

Carotenoid metabolism as a preparation for function

Brian H Davies

Department of Biochemistry, The University College of Wales, Penglais, Aberystwyth, Dyfed, SY23 3DD, U.K.

Abstract - Much is now known of the functions, and possible functions, of natural carotenoids and of how these relate to carotenoid structure. Becoming better understood is the dependence of many of these functions in both plants and animals on the generalized structure of carotenoids, and of specialized functions in many species on the structures of specific carotenoids. Much is understood, too, of carotenoid biosynthesis in plants and microorganisms, and of how carotenoids are metabolized in animals.

It is therefore now appropriate to consider the extent to which carotenoid biosynthesis in the plant kingdom, and the further modifications which dietary carotenoids undergo in the animal kingdom, have evolved in order to form a wide variety of functional molecules. The formation of their structural characteristics will be surveyed against a background of different species and the functions of carotenoids in those species, presenting a picture of carotenoid metabolism as being a deliberate preparation for carotenoid function.

INTRODUCTION

The natural functions of carotenoids which have so far been identified are proving to be of three types: those which are common to most carotenoids, those which are functions of particular carotenoids, and those in which the carotenoids act as precursors of specific functional metabolites. The features of carotenoid structure upon which the various functions depend are the length and rigidity of the molecule, the length of the conjugated chromophore and hence its quenching and light-absorbing properties, the cyclized or acyclic nature of the end group(s), and the presence of contrastingly more polar substituents in these predominantly hydrocarbon molecules; the mode of this substitution may be crucial to the binding of the carotenoid with other molecules, often in functional membranes.

The following review is an assessment, largely in terms of comparative biochemistry, of the extent to which carotenoid metabolism, comprising both biosynthesis and further catabolism, can be seen to have evolved to enable this class of some 600 yellow, orange and red pigments to perform such a variety of functions throughout Nature. In taking so wide a view, it is necessary to differentiate between the metabolic capacities of members of the plant and animal kingdoms. Higher plants, fungi, algae and bacteria are all capable of carotenoid biosynthesis (see ref. 1); different permutations and combinations of known metabolic transformations allow this *de novo* biosynthesis to produce an enormous range of structural types. Animals, on the other hand, are not capable of carotenoid biosynthesis (see ref. 2), so that every animal carotenoid has to be derived, ultimately, from a provenance within the plant kingdom. The nature of biological food chains is such that the plant origins of animal carotenoids are, more often than not, in higher plants or algae, although animal carotenoids suspected of arising from symbiotic relationships with bacteria or fungi have been recognized. In contrast to plant species, which enjoy the evolutionary capacity to adapt *de novo* biosynthesis to their own individual requirements, animals have to manage by absorbing and accumulating such dietary carotenoids as are available, and modifying them, where necessary and as best they can, by a limited repertoire of metabolic transformations, to their own needs.

STRUCTURAL REQUIREMENTS OF A FUNCTIONAL CAROTENOID

In so far as the main functions of the carotenoids as a class are either those of light absorption (as accessory pigments in photosynthesis and as light filters in some animals) or that of protecting living organisms, and particularly their membranes, against some toxic form of oxygen (ref. 3; including photoprotection, in which carotenoids quench photochemically produced

singlet oxygen), the structural characteristics of a carotenoid which is functional *per se* are easy to define.

For the absorption of visible light, a chromophore of suitable length is required; this will contain sufficient conjugated olefinic bonds to render the carotenoid yellow (7-11 such bonds), orange (9-12) or red (11-13); bathochromic spectral shifts due to complexing with protein can extend absorption beyond the normal upper wavelength limit of ca. 550nm. For an effective photoprotective role (which depends upon the quenching of singlet oxygen, 1O_2), a carotenoid requires at least 9 conjugated double bonds (refs. 4, 5), and functions better on a molar basis with 11 or more. The whole molecule has to be long enough to accommodate conjugated chromophores of these lengths, and a degree of rigidity (of the chromophore) can impart structural strength to any membrane of which the carotenoid is a component part. In addition to a minimum length, the molecule has to have sufficient apolar (e.g. methyl) substituents to give it a lipophilic affinity for the components of the natural bilayer membranes, and some carotenoids may require, in addition and in contrast, more polar regions (oxygen substituents) at one or both ends, if a degree of binding is necessary to anchor the molecule, as in protein complexes. The structures of natural carotenoids meet these requirements to perfection.

TERPENOID BIOSYNTHESIS IN PLANTS AND ANIMALS

Lipid-soluble functional molecules in both the plant kingdom and in animals are, more often than not, terpenoid (isoprenoid) in nature. Some are pure terpenoids (carotenoids, steroids, hopanoids, dolichols etc.) and others are 'mixed', a terpenoid side chain imparting their predominantly lipophilic properties (to chlorophylls, tocopherols, menaquinones, ubiquinones etc.). Although animals are incapable of the *de novo* formation of carotenoids, the early steps of carotenoid biosynthesis in plants involve intermediates of isoprenoid formation which are common to both plants and animals.

