tocopherol results in an inhibition of lipid peroxidation significantly greater than the sum of the individual inhibitions, providing evidence that β-carotene can act synergistically with α-tocopherol as an effective radical-trapping antioxidant in membranes (Palozza and Krinsky, 1992). At high oxygen concentration (760 mm Hg pO2), β-carotene loses its antioxidant activity and shows a prooxidant effect, increasing lipid peroxidation products (Palozza et al., 1995). In such conditions α-tocopherol prevents the prooxidant effect of β-carotene in a dose-dependent manner confirming the existence of cooperative interactions between β-carotene and α-tocopherol (Palozza et al., 1995).

The presence of carotenoids (β-carotene or astaxanthin) in the microsomal membrane partially inhibited the loss of α-tocopherol, especially during the later phase of oxidant stress (Nakagawa et al., 1997). Furthermore, when lipid peroxidation is generated by membrane-bound cytochrome P450, the presence of carotenoids provides effective antioxidant protection (Nakagawa et al., 1997).

The action and activity of β-carotene, as a radical-scavenging antioxidant, in comparison to other antioxidants have also been studied (Tsuchihashi et al., 1995). β-Carotene suppressed the free-radical-mediated oxidations of methyl linoleate in benzene solution and soybean phosphatidylcholine liposomal membranes in aqueous
dispersions in a dose-dependent manner, but the antioxidant activity of \( \beta \)-carotene was much smaller than that of \( \alpha \)-tocopherol. On the other hand, when \( \alpha \)-tocopherol and \( \beta \)-carotene were incorporated simultaneously into the same dimyristoyl phosphatidylcholine liposomal membranes and the radicals were generated within the lipophilic compartment of the membranes, \( \beta \)-carotene was consumed faster than \( \alpha \)-tocopherol, implying that \( \beta \)-carotene is relatively more favourable than \( \alpha \)-tocopherol for scavenging lipophilic radicals within the membranes. Therefore \( \beta \)-carotene has lower reactivity toward radicals than does \( \alpha \)-tocopherol and acts as a weak antioxidant in solution. It is more lipophilic than \( \alpha \)-tocopherol and is assumed to be present at the interior of membranes or lipoproteins, which enables it to scavenge radicals within the lipophilic compartment more efficiently than does \( \alpha \)-tocopherol (Niki et al., 1995).

Although the results showed that both \( \beta \)-carotene and \( \alpha \)-tocopherol suppress the peroxidation of microsomal membranes, their antioxidant efficacy is influenced by several factors, including the type of radical initiator involved and the site and rate of radical production (Palozza et al., 1992). In addition the fatty acid profile of the model system could be a crucial factor in the efficiency of tested antioxidants including carotenoids. For example, different antioxidant roles for \( \beta \)-carotene and \( \alpha \)-tocopherol have been shown in an induced lipid peroxidation suggesting that \( \beta \)-carotene can be a very effective antioxidant in highly unsaturated membranes, such as those enriched with n-3 polyunsaturated fatty acids (Palozza et al., 1995). Oxygen concentration is another factor determining rate of lipid peroxidation and efficiency of antioxidant defence. Iron-dependent peroxidation of rat liver microsomes, enhanced by adriamycin, was measured in the presence of increasing concentrations of \( \alpha \{\text{-}}tocopherol, \( \beta \)-carotene and retinol at low and high oxygen concentration (Vile and Winterbourn, 1988). \( \beta \)-Carotene and \( \alpha \)-tocopherol inhibited lipid peroxidation by more than 60% when present at concentrations greater than 50 nmol/mg microsomal protein at both high and low \( \text{pO}_2 \). However, \( \beta \)-carotene was more effective than \( \alpha \)-tocopherol at a low \( \text{pO}_2 \), whereas \( \alpha \)-tocopherol was more effective under more aerobic conditions.

The basic understanding of carotenoid-vitamin E interaction came from the recent experiments of Bohm et al. (1997) where electron transfer from carotenoids (CAR) to \( \alpha \)-tocopheroxyl radical (TO*) has been observed:

\[
\text{TO}^* + \text{CAR} = \text{TOH} + \text{CAR}^*^+ 
\]

High values of the rate constants for the above reaction obtained by Bohm et al. (1997) indicate the direction of electron transfer, from carotene to TO* indicating the repair of the \( \alpha \)-tocopheroxyl radical by carotenoids. Recently, it has also been shown that one-electron-oxidised lycopene can be reduced by \( \alpha \)-tocopherol whereas one-electron-oxidized \( \delta \)-tocopherol can be reduced by lycopene (Mortensen and Skibsted, 1997) and \( \beta \)- and \( \gamma \)-tocopherols are in equilibrium with lycopene, i.e. they can reduce lycopene radical or can be reduced by lycopene depending on the conditions. Li et al. (1995) showed that the combination of \( \alpha \)-tocopherol and \( \beta \)-carotene exhibited a significant synergistic effect during oxidation of linoleic acid in tert-butyl alcohol and the decay of \( \alpha \)-tocopherol was decreased by about the half in the presence of \( \beta \)-carotene. These results could be a reflection of \( \alpha \)-tocopherol regeneration by \( \beta \)-carotene (Yanishlieva et
The fundamental finding of the work showing that all tested carotenoids except astaxanthin are able to recycle vitamin E has had a great impact on the understanding of the role of carotenoids as an integral part of the antioxidant system of the cell. Indeed enhanced protection of human cells against ultraviolet light by antioxidant combinations involving dietary carotenoids was shown (Bohm et al., 1998) and further both in vivo and in vitro cell protection from the peroxynitrite anion and nitrogen dioxide is demonstrated for \( \beta \)-carotene in the presence of vitamin E and vitamin C (Bohm et al., 1998a). In the experiment, a synergistic protection was observed compared to the individual anti-oxidants and this was explained in terms of a possible electron transfer reaction in which the \( \beta \)-carotene radical is repaired by vitamin C. The results of those recent experiments become a necessary connecting link between carotenoids and other (vitamin E and C) antioxidants explaining some old results and providing an insight into new directions of research in this field.

Therefore, in addition to its singlet oxygen quenching properties, \( \beta \)-carotene and probably other carotenoids have good radical-trapping properties at low partial pressures of oxygen, which prevail in healthy tissues (Frankel, 1989). In biological systems, \( \alpha \)-tocopherol and carotenoids exhibit synergism by reinforcing their mutual activities. In general synergistic and co-operative interactions of antioxidants rely on the sequential degradation of peroxides and free radicals as well as on mutual protection by enzymes. This antioxidant network can induce metabolic deviations and plays an important role in the regulation of protein expression and/or activity at the transcriptional or post-translational levels (Chaudiere and Ferrari-Iliou, 1999).

**Modulation of antioxidant systems by carotenoids**

As mentioned above, carotenoids modulate antioxidant systems of the body by interacting with other antioxidants including vitamin E. Evidence has accumulated that carotenoids could modulate other parts of the antioxidant systems as well. For example the administration of lycopene to female rats increased the activities of antioxidant enzymes including SOD, GSH-Px and Glutathione reductase (Breinholt et al., 2000). Dietary lycopene supplementation also prevented changes in antioxidant enzyme activities and protected cellular glutathione from oxidation caused by T-2 toxin in the chicken diet (Leal et al., 1999). Consumption of spinach by humans resulted in greater erythrocyte glutathione reductase activity and lower erythrocyte catalase activity and serum \( \alpha \)-tocopherol concentration compared with the control group which can be attributed to an increased carotenoid (lutein and zeaxanthin) intake or to other compounds of spinach including flavonoids (Castenmiller et al., 1999). Chicken embryo fibroblasts incubated with 0.25 mM paraquat (PQ) for 18 h exhibited increased SOD and CAT activities and decreased GSH-Px activity compared with the control. Incorporation of \( \beta \)-carotene (0.1 microM) into this system returned SOD activity to that seen in the control cells. \( \beta \)-Carotene (0.1 microM) reduced the CAT activity from that seen in PQ-treated cells and returned the GSH-Px activity to its control value thus protecting the cells against PQ-induced oxidative stress (Lawlor and O’Brien, 1995).
The 15-day canthaxanthin dietary treatment of mice resulted in a 35% decrease of GSH-Px activity in the liver and increased catalase and manganese superoxide dismutase (MnSOD) activities. Moreover, carotenoid feeding caused a significant overexpression of the MnSOD gene; mRNA levels of the enzyme were greater in treated mice than in controls (Palozza et al., 2000).

β-carotene prolongs survival, decreases lipid peroxidation and enhances glutathione status in transplantable murine lymphoma in mice (Basu et al., 2000). In contrast the dietary β-carotene did not affect the activity of GSH-Px in liver, kidney and brain of mice (Andersen and Andersen, 1993). In N-nitrosodimethylamine treated rats, β-carotene dietary supplementation prevented the decrease in GSH-Px and SOD activity in the liver (Chen et al., 1998). Similarly dietary β-carotene (10 or 30 mg/kg/day) reduced the cardiotoxicity induced by doxorubicin, diminished the myocardial malondialdehyde (MDA) production and protected the activities of SOD and GSH-Px (Lu et al., 1996). β-carotene also suppressed induction of SOD in rats treated with corn oil (Blakely et al., 1988). In rats exposed to a total dose of 0.75 Gy of gamma radiation the contents of total glutathione and GSH/GSSG ratio were considerably decreased, whereas the NAD/NADH ratio was increased (Slyshenkov et al., 1999) and all these effects were alleviated in animals supplied with β-carotene. In the same experiment dietary supplementation with β-carotene prevented a decrease in glutathione reductase, GSH-Px and catalase in liver.