In both plants and animals, isopentenyl diphosphate (IDP, C_5), formed on the decarboxylation of diphosphomevalonic acid (C_6), isomerises to dimethylallyl diphosphate (DMADP) which is the primer for the chain elongation reactions catalysed by prenyl transferases (ref. 6). Successive C_5 fragments are added from further molecules of IDP to yield, in sequence, geranyl (C_{10}), farnesyl (C_{15}) and geranylgeranyl (C_{20}) diphosphates (GDP, FDP and GGDP). Further transprenylations with IDP yield solanesyl ($C_{25,50}$) and longer (C_{25-110}) diphosphates which provide, respectively, ubiquinone side chains and the dolichols. These represent the end products of prenyl biosynthesis in animals (ref. 7); in plants, polyterpenoids such as rubber (*cis*) and gutta (*trans*) arise from even longer prenyl diphosphate molecules.

PHYTOENE FORMATION IN PLANTS

Much of terpenoid biosynthesis in both eukaryotic plants and animals is channeled into sterol formation. A reductive dimerization of FDP yields the C_{30} symmetrical squalene, the epoxide of which (2,3-epoxide; squalene numbering) cyclizes to one of the tetracyclic sterol intermediates, lanosterol or cycloartenol. The hopanoids, formed predominantly by prokaryotes, are pentacyclic products of squalene cyclization (ref. 8).

In plants, carotenoids are formed by dimerization, too, but this is an oxidative dimerization of GGDP to yield the C_{40} symmetrical phytoene (7,8,11,12,7',8',11',12'-octahydro- ψ,ψ -carotene), the first hydrocarbon precursor of the carotenoids. Phytoene differs from squalene not only in molecular size, but also in having, at the centre of the molecule and resulting from the oxidative nature of the dimerization, a chromophore of three conjugated olefinic bonds, thus denying the molecule the overall flexibility necessary for any extensive cyclization (cf. sterol and hopanoid formation), although a phytoene 1,2-epoxide (carotenoid numbering) is known (ref. 9).

A similar oxidative dimerization, but of FDP, yields the C_{30} 4,4'-diapocarotenoids of some non-phototrophic bacteria (ref. 10). Although animals can form both FDP (for sterol formation) and GGDP (for the cytochrome a_{33} side chain) and use them in other metabolic reactions, it is the essential oxidative dimerization step which is lacking (ref. 11). This is why animals cannot form carotenoids *de novo* and why all animal carotenoids are derived, necessarily and ultimately, via their diet from plant sources.

CHROMOPHORE DEVELOPMENT

The functionally-essential and characteristic chromophore of all carotenoids is developed during the early stages of their biosynthesis as carotenes (hydrocarbons) in higher plants, algae, fungi and bacteria. A sequence of didesaturations acting on alternate sides of the phytoene chromophore brings into conjugation some of the previously isolated double bonds so that the successive products, phytofluene (7,8,11,12,7',8'-hexahydro- ψ,ψ -carotene), ζ -carotene (7,8,7',8'-tetrahydro- ψ,ψ -carotene), neurosporene (7,8-dihydro- ψ,ψ -carotene) and lycopene (ψ,ψ -carotene), have

5, 7, 9 and 11 conjugated olefinic bonds respectively. This Porter-Lincoln sequence of desaturation is typical of most higher plant tissues, but there are variations in some mutant fruits and in some microorganisms. In the non-phototrophic bacteria, *Streptococcus faecium* and *Staphylococcus aureus*, an analogous desaturation sequence operates on 4,4'-diapophytoene to form 4,4'-diaponeurosporene (ref. 12). In some tomato fruits, *cis* rather than *trans* intermediates lead to the formation of the tetra-*cis* prolycopene (ref. 13). In some fungi, the degree of conjugation is extended by further desaturation, e.g. to 3,4-didehydrolycopene (ref. 14), while some phototrophic bacteria and fungi have an unsymmetrical conjugated heptaene as intermediate (ref. 15).

The completion of the carotene chromophore generally precedes further reactions such as carotene cyclization, although the existence of such monocyclic carotenes as β -zeacarotene (7',8'-dihydro- β , ψ -carotene) and 7',8',11',12'-tetrahydro- γ -carotene (7',8',11',12'-tetrahydro- β , ψ -carotene) indicates that, in some fungi at least, such reactions can occur at the fully desaturated end of an acyclic carotene with less than 11 conjugated double bonds. In phototrophic bacteria (e.g. *Rhodospirillum rubrum*), 7,8-desaturation at either end of the molecule is also a prerequisite of 1,2-hydration which can then be followed by methylation and further (3,4) didehydrogenation in either order (ref. 16). These reactions, occurring at both ends of a lycopene molecule, lead to the formation of spirilloxanthin (1,1'-dimethoxy-3,4,3',4'-tetrahydro-1,2,1',2'-tetrahydro- ψ , ψ -carotene); this carotenoid, with an extended chromophore of 13 conjugated olefinic bonds in an acyclic molecule, exemplifies the greater range of chromophore length possible in these bacteria.

Any interference with the carotene desaturation reactions blocks the formation of an adequately photoprotective chromophore. This is the mode of action of the herbicides metflurazon, norflurazon and fluridon, all of which block phytoene desaturation and leave plants vulnerable to the combined lethal effects of light and oxygen (photodynamic action; ref. 17).

CYCLIC END GROUPS AND HOMOCAROTENOID FORMATION

Further metabolism of the acyclic (ψ) end-group (e.g. of lycopene) is initiated by attack at C-2 (ref. 18). The attacking species may be a proton, in which case the resulting carbonium ion can be stabilized either by cyclization followed by the loss of another proton (from C-6, C-4 or C-18 respectively) to yield a β -, ϵ - or γ -end group, or by hydroxyl anion addition at C-1, when the overall reaction is a 1,2-hydration yielding a C₄₀ xanthophyll (oxygenated carotenoid). Alternatively, and typically in some non-phototrophic bacteria, the attacking species may be a C₅ electrophile resulting from pyrophosphate loss from IDP or DMADP. Stabilization of the resulting carbonium ion by the same variety of analogous means results in the formation of C₄₅ or C₅₀ homocarotenoids which may be acyclic or have β -, ϵ - or γ -rings (see ref. 19).

This is clearly the stage of carotenoid biosynthesis at which molecular dimensions are established and where the length of the chromophore can again be modified. In the C₄₀ series, the overall lengths of carotenoid molecules are in the sequence acyclic > monocyclic > bicyclic, whilst the homocarotenoids are correspondingly longer by one or two acyclic C₅ fragments. The molecular dimensions of the carotenoids must be relevant to those of the membranes in which they function. Cyclization results also in chromophore modification. The olefinic bonds of the ϵ - and γ -rings are no longer in conjugation with the chromophore, which is correspondingly reduced in length by one conjugated double bond for each such ring. The double bond (5,6) of each β -ring, though still conjugated, is forced out of planarity by steric hindrance with a resulting hypsochromic shift and some loss of spectral persistence.

FORMATION AND METABOLISM OF XANTHOPHYLLS

Much of the early evidence that xanthophylls (oxygen-containing carotenoids) are formed from the hydrocarbon carotenes was indirect and came from observations on green algae; the first direct proof of the relationship came from bacterial studies (see ref. 19). The commonest types of xanthophyll are those in which a hydroxyl group is introduced at C-3 of a cyclic (β or ϵ), or at C-1 of an acyclic (ψ), end-group. Geometrically, these are the most exposed positions at the ends of otherwise lipophilic molecules and such hydroxyls will facilitate binding; also their polarity can readily be modified, slightly attenuated by O-methylation (e.g. in some phototrophic bacteria) or increased by glycosylation (e.g. in some non-phototrophic bacteria).

Xanthophyll formation in the green tissues of higher plants is presumed to occur by the stereospecific introduction (mixed-function oxygenase) of oxygen as hydroxyl groups at C-3 (and 3') of β - or α -carotene. Of the two, the oxygenation of α -carotene (β , ϵ -carotene) seems to be the more complete, since lutein (β , ϵ -carotene-3,3'-diol) is a major xanthophyll of leaves while α -carotene remains only in traces. An analogous hydroxylation converts about half the β -carotene (β , β -carotene) into zeaxanthin (β , β -carotene-3,3'-diol), leaving the rest as the main carotene. That little zeaxanthin normally persists is due to its ready epoxidation via antheraxanthin (5,6-epoxy-5,6-dihydro- β , β -carotene-3,3'-diol) to violaxanthin (5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro- β , β -carotene-3,3'-diol). These epoxidations by atmospheric oxygen are enzymic and stereospecific (in contrast to epoxides produced, presumably by ¹O₂, as a result of herbicide treatment; ref. 20),

and their reversibility is crucial to the operation of the xanthophyll (violaxanthin) cycle (ref. 21). Lutein, in contrast to zeaxanthin, is subject to only limited (5,6- β) epoxidation.

The xanthophyll epoxides have been recognized for some time as precursors of specific carotenoids in other tissues, e.g. in *Capsicum* fruits (ref. 22) and *Eschscholtzia* flowers, and violaxanthin is known as a precursor of the allenic neoxanthin (5',6'-epoxy-6,7-didehydro-5,6,5',6'-tetrahydro- β,β -carotene-3,5,3'-triol) in green tissues (see ref. 19). Only recently, however, has the observation (ref. 23) that abscisic acid (ABA) is produced from xanthophylls in water-stressed etiolated bean leaves been extended in specific terms to a pathway, supported by labelling evidence (ref. 24). In this pathway, 9'-*cis*-neoxanthin, formed from violaxanthin, is cleaved to xanthoxin, the precursor of ABA aldehyde and ABA; the essential *cis* bond of the ABA is thus introduced in the context of a 9-*cis* carotenoid.