Possible interactions between carotenoids and ascorbic acid have not been studied in detail and there are only a few publications in this area. For example in middle age subjects supplemented with β-carotene or ascorbic acid, no statistically significant interaction between β-carotene and vitamin C supplementations was observed either for serum β-carotene or for serum ascorbic acid (Sasaki et al., 2000). In contrast β-carotene supplementation of the human diet increased plasma ascorbate level (Davison et al., 1993). Based on the localisation of carotenes including β-carotene deep within the lipid bilayer of membrane or core of lipoproteins, it has been suggested that cooperative interactions between ascorbic acid and β-carotene are unlikely (Niki et al., 1995). On the other hand, taking into account the different localisation of xanthophylls (lutein and zeaxanthin) in the membrane the interactions with water soluble antioxidants (ascorbic acid and reduced glutathione) are more probable and this question warrants further investigations.

Carotenoids and chick embryo development

In 1919, Palmer and Kempster showed that all carotenoids of egg yolk were derived from ingested feed. During embryogenesis, carotenoids are transferred from the yolk to the developing tissues and it was shown that day-old chicks contained 76% of the mass of carotenoids present in eggs laid by the hen (Plack, 1963).

The embryo of the chicken develops within a closed system, the egg, which contains all the nutrients required for the 21-day developmental process (Speake et al., 1998). The lipid-rich yolk contains the lipid-soluble vitamins E and A, and also a range of carotenoids (Griffin et al., 1984). Current evidence suggests that during development,
the lipids and associated components are transferred from the yolk to the surrounding yolk sac membrane (YSM) by phagocytosis (Noble and Cochi, 1990). Within the YSM, lipids are assembled together with vitamin E, carotenoids, and apoproteins to form lipoprotein particles, which are released into the embryonic circulation (Maldjian et al., 1995; Surai et al., 1996). Following de-lipidation by the action of lipoprotein lipase in the capillaries of the embryo’s adipose tissue and muscle (Speake et al., 1993), the lipoprotein remnants which consist mainly of cholesteryl ester but which also contain vitamin E and carotenoids are deposited in the embryonic liver (Shand et al., 1994; Surai et al., 1996).

In a newly hatched chick, the highest concentration of the two major yolk-derived carotenoids lutein + zeaxanthin was found in the liver (30.8 µg/g) and yolk sac membrane also contained a high level of carotenoids (18.6 µg/g). Other tissues (kidney, heart, lung and muscles) contained much lower carotenoid concentrations (2.0–3.3 µg/g) and in the brain carotenoids were not detected (Surai et al., 1999a). In another experiment, using eggs which initially contained 32 µg carotenoids/g the highest carotenoid concentration (120.6 µg/g) in tissues of the day-old chicks was found in the liver and a similar concentration was in the yolk sac membrane (117.7 µg/g). However, adipose tissue contained a much lower concentration of carotenoids (28.5 µg/g) and in the heart, kidney, lung and muscles carotenoid concentration was yet lower (6.6–6.7 µg/g) (Surai and Sparks, 2000). These data also indicate a specific accumulation of carotenoids in the YSM and liver since the concentration of the carotenoids (mainly lutein and zeaxanthin) was 3–4 times higher compared to the initial yolk.

Thus, high levels of vitamin E and carotenoids accumulate in the liver, particularly during the final few days of development (Surai et al., 1996; Table 1). The liver therefore functions as a site of accumulation of carotenoids during embryonic life and then as a centre of their redistribution after hatching. It was shown that for the first 9 days of postnatal development the carotenoid concentration in the liver of chicken, turkey, duck and goose decreased approximately 3 times (Surai et al., 1998; Table 2) indicating the importance of the posthatch carotenoid reserves which were accumulated in the liver during embryonic development. When laying hens were fed on a diet

| Table 1. Carotenoid accumulation in tissues of the developing chick embryo, µg/g (Surai and Speake, 1998) |
|---|---|---|---|---|---|
| Tissue | 15  | 17  | 19  | 21  | 22  |
| Liver  | 7.13| 13.68| 18.12| 27.19| 35.12|
| YSM    | 15.33| 30.11| 36.67| 40.11| 43.68|
| Heart  | 0.68| 0.93| 1.15| 1.44| 1.87|
| Kidney | 0.48| 0.69| 0.95| 1.71| 2.23|
| Lung   | 0.55| 0.74| 0.96| 1.88| 2.35|
| Muscle | 0.59| 0.82| 1.28| 1.32| 1.40|
| Plasma | 0.14| 0.69| 0.95| 1.92| 3.12|
supplemented with lutein, the concentration of this carotenoid in plasma of newly hatched chicks was elevated and this difference compared to the control chickens was maintained for up to 5 weeks posthatch (Haq et al., 1995). Therefore the maternal diet can determine the carotenoid concentration in the plasma of the chicks for some time after hatching.

In order to address the question of carotenoid transfer from the hen’s feed into egg yolk and subsequently to the developing embryonic tissues, an experiment was conducted (Surai and Speake, 1998) in which laying hens at 30 weeks of age were fed on a high carotenoid diet with the final concentration of the various carotenoids being (mg/kg feed): lutein, 24.5; citranaxanthin, 45.0; canthaxanthin, 3.0 and β-apo-8′-carotenolic acid, 24. This contrasts with the control diet which contained (mg/kg feed): lutein, 8.8 and citranaxanthin, 6.5. It was observed that the concentration of total carotenoids in the yolk responded rapidly to the changes in the level of carotenoid provision to the hen. Thus, 23 days after the entry of the hens into the high-carotenoid dietary regime, a new steady state was established in which the yolk carotenoid levels were about 3 times higher than in the control.

Differences in the concentrations of total carotenoids in the yolks were at least partly reflected in the carotenoid levels measured in the tissues of the day-old chicks, which developed within the eggs obtained from the two dietary groups. With regard to the chicks from the high-carotenoid group, carotenoid levels in the YSM, liver, and plasma were significantly higher than in the control group to an extent (3-fold), which reflects the difference in the carotenoid levels in the yolk. However, carotenoid concentrations achieved by the heart, lung, and thigh muscle of the chicks were not significantly greater in the high-carotenoid group than in the controls. This may be partly due to the high lipid content of tissues such as the liver and YSM (Speake et al., 1998) which may permit the accumulation of large amounts of lipid-soluble carotenoids. By contrast, tissues with a more modest lipid content at the time of hatching may be limited in their capacity to accumulate carotenoids, even when the supply of carotenoids from the yolk is increased. The higher responsiveness of the YSM, plasma and liver of the chick to the elevated levels of carotenoids in the initial yolk may also reflect the transport mechanisms functioning in the embryo, particularly with regard to the hepatic uptake of carotenoid-rich lipoprotein remnants (Surai et al., 1996).
The observation that the chick’s liver, but not the initial yolk, contained a greater concentration of vitamin A in the high-carotenoid group than in the controls (Surai and Speake, 1998) suggests that the embryo may have some ability to convert appropriate carotenoids to vitamin A. The level of vitamin E in the liver of the day-old chicks was also significantly elevated in the high-carotenoid group. This may reflect the antioxidant properties of carotenoids, preventing depletion of vitamin E levels during periods of high oxidative stress such as the hatching process (Surai, 1999). Carotenoid supplementation in humans, rats (Furr and Clark, 1977) and chickens (Woodall et al., 1996) have previously been reported to decrease, increase, or have no effect on plasma vitamin E levels.

**Carotenoid discrimination in avian tissues**

For the both the control and the high carotenoid dietary groups, the relative proportions of the various carotenoids present in the initial yolk were very similar to those displayed by the YSM of the day old chicks (Surai and Speake, 1998). Thus, there is no evidence for any selective uptake of particular carotenoids from the yolk by the YSM during embryonic development. This is consistent with the view that lipid components are transferred from the yolk to the YSM by nonspecific phagocytosis (Noble and Cocchi, 1990). However, the proportion of lutein present in the liver of the day old chick was far lower than in the yolk and YSM, whereas the proportions of the other carotenoids in the chick liver were higher than in the yolk and YSM.

By contrast, the proportions of lutein in the non-hepatic tissues of the day-old chick tended to be higher than in the yolk, with commensurate decreases in the proportions of citranaxanthin and β-apo-8′-carotenoid acid in these tissues. Comparison of the lutein/citranaxanthin ratio between the different tissues provided a further illustration of the selective utilization of the various carotenoids by the embryo. In the case of both the control and the high-carotenoid groups, the ratio was the same for the yolk and the YSM, was significantly lower in the liver of the chick than in the initial yolk, and was significantly higher in the chick’s heart, kidney, lung, thigh muscle, and plasma than in the yolk (Table 3).