CAROTENOIDS IN PHOTOSYNTHETIC ORGANISMS

Higher plant chloroplasts, apparently irrespective of the type and habitat of the plants (ref. 25), contain β -carotene and the unesterified xanthophylls, lutein, violaxanthin and neoxanthin. Minor components include α -carotene, β -cryptoxanthin (β,β -caroten-3-ol), zeaxanthin and antheraxanthin. It is thought not only that this constancy of distribution pattern reflects the common ancestry of green plants but also that any mutation which causes a departure from this pattern is lethal (ref. 26). Mutations have been observed, both in higher plants and green algae (see ref. 27), in which acyclic precursor carotenes more saturated than α - or β -carotene accumulate; these confer no photoprotection on the mutants which are therefore vulnerable to illumination (and to oxygen evolved within the tissues of such oxygenic photosynthesizers). It was such a mutation, but in the phototrophic bacterium *Rhodobacter sphaeroides* (formerly *Rhodospseudomonas sphaeroides*), which, historically, first led to the recognition of a photoprotective role for carotenoids in organisms in which the excess energy from light absorbed by a photosensitizer is dissipated in the formation of toxic 1O_2 (ref. 28).

The main carotenoids of the chloroplast are located within the thylakoid membranes in the form of pigment-protein complexes (PPCs). They occur in both the reaction centre (RC) complexes of photosystems I and II, where β -carotene and chlorophyll *a* are the predominant carotenoid and chlorophyll, and also, along with chlorophylls *a* and *b*, in the light-harvesting chlorophyll proteins (LHCP), where the xanthophylls, lutein, violaxanthin and neoxanthin, are the main carotenoids (ref. 29). Their role as accessory antenna pigments is mediated in the LHCP complex, but they are presumed to exert their photoprotective role in both the RC and LHCP situations; a minor role, associated with electron transport, has been inferred for the RC carotene (ref. 30).

It is unclear as yet how the carotenoids of the PPCs are held in the correct orientation in the membranes in green tissues, or what structural features are important for their binding. It is clear, however, that lycopene, with a chromophore long enough to be photoprotective but with a molecule (acyclic) perhaps too long to fit the membrane, is not an effective photoprotectant. Seedlings in which carotene cyclization is inhibited by CPTA [2(4-chlorophenylthio)-triethylammonium chloridel] accumulate lycopene which is not photoprotective (ref. 31).

The nature of the binding of carotenoids in PPCs is a little clearer in the purple, non-sulphur, phototrophic bacteria. In such bacteria (*Rhodospirillaceae*), the carotenoids are again associated with RC complexes and with the LHCP antenna systems, where 15-*cis* and all-*trans* chromophores, respectively, are present (ref. 32). The use of surface-enhanced resonance Raman scattering (SERRS) spectroscopy has shown that in *Rhodobacter sphaeroides*, which, under anaerobic conditions, has spheroidene (1-methoxy-3,4-didehydro-1,2,7',8'-tetrahydro- ψ,ψ -carotene) as its main carotenoid which is converted into its 2-one (spheroidenone) on oxygenation, the monomethoxycarotenoids have their methoxy groups close to the cytoplasmic surface of the intracytoplasmic membrane, and the other end deep within it (ref. 33). A similar model, based again on orientational inferences from SERRS spectroscopy, is proposed for *Rhodospirillum rubrum* (ref. 34), where one end of the symmetrical dimethoxycarotenoid, spirilloxanthin, is coincident with the cytoplasmic surface of the membrane.

The carotenoids of the phototrophic bacteria have a wide range of structures (ref. 35) but, from the point of view of antenna function, the acyclic carotenoids of the *Rhodospirillaceae* have the greatest potential for forming chromophores of different lengths. Thus in *Rhodobacter sphaeroides*, the main carotenoid, spheroidene, is based biosynthetically upon neurosporene, from which it is formed by 1,2-hydration, O-methylation and 3,4-didehydrogenation. The same sequence of reactions, but operating at both ends of lycopene forms the spirilloxanthin of *Rhodospirillum rubrum*. Such carotenoids are capable of absorbing maximally *in vivo* at higher wavelength ranges than the higher plant carotenoids (420-500nm). Thus spheroidene (430-520nm) and spirilloxanthin (450-550nm) are potentially superior accessory light-harvesting pigments, filling better the gap between the main and the Soret absorption bands of chlorophylls, although they are not nearly as good in this regard as the red and blue phycobilins of certain other classes of photoautotrophs.

CAROTENOIDS IN NON-PHOTOTROPHIC BACTERIA

Many non-phototrophic bacteria are capable of producing carotenoids, the molecules of which are often glycosylated at the end(s). The range of molecular size is wide; in addition to the conventional C₄₀ carotenoids, there are also (in *Streptococcus faecium* and *Staphylococcus aureus*; ref. 12) the C₃₀ diapocarotenoids formed as a result of FDP dimerization. A number of other bacteria extend C₄₀ carotenoids by an additional isoprenoid unit at each end to yield the C₅₀ homocarotenoids; these may be acyclic or may have β-, ε- or γ-rings (see ref. 36). The range of molecular lengths is from ca. 28Å (C₃₀) to ca. 38Å (C₅₀; see ref. 8).