The mechanism of this apparent discrimination between the different carotenoids in the chick embryo is not clear. One possibility is that lutein may transfer more readily from the YSM-derived lipoproteins to high-density lipoprotein in the embryo’s plasma, whereas the other carotenoids may exhibit a greater tendency to be delivered to the liver as components of the lipoprotein remnants. Studies on the absorption and transport of carotenoids by the mammalian intestine have suggested that the more polar carotenoids, such as lutein, tend to partition into the amphipathic surface layer of the chylomicrons and are therefore more likely to be transferred to high density lipoprotein prior to the uptake of the chylomicron remnants by the liver (van Vliet, 1996; Parker, 1997; Bierer et al., 1995). An alternative explanation is the possibility that the liver of the embryo may selectively incorporate lutein into VLDL for export to the non-hepatic tissues. This could possibly be achieved by the action of a lutein-binding protein in the hepatocytes, which could specifically transfer this carotenoid to the site of VLDL
Table 3. Carotenoid profile of the initial yolk and the tissues of the day-old chicks derived from hens on the high-carotenoid diet (Surai and Speake, 1998)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Lutein</th>
<th>Citranaxanthin</th>
<th>Carotenoic acid</th>
<th>Canthaxanthin</th>
<th>Lutein/Citranaxanthin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent’s feed</td>
<td>23.1</td>
<td>42.4</td>
<td>22.6</td>
<td>2.8</td>
<td>0.54</td>
</tr>
<tr>
<td>Initial yolk</td>
<td>29.5</td>
<td>34.1</td>
<td>29.5</td>
<td>2.2</td>
<td>0.87</td>
</tr>
<tr>
<td>Day old chick</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YSM</td>
<td>26.2</td>
<td>28.8</td>
<td>25.8</td>
<td>2.2</td>
<td>0.92</td>
</tr>
<tr>
<td>Liver</td>
<td>11.5</td>
<td>30.4</td>
<td>32.6</td>
<td>3.5</td>
<td>0.39</td>
</tr>
<tr>
<td>Heart</td>
<td>43.0</td>
<td>24.9</td>
<td>15.9</td>
<td>4.2</td>
<td>1.77</td>
</tr>
<tr>
<td>Kidney</td>
<td>35.7</td>
<td>23.0</td>
<td>13.3</td>
<td>—</td>
<td>1.55</td>
</tr>
<tr>
<td>Lung</td>
<td>53.7</td>
<td>16.9</td>
<td>10.5</td>
<td>—</td>
<td>3.25</td>
</tr>
<tr>
<td>Thigh muscle</td>
<td>33.3</td>
<td>28.8</td>
<td>18.6</td>
<td>—</td>
<td>1.18</td>
</tr>
<tr>
<td>Plasma</td>
<td>54.3</td>
<td>13.2</td>
<td>—</td>
<td>—</td>
<td>4.18</td>
</tr>
</tbody>
</table>

Table 4. Carotenoid distribution in the tissues of laying hens, µg/g (Surai et al., 1999)

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Low-carotenoid diet</th>
<th>High-carotenoid diet</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>6.17±0.54</td>
<td>18.42±1.25</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.66±0.06</td>
<td>0.98±0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.54±0.04</td>
<td>1.24±0.13</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Abdominal fat</td>
<td>0.76±0.07</td>
<td>1.01±0.12</td>
<td>0.3</td>
</tr>
<tr>
<td>Heart</td>
<td>0.46±0.03</td>
<td>1.46±0.18</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lung</td>
<td>0.38±0.05</td>
<td>1.05±0.11</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.24±0.02</td>
<td>0.46±0.06</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Blood plasma</td>
<td>0.73±0.09</td>
<td>2.96±0.31</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

In order to expand the data on possible carotenoid discrimination by poultry we studied their distribution in the tissues of laying hens fed on low and high carotenoid diets (Surai et al., 1999; Table 4). In the tissues of the laying hens fed on the low-carotenoid diet (which contained mainly lutein), lutein comprised 82.6; 86.4; 88.9; 81.6; 82.6; 81.6; 83.3 and 89.0% of total carotenoids in the liver, kidney, spleen, fat, heart, lung, muscle and blood plasma respectively. On the other hand, enrichment of the diet with a greater range of carotenoids present in the high-carotenoid diet was associated with their accumulation in the tissues. In the hens fed the high-carotenoid diet (which contained citranaxanthin, carotenoic acid and canthaxanthin in addition to lutein) lutein comprised 28.9%, citranaxanthin 30.3%, carotenoic acid 22.0% and canthaxanthin 3.5% of the total carotenoids of the liver.
In the kidney, the distribution of the carotenoids was as follows: lutein 65.3%; citranaxanthin 18.4%; carotenoic acid 10.2%; in the spleen, lutein 49.2%; citranaxanthin 21.8%; carotenoic acid 18.6%; in the heart, lutein 41.8%; citranaxanthin 24.0%; canthaxanthin 4.1%; carotenoic acid 15.8%; in the plasma, lutein 38.9%; citranaxanthin 22.6%; canthaxanthin and carotenoic acid both 27.7%.

The results indicate a tissue-specific response and distribution of different carotenoids. For example, in spite of the higher supplementation of the diet by citranaxanthin in the high carotenoid diet, in most of tissues lutein remained the major carotenoid. The accumulation of citranaxanthin, canthaxanthin and carotenoic acid in the liver (as % of total) was much higher compared to the other tissues. Possibly the liver accumulated all four carotenoids, but released lutein preferentially into the blood, associated with VLDL.

The next illustration of carotenoid discrimination in avian species came from our work with free living lesser black-backed gulls (Surai et al., 2000). The gull tissues are characterised by comparatively high carotenoid levels with the liver having the highest carotenoid concentration (Table 5). The most striking feature of the liver is the very high proportion of β-carotene which comprises 51.2% of total carotenoids. The second important feature of carotenoid accumulation in gull tissues is the evidence of discrimination between individual carotenoids. For example, the liver was characterised by the highest proportion of β-carotene but the lowest proportions of lutein and zeaxanthin compared with other tissues studied. This picture is very similar to that in the tissues of adult laying hens fed on the diet supplemented with carotenoid mixture and in the tissues of newly hatched chicks obtained from the eggs of those hens. Again this could possibly suggest the existence of a lutein/zeaxanthin-binding protein in avian liver. In this case, a mixture of carotenoids is absorbed in the intestine and delivered with portomicrons to the liver. In the liver, carotenoids are incorporated in newly synthesised VLDL which are responsible for their delivery to peripheral tissues. Because of the action of the postulated lutein or/and zeaxanthin-binding protein, these

Table 5. Carotenoid concentration and composition in tissues of adult gulls (Surai et al., 2000)

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Total carotenoids, µg/g</th>
<th>Lutein, %</th>
<th>Zeaxanthin, %</th>
<th>Canthaxanthin, %</th>
<th>β-Cryptoxanthin, %</th>
<th>Echinone, %</th>
<th>β-carotene, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>45.01</td>
<td>12.35</td>
<td>4.24</td>
<td>3.40</td>
<td>2.31</td>
<td>8.11</td>
<td>51.17</td>
</tr>
<tr>
<td>Kidney</td>
<td>8.17</td>
<td>40.15</td>
<td>12.61</td>
<td>7.10</td>
<td>3.30</td>
<td>8.57</td>
<td>11.63</td>
</tr>
<tr>
<td>Heart</td>
<td>2.75</td>
<td>37.45</td>
<td>7.64</td>
<td>8.36</td>
<td>3.27</td>
<td>4.36</td>
<td>2.11</td>
</tr>
<tr>
<td>Lung</td>
<td>2.10</td>
<td>47.14</td>
<td>16.19</td>
<td>7.62</td>
<td>3.33</td>
<td>5.24</td>
<td>3.19</td>
</tr>
<tr>
<td>Breast muscle</td>
<td>2.05</td>
<td>51.22</td>
<td>11.71</td>
<td>18.54</td>
<td>1.41</td>
<td>2.93</td>
<td>3.07</td>
</tr>
<tr>
<td>Pancreas</td>
<td>6.50</td>
<td>58.46</td>
<td>17.54</td>
<td>1.69</td>
<td>0</td>
<td>3.08</td>
<td>4.15</td>
</tr>
<tr>
<td>Abdominal fat</td>
<td>2.54</td>
<td>28.74</td>
<td>9.45</td>
<td>23.23</td>
<td>4.33</td>
<td>4.33</td>
<td>3.43</td>
</tr>
</tbody>
</table>
carotenoids could be preferentially incorporated into VLDL, in a similar fashion to α-tocopherol (Traber and Sies, 1996), and delivered to peripheral tissues which become enriched with these particular carotenoids. Other carotenoids, mainly β-carotene, are retained in the liver and this organ therefore becomes enriched with this carotenoid compared to peripheral tissues. Recently, a cellular carotenoid-binding protein has been purified to homogeneity from β-carotene-fed ferret liver and except for α-carotene and cryptoxanthin none of the model carotenoids competed with β-carotene for binding to the protein (Lakshman and Rao, 1999). Furthermore, specific binding proteins for oxycarotenoids have been identified and characterised in crustacea (Olson, 1993).

The mechanisms responsible for β-carotene absorption and accumulation in gulls are not known at present. Judging by the vitamin A level in the gull liver, which is similar to that in laying hens (Surai et al., 1998a), we can exclude the possibility of a low efficiency of β-carotene conversion into vitamin A. Therefore, gull eggs (Royle et al., 1999) and tissues (Surai et al., 2000) differ from those of poultry in being able to accumulate β-carotene. This question warrants further investigation.

It is interesting to underline that different carotenoids have different affinity to lipoproteins. In human plasma lutein + zeaxanthin was found mainly in HDL (53%) and LDL (31%) and much less (16%) in VLDL. A similar distribution was characteristic for β-cryptoxanthin and 39, 42 and 19% of this carotenoid were respectively found in HDL, LDL and VLDL (Clevidence and Bieri, 1993). In contrast, carotenines were found mainly in LDL: lycopene 73%, β-carotene 67% and alpha-carotene 57%, whereas the content of these carotenoids in HDL was 17, 22 and 26% respectively (Clevidence and Bieri, 1993). It has also been shown that β-carotene and lycopene were located in different LDL subfractions (Lin et al., 2000). Lycopene, β-carotene and β-cryptoxanthin were mainly located in the larger, less-dense LDL particles whereas lutein and zeaxanthin were found preferentially in the smaller, more dense LDL particles (Lowe et al., 1999). There is no data available on carotenoid distribution in the plasma of avian species. However, preliminary data indicate that LDL (d = 1.063) was associated with the carotenoids of gut origin, whereas the HDL fraction (d = 1.21) was associated with various metabolically modified carotenoids of hepatic origin (Brush, 1981). There is also some indications that in the roseate spoonbill there is a specificity in the distribution of different carotenoids between LDL and HDL (Brush, 1981). This information could be also crucial for understanding carotenoid discrimination described above. Our data (Table 6) indicate that in newly hatched chicks lutein and zeaxanthin are exclusively associated with HDL and only a small proportion of these carotenoids were found in the other lipoprotein fractions.