Since the metabolism of these bacteria is not photosynthetic, such species are not as vulnerable as others to photodynamic action, but they may still require an element of photoprotection. All the main carotenoids of non-phototrophic bacteria have chromophores which are adequate in this regard, and it may be noted that even the C₃₀ diapocarotenoid chromophores are sufficiently long to quench ¹O₂. Oxygen toxicity, though, may not arise from photosensitization alone. A carotenoidless mutant of *Sarcina lutea* (now *Micrococcus luteus*) is killed more rapidly by human polymorphonuclear leukocytes than is the pigmented wild-type; this has been explained (ref. 37) as a protective effect of the carotenoid in quenching ¹O₂ which is produced during phagocytosis and which may arise either by the spontaneous dismutation of the superoxide radical or from a myeloperoxidase-hydrogen peroxide reaction.

It has also been suggested that carotenoids may play a part in the structure of bacterial membranes (ref. 38). Here it is not so much the length of the chromophore but, rather, the rigidity imparted by it which is the more important. Lipid bilayer membranes contain inclusions not only of protein, but also of terpenoids. Thus cholesterol (animals), ergosterol (fungi), β-sitosterol, campesterol and stigmasterol (green plants) all have roles in membrane structure whilst, in prokaryotes, hopanoids replace the sterols (ref. 39). In all cases, the hydroxyl of the triterpenoid represents the polar end of the molecule, and has an affinity for the polar heads of the phospholipids of the bilayer. The role postulated for a bacterial carotenoid (ref. 8) is that of a rigid rivet, traversing the bilayer with the rigid and lipophilic part of its molecule lying alongside the apolar fatty acid chains of the phospholipid, and with its polar end-groups (hydroxylated, often modified by glycosylation) associated with the polar phospholipid heads at each face of the membrane bilayer. In this context, it is worth recalling the spheroidenone of the phototrophic *Rhodobacter sphaeroides*, where the more polar (methoxylated) end of the molecule has been identified at the cytoplasmic face of the intracytoplasmic membrane (ref. 33).

Indirect studies on the inclusion of carotenoids in artificial membranes are supportive of such a structural role. The incorporation of carotenoids into experimental vesicles has a reinforcing effect in terms both of decreased membrane permeability and of enhanced rigidity (ref. 40). Comparisons of C₄₀ and C₅₀ xanthophylls in vesicles prepared from either dimyristoyl (C₁₄) or dipalmitoyl (C₁₆) phosphatidyl choline showed a correlation of carotenoid/phospholipid lengths in membrane reinforcement (ref. 41). Artificial membranes made from *Halobium* lipid (diphytanyolphosphatidyl choline) showed a more effective incorporation of the acyclic C₅₀ xanthophyll from *Halobium* [bacterioruberin: 2,2'-bis(3-hydroxy-3-methylbutyl)-3,4,3',4'-tetrahydro-1,2,1',2'-tetrahydro-ψ,ψ-carotene-1,1'-diol] than they did of the bicyclic C₅₀ decaprenoxanthin [2,2'-bis(4-hydroxy-3-methyl-2-butenyl)-ε,ε-carotene], possibly because of the bulky nature of the cyclized end-groups of the decaprenoxanthin in an environment where the phospholipid side-chains already carry lateral methyl groups (ref. 42). Clearly, chromophore length, molecular length and rigidity, the nature of the end-groups, and the extent of their polar substitution, are all important in bacterial carotenoids.

CAROTENOID DEGRADATION IN THE PLANT KINGDOM

In addition to the cleavage of 9'-*cis*-neoxanthin to form abscisic acid (see above), a number of other degradation reactions which carotenoids undergo in the plant kingdom are worthy of comment. The formation of the mating hormones, the trisporic acids, in the Mucorales fungi is one of these; β-carotene is converted, via retinal, into trisporic acid C (ref. 43). The detection of minute quantities of retinal in higher plants was one of the early achievements of thin-layer chromatography (ref. 44), but it occurs in larger amounts in the membrane retinal-protein complex, bacteriorhodopsin, in *Halobacterium* spp. (ref. 45). Oxidative degradations of higher plant carotenoids lead to a number of apocarotenoids which are characteristic of ripened fruit (see ref. 46), but little specific function can be ascribed to these other than that suggested for all fruit pigments, namely that they attract animals which aid seed dispersal. A more complete degradation of carotenoids, yielding many low molecular mass fragments, occurs in the curing of tobacco leaves (ref. 47). The value of some plant materials as food additives (colorants) stems from the presence of diapocarotenoids such as bixin (methyl hydrogen 9'-*cis*-6,6'-diapocarotene-6,6'-dioate) in annatto extracted from the seeds of *Bixa orellana*, or crocin (the digentiobioside of crocetin, 8,8'-diapocarotene-8,8'-dioic acid) in saffron, the dried stigmas of a *Crocus* sp. Crocetin is formed in the crocus by oxidation at each end of a C₄₀ carotenoid, probably zeaxanthin (ref. 48); its specific function in the crocus stigma is unclear, but it has recently been shown to have some medical potential, in anaesthesia, in increasing the partial

pressure of oxygen in arterial blood during mild hypoxia (ref. 49). A curious ability of some cyanobacteria, *Microcystis* spp., is their capacity for converting β -carotene or zeaxanthin into crocetinial (8,8'-diapocrotene-8,8'-dial), the ends of the carotenoid molecules yielding β -cyclocitral and 4-hydroxy- β -cyclocitral, respectively (ref. 50).