It is important to mention that there is also a tissue specificity in carotenoid distribution in human tissues: storage in fat tissue was lowest for β-carotene and highest for zeaxanthin and the opposite was found in the liver (Kaplan et al., 1990). Whether these differences are due to different carotenoid distribution between lipoproteins (van Vliet, 1996) remains to be established.
Table 6. The distribution of major carotenoids in plasma lipoproteins of newly hatched chick, % (Surai and Speake, unpublished)

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>VLDL</th>
<th>IDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lutein</td>
<td>2.72</td>
<td>1.16</td>
<td>5.95</td>
<td>90.82</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>2.61</td>
<td>0.98</td>
<td>5.62</td>
<td>90.79</td>
</tr>
</tbody>
</table>

Antioxidant properties of carotenoids and avian embryo

The embryo requires an effective array of antioxidant component in order to protect the developing tissues from peroxidative damage (Allen, 1991). Oxidative stress arising from the production of free radicals is likely to be most prominent in individuals with high metabolic activity, but also in rapidly growing embryos because of their high levels of oxidative metabolism (Vleck and Bucher, 1998). It may be relevant that the antioxidant activity of carotenoids is mainly expressed at the low oxygen tension (Burton, 1989) that prevail in embryonic tissues (Ar and Mover, 1994).

The elevated carotenoid levels in the yolk and chick tissues from the high-carotenoid dietary group were associated with significantly reduced susceptibility to in vitro peroxidation (Surai and Speake, 1998; Figure 1). These results suggest that carotenoids are able to function effectively as antioxidants during incubation of these extracts, even in the presence of atmospheric oxygen concentrations. Because all the extracts were rich in vitamin E, it is feasible that these antioxidant effects may be brought about by synergistic interactions between carotenoids and vitamin E, as observed in other systems (Edge et al., 1997).

Carotenoid protection against lipid peroxidation could have a general biological relevance for developing organisms. For example in sea-urchin embryos only one carotenoid, all-trans β-echinenone was found and during development its content decreased by 50% and an antioxidant role of this carotenoid in the embryo was therefore proposed (Vershinin and Lukyanova, 1993). It has also been suggested that carotenoids which were transferred from the egg to the newly hatched chicken could have a profound effect on their future viability and survival with further implications for the functional ecology of birds (Blount et al., 2000).

Based on the review of recent information it has been suggested that there are different strategies for male and female birds in relation to carotenoid use (Negro et al., 1998; Moller et al., 2000). The female heavily invests in its progeny by transferring carotenoids into the egg, since the hatching process is considered as a period of oxidative stress (Surai, 1999) and antioxidant protection is therefore vital at this stage of development. It is interesting that, in laying hens, 50% of the total body zeaxanthin and 80% of the canthaxanthin are located in the ovary (Nys, 2000). On the other hand, in males of many avian species, carotenoids are often used for visual display (Negro et al., 1998; Moller et al., 2000) and male coloration is regarded as an honest signal of health or of “good” genes (Saino et al., 1999). In this respect, an interesting
finding is that carotenoid concentrations in the embryonic tissues of four avian species reach the maximal level at hatching (Surai et al., 1998) providing maximal antioxidant protection to tissues with high levels of polyunsaturated fatty acids (Speake et al., 1998) and which are vulnerable to free radical attack (Surai, 2000). Since carotenoids are not accumulated in the body to any great extent (Surai et al., 2000), it is important for the female to transfer them effectively to the egg yolk. For example, in the lesser black-backed gull the second and third eggs of a clutch usually contain less carotenoids than the first egg laid (Royle et al., 1999).

**Conclusions**: **Future developments**

Carotenoids together with other antioxidants (vitamin E, ascorbic acid, reduced glutathione or antioxidant enzymes) build an effective antioxidant system responsible for maintaining protection against damaging effects of free radicals and products of their metabolism (Surai, 1999). Therefore the antioxidant effect of carotenoids in embryonic tissues could be expressed directly or indirectly via antioxidant interactions but in both cases antioxidant protection could be the major advantage for the chick liveability and survival in postnatal development.

Thus in the lipid environment of biological membranes, a combination of carotenoids and other antioxidants, especially tocopherols, may provide better antioxidant protection than tocopherols alone. Furthermore, even low tocopherol concentrations are able to prevent carotenoids from destruction and inhibit their possible prooxidant action. In accordance with Britton (1995), in order to act as an antioxidant *in vivo*, a carotenoid would need to be incorporated into tissues in the correct location and at a suitable concentration relative to the oxidizable substrate. We hypothesise that antioxidant properties of carotenoids are not the major physiological players themselves but rather are an important part of the antioxidant system of the cell where antioxidant interactions including their recycling provide an effective and reliable system of defence from free radicals and toxic products of their metabolism.