ABSORPTION OF DIETARY CAROTENOIDS BY ANIMALS

Since animals are not capable of the synthesis of carotenoids *de novo*, it is clear that any metabolic reactions which carotenoids undergo in animals are limited first by the nature of the carotenoids available in the diet, and then by the extent to which the dietary carotenoids are actually absorbed by the animal. Animals differ considerably in the latter regard, and can be categorized accordingly. Some absorb carotenes in preference to xanthophylls, and some (fish, birds; ref. 51) absorb predominantly xanthophylls; some species are indiscriminate and absorb all dietary carotenoids, while others absorb little or no unmetabolized carotenoid into their tissues.

Studies on carotenoid absorption have, naturally enough, been carried out mostly on those animals where carotenoid levels are of particular significance for health or nutritional reasons, on those which are readily amenable to the laboratory situation, or on those which are reared for food and when an appropriate colour of the food product is a criterion for market acceptability. Studies of carotenoid absorption have shown that mammals can be divided into three of the above categories (ref. 2). Some (e.g. rats) metabolize β -carotene to retinal and hence to other retinoids, but accumulate little unmetabolized carotenoid. Dairy cattle are typical of the carotene accumulators, for their fatty tissues can be particularly rich in carotene. Humans are among the more indiscriminate of the mammalian carotenoid accumulators, absorbing both carotenes and xanthophylls.

HPLC analyses have confirmed the indiscriminate nature of man's carotenoid absorption. Levels of β -carotene, lycopene and dihydroxycarotenoids (lutein or zeaxanthin) in either plasma (ref. 52) or in various organs (ref. 53) seem to reflect the carotenoid composition of the (U.S.) subjects' diets, but there was some unevenness of distribution noted in different organs. In general, those organs (adrenal, testes, liver) with the highest rates of lipoprotein uptake and greatest number of LDL (low-density lipoprotein) receptors had the highest carotenoid levels. This is what might be anticipated, for most carotene is carried by the LDL fraction whereas xanthophylls are transported both by the LDL and HDL (high-density lipoprotein) fractions in plasma (ref. 54).

METABOLISM AND ROLES OF MAMMALIAN CAROTENOIDS

In contrast to many other animals (birds, fish, crustacea, insects etc.), mammals are limited (ref. 2) to the breakdown of appropriate carotenoids (β -carotene and carotenoids with an unsubstituted β -ring) to yield retinoids (vitamin A and related compounds). The first retinoid formed is retinal, which is reduced to retinol and then transported from the intestinal wall as retinyl esters. In spite of the early characterization of an enzyme, carotene-15,15'-dioxygenase, which catalyzes the central cleavage of β -carotene to yield retinal (refs. 55-57), there still persist reports that excentric cleavage (refs. 58, 59), in which longer β -apocarotenoids are formed first and then metabolized to retinal, may have a role in the formation of retinal (ref. 60).

This metabolism to retinal (for vision) and vitamin A (for reproduction, cellular differentiation and epithelial tissue maintenance) is clearly a crucial role for carotenoids. But carotenoids *per se* are also known to have a wide variety of other effects throughout the animal kingdom, and not least in humans. A recent symposium on the biological actions of carotenoids (ref. 61) defined these as (a) functions (as provitamins A), (b) actions (as protectants against toxic forms of oxygen, in reducing photoinduced effects, in inhibiting mutagenesis, in enhancing immune responses and fertility), and (c) associations (e.g. with cancer prevention).

β -CAROTENE AND MAMMALIAN REPRODUCTION

The particularly high concentration of β -carotene which is characteristic of corpus luteum tissue (ref. 2) has led to considerations of (a) whether β -carotene can actually be biosynthesized in such an animal tissue, and (b) whether β -carotene *per se* (rather than the retinol or retinal to which it is metabolized in this tissue; refs. 62, 63) has a specific reproductive function. In spite of an early report (ref. 64) that bovine corpus luteum slices are capable of forming β -carotene from labelled acetate, our own experience with an extremely efficient terpenoid synthesizing enzyme system from bovine corpora lutea is that no β -carotene, but a considerable amount of squalene, is formed from labelled mevalonic acid. This work was done by Alan Akers, who could find no intermediate of carotene biosynthesis (phytoene, lycopene) in the bovine tissue.

The conclusion has to be that the β -carotene in the corpus luteum is of dietary origin and is concentrated, presumably for a purpose, in this particular tissue. Both indirect and direct

evidence supports a reproductive role for β -carotene *per se*. The fertility of both dairy cattle (see ref. 65) and horses responds to β -carotene; the onset of reproductive activity in experimental Welsh pony mares and thoroughbred mares is advanced by increased levels of β -carotene (ref. 66). Steroidogenesis in cultures of bovine luteal cells is influenced by β -carotene (ref. 67) while, in porcine luteal cells, β -carotene stimulates progesterone secretion to a far greater extent than can be accounted for by its metabolism to retinoids (ref. 68).

The effect of β -carotene in the corpus luteum (and in the adrenals where it also accumulates; ref. 2) is probably more than just protection of progesterone formation against the deleterious effects of a form of oxygen, for the formation of progesterone (in both tissues) is a series of essentially oxidative steps, in which cholesterol is first converted into its 20,22-diol and then cleaved (cholesterol 20,22-desmolase, cytochrome P-450; refs. 69, 70) to pregnenolone and isocaproic aldehyde; progesterone is formed from pregnenolone by dehydrogenase and isomerase reactions.

FORMATION AND FUNCTION OF 4,4'-DIOXOCAROTENOIDS

Although in mammals carotenoid metabolism is confined to their cleavage to form retinoids, other animals can metabolize carotenoids in different ways, notably by oxidizing their end-groups and, more recently recognized in a few species, by a form of reductive metabolism which can sometimes change the nature of the end-groups (e.g. β to ϵ ; refs. 71, 72). In many animal phyla, the commonest modification of a dietary carotenoid is an oxidation to carbonyl of C-4 (and 4') of the β -ring. Although the appropriate monoketones are recognized as intermediates, the products are 4,4'-dioxocarotenoids such as canthaxanthin (β,β -carotene-4,4'-dione), when the oxidation substrate is β -carotene (brine shrimp, flamingo; refs 73, 74), or astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) if zeaxanthin is so oxidized. Astaxanthin is a common carotenoid of fish and marine invertebrates, and it can also be formed by some of them from β -carotene if 3- (and 3'-) hydroxylation precedes or follows the formation of the oxo-groups; a large number of possible intermediates are known (ref. 11). In my own laboratory, Bethan Davies showed that the goldfish, *Carassius auratus*, could form labelled astaxanthin if any of the separate radioactive dietary carotenoids, β -carotene, zeaxanthin or canthaxanthin, was fed (ref. 75). It is thought that the natural astaxanthin of trout and salmon is of direct dietary origin (ref. 71) since zeaxanthin is absorbed but poorly and there is virtually no absorption of dietary β -carotene (ref. 76). Astaxanthin can be present in the food chain at an early stage because of its formation as a secondary carotenoid by green algae (ref. 77); its chirality also depends on its biosynthetic origin (ref. 11).

The introduction of carbonyl (C=O) bonds which are conjugated with the polyene chain means that these (red) 4,4'-dioxocarotenoids absorb at longer wavelengths. In so doing, they may be visually more significant and have an episeomatic role in animal behaviour. The first successful breeding of flamingos in captivity coincided with the introduction of feeds containing canthaxanthin (ref. 78), although other factors in addition to plumage colour are important to a breeding colony (ref. 79). Such colours may have a role, too, in fish reproduction; some progress has been made in identifying the biochemical factors which govern pigment organelle translocation in fish chromatophores (ref. 80). A more chemical role, as an antioxidant, may also be anticipated; a recent comprehensive study of the 1O_2 -quenching properties of a number of carotenoids shows canthaxanthin and astaxanthin to be more effective than β -carotene or zeaxanthin (ref. 81).

In the marine invertebrates, astaxanthin has a more obvious role as the prosthetic group of a number of carotenoproteins such as lobster crustacyanin and the starfish (*Asterias*) carotenoprotein; in the latter, astaxanthin is accompanied by acetylenic analogues (ref. 82). The native α -crustacyanin is an octamer of β -crustacyanin units, each of which contains two dimerized apoprotein molecules (ref. 83). Each apoprotein contains an astaxanthin molecule within a hydrophobic pocket and associated with a tryptophan residue at the bottom of the calyx (ref. 84); the dimerization of the apoproteins protects the astaxanthin from the aqueous environment. Reconstitution studies have shown that the 4-oxo group is important for binding with the apoprotein, and apoprotein dimerization is optimal and the concomitant bathochromic spectral shift greater with 4-oxo groups at each end of the carotenoid molecule (ref. 82). The importance of the formation of such carotenoproteins is the variety of large bathochromic shifts which are possible, dependent both on carotenoid composition and on variation in apoprotein structure; these shifts produce potentially cryptic colours which may be the basis of colour polymorphism in a number of species.

XANTHOPHYLLS AS PROVITAMINS A

The metabolic progression of carotenoids in plants is oxidative; carotenes are formed first, followed by the xanthophylls, many highly oxidized. As the carotenoids progress through the food chain into animals, oxidation continues so that animals which have little dietary access to fresh plant material, and therefore exist on diets in which the carotenoids are largely xanthophylls, will have a limited intake of β -carotene and other conventional provitamins A to act as

precursors of retinal for vision and of other essential retinoids. There seem to be two ways which have evolved in some animals which enable them to overcome this difficulty.