It has been suggested that vitamin E acts as a catalyst, being effectively recycled from its free radical (chromanoxyl) form (Packer, 1996). The recycling of vitamin E takes place through the interaction between water- and fat-soluble antioxidants by nonenzymatic and/or enzymatic mechanisms, that regenerate vitamin E from its...
tocotrienoxyl or tocopheroxyl radical back to the active reduced forms, tocotrienol and tocopherol, respectively (Packer, 1996). Thus, the major finding in recent years is the possibility of direct or indirect vitamin E recycling from its oxidised radical form by means of ascorbate (Chan et al., 1991; Chan, 1993), glutathione (Niki et al., 1982; Chan, 1993), cysteine (Motoyama et al., 1989), ubiquinols (Freisleben and Packer, 1993; Chan, 1993), lipoic acid (Packer, 1998) and carotenoids (Palozza and Krinsky, 1992; Bohm et al., 1997). Enzymatic regeneration of α-tocopherol has been also described (Maguire et al., 1989). The rate of reduction of phenoxyl radical in the membrane decreased in the order of ascorbic acid > cysteine > glutathione (Niki, 1996). The rate of regeneration, or recycling, of the vitamin E radicals that form during their antioxidant action may affect both its efficiency in antioxidant action and its lifetime in biological systems and high recycling activity is associated with increased inhibition of lipid peroxidation (Packer, 1995). Whether all the mentioned regeneration reactions take place in vivo await investigation. Due to incomplete regeneration (the efficiency of recycling is usually less than 100%) in biological systems, the antioxidants have to be replenished from the diet (vitamin E and carotenoids) or synthesised in the tissues (ascorbic acid and glutathione).

Under conditions where these auxiliary systems act synergistically to keep the steady state concentration of vitamin E radicals low, the loss or consumption of vitamin E is prevented (Packer, 1992). For example, this was the case in chick embryo brain where a very low vitamin E concentration in combination with a high ascorbic acid content is able to prevent lipid peroxidation in physiological conditions (Surai et al., 1996). Vitamin E recycling is ultimately dependant on the reducing power of NADPH generated by the pentose phosphate cycle of carbohydrate metabolism (Surai, 1999). In this case, as a result of the antioxidant action of vitamin E, the tocopheroxyl radical is formed. This radical can be reduced back to active form of α-tocopherol by coupling with ascorbic acid oxidation. Ascorbic acid can be regenerated back from the oxidised form by recycling with glutathione which can receive reducing potential from NADPH in the glutathione reductase reaction. Therefore, this scheme connects antioxidant system regulation with general metabolism in the cell. If carotenoids are proven to be an integral part of this unique self-regulated biological system then their biological role will be more understandable.

This question needs to be addressed in specially designed experiments where antioxidant composition and lipid environment would be a reflection of the physiological situation. In this respect, tissues of the avian embryo, especially liver and yolk sac membrane which are very rich in unsaturated lipids, carotenoids, tocopherols, tocotrienols and contain quite high concentrations of ascorbic acid and reduced glutathione, provide a unique opportunity for such studies.

Carotenoid research is progressing rapidly but there are many biological properties of carotenoids for which a causal relationship has not been adequately demonstrated (Krinsky, 1994) and therefore future research efforts are required to answer those questions.
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References


Burton GW and Ingold KU. beta-Carotene: an unusual type of lipid antioxidant. Science


Kim HP, Kim SY, Lee EJ and Kim YC. Zeaxanthin dipalmitate from Lycium chinense has hepatoprotective activity. Research Communications in Molecular Pathology and Pharma-


Lu HZ, Geng BQ, Zhu YL and Yong DG. Effects of beta-carotene on doxorubicin-induced...


Palozza P, Luberto C and Bartoli GM. The effect of fatty acid unsaturation on the antioxidant


Soffers AE, Van Haandel MJ, Boersma MG, Tyrkowska B, Laane C and Rietjens IM. Antioxidant activities of carotenoids: quantitative relationships between theoretical calcula-


Surai P, Ionov I, Kuchmistova E, Noble RC and Speake BK. The relationship between the levels of α-tocopherol and carotenoids in the maternal feed, yolk and neonatal tissues: Comparison between the chicken, turkey, duck and goose. Journal of Science in Food and Agriculture, 76: 593–598, 1998.

Surai PF, Ionov IA, Kuklenko TV, Costjuk IA, MacPherson A, Speake BK, Noble RC and Sparks NHC. Effect of supplementing the hen’s diet with vitamin A on the accumulation of vitamins A and E, ascorbic acid and carotenoids in the egg yolk and in the embryonic liver. British Poultry Science, 39: 257–263, 1998a.