Over a number of years, reports appeared which claimed, contrary to contemporary dogma, that xanthophylls could act in freshwater fish as precursors of vitamin A₁ (retinol) and vitamin A₂ (3,4-dehydroretinol). Astaxanthin and canthaxanthin could be converted by such fish into A₁ and A₂ (refs. 85, 86) and even lutein, not a β -carotene derivative, was claimed as a precursor of A₂ (ref. 87) in a pathway where anhydrolutein (3,4-didehydro- β,ϵ -caroten-3'-ol) was a likely intermediate (ref. 88). The results of two more recent studies, in which radiolabelled carotenoids were fed, confirm that reductive metabolism and cleavage do take place in freshwater fish. In trout depleted of vitamin A, astaxanthin, zeaxanthin and canthaxanthin (ref. 76), and in goldfish, β -carotene, zeaxanthin, canthaxanthin and lutein (ref. 89), were all converted into A₁ and A₂. In all cases, the specific radioactivities of the products were consistent with retinol being formed first and then converted into 3,4-dehydroretinol; this is particularly significant in the case of lutein since anhydrolutein is not necessarily involved.

The recognition of a new visual pigment chromophore in the eyes of the insect *Calliphora erythrocephala* as 3-hydroxyretinal (ref. 90) was followed by a number of similar observations in insects of the Diptera and Lepidoptera (see ref. 91). A rigorous confirmation of the structure of 3-hydroxyretinal in *Drosophila melanogaster* (ref. 92) was complemented by nutritional studies to establish its metabolic origin. Low levels in the eyes of insects maintained on retinoid- and carotenoid-deficient diets were increased by the addition to the food of either zeaxanthin or lutein, but the addition of astaxanthin had no such effect (ref. 93). In a recent study by Victoria Parker in my own laboratory, dietary cornmeal for a *Drosophila* diet was lipid-extracted and then reconstituted using colourless vegetable oil containing radiolabelled zeaxanthin. 3-Hydroxyretinal isolated from the flies as its oxime was radioactive. Clearly, the metabolic source of 3-hydroxyretinal is a xanthophyll containing the 3-hydroxy- β end-group.

CAROTENIDS IN VISION

The universality of (11-*cis*)-retinal (and its analogue in insect xanthopsin) as the photoreceptor of visual pigments is sufficient evidence of the importance of carotenoids to vision. This represents their main precursor function, but there are also instances where carotenoids themselves play an important part in vision, acting as colour filters. The presence of lutein and zeaxanthin in the macular and adjacent regions of human and other primate retinas (ref. 94), and the pigmentation of fish corneas (ref. 95), can be rationalized in terms of blue-light filters, which reduce scattering and minimize the effects of low-wavelength lens aberration. It has been argued that the perception of colour by vertebrates reaches its height in the eyes of birds (ref. 96). As in other vertebrates capable of colour vision, a number of different photoreceptors (iodopsins) are sensitive to blue, red and green light, but the variously coloured oil droplets (red, yellow, greenish yellow etc.) of the individual avian retinal cone cells, through which light has to pass to reach the iodopsins in the cone outer segments, are a more flexible and selective means of restricting short wavelengths than are the macular or lens filters of other vertebrates.

Detailed chemical studies of the carotenoids of the turkey retina (ref. 75) have now identified some 12 carotenoids, of which the three main components, astaxanthin (66% 3S,3'S), (3R)-galloxanthin (10'-apo- β -carotene-3,10'-diol) and (6S,6'S)- ϵ,ϵ -carotene (the sole carotene), together comprise 65% of the total pigment. Their very different absorption spectra can be correlated with those of the individual oil droplets, obtained by microspectrophotometry (MSP) of turkey retinas. The droplet spectra show that none of the carotenoids occurs in pure form in any of the main droplet types, but three types of droplet can be identified in which one of the three main carotenoids predominates, giving a selection of three filter cut-off wavelengths over a range of some 100nm. That the three main carotenoids are kept apart so well points to a differential transport mechanism; in this context it is worth noting the vastly different polarities of the three components which are well-known to carotenoid chromatographers, astaxanthin and ϵ,ϵ -carotene being a very polar xanthophyll, and the least polar carotene, respectively. The three main carotenoids are known to be all formed from zeaxanthin; the evidence for this comes from embryos developing in fertile chicken eggs in which part of the yolk has been replaced by a mixture of radiolabelled zeaxanthin and L- α -phosphatidyl choline (ref. 75). All three types of carotenoid metabolism envisaged in animals (ref. 11) are evident in the formation of the avian retina carotenoids from zeaxanthin, cleavage to galloxanthin, 4,4'-oxidation to astaxanthin, and a reductive end group modification to form ϵ,ϵ -carotene.

Some of these reactions must be carried out, too, by some reptiles. The sea turtle, *Chelonia*, has retinal oil droplets, the published MSP spectra of which (ref. 97) appear to be consistent with the presence of at least astaxanthin and ϵ,ϵ -carotene; indeed, the identity of the ϵ,ϵ -carotene has been confirmed by HPLC (ref. 98). Reptiles and birds have a common ancestry in the *Cotylosauria* (ref. 99), which predated the dinosaurs, which may therefore also have had carotenoid-containing oil droplets. What is clear is that such sophisticated metabolic reactions, which are able so precisely to modify dietary carotenoids for functional needs, have been operating for some 310 million years!

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