CHAPTER 1
CAROTENOIDS AND VITAMIN A:
A BRIEF REVIEW

1.1 Introduction

Vitamin A is a term denoting any compound with a beta-ionone structure having the biological activity of retinol. In the diet, preformed vitamin A is found exclusively in animal products. Vitamin A precursors are carotenoids that have the biological activity of vitamin A after intestinal conversion to retinol (Gerster, 1997). Role of vitamin A in vision, reproduction, development, differentiation and gene expression has been well established. Recently, new functions of vitamin A such as maintaining the integrity of the immune response, cell differentiation and proliferation have emerged. Vitamin A deficiency is a serious problem in many parts of the world especially in many developing countries (Beaton et al., 1993). In South East Asia, it is estimated that a quarter of a million children go blind each year because of this nutritional deficiency (Sommer, 1982). Vitamin A deficiency increases the susceptibility to diseases such as diarrhoea, respiratory diseases and childhood diseases such as measles (Grant, 1991 and West et al., 1989). It is estimated that about 124 million children worldwide are deficient in vitamin A (Humphrey et al., 1992).

Since animals are unable to synthesize vitamin A de novo from endogenous sources, they must derive it from dietary components, the provitamin A carotenoids. Hence the availability of this micronutrient is always related with nutritional status. Carotenoids which are of plant origin, represent the most wide spread group of naturally occurring pigments in nature. Of the 600 carotenoids from natural sources that have been characterized, fewer than 10% serve as precursors of vitamin A. Vitamin A is formed primarily by the oxygen-dependent central cleavage of β-carotene and other provitamin A carotenoids (Bendich and Olson, 1989). The relationship between dietary carotenoids and vitamin A status in human beings is a major topic of discussion. Several questions remain unanswered except the remarkable development regarding the
elucidation of molecular mechanism of action of carotenoid cleaving enzyme (von Lintig and Vogt, 2000). Assessment of vitamin A deficiency is still problematic especially because the serum levels may not be a good indicator due to many reasons. Therefore it is important to develop acceptable tools for the assessment of vitamin A status in human beings. Use of labeled precursors is one of the approaches. Results of investigations carried out on the production of intrinsically labeled carotenoids particularly β-carotene and its bioavailability studies using exfoliated human colonic epithelial cells form the subject matter of this thesis. A brief review of recent developments on the biological function and metabolism of vitamin A, methods for assessment of vitamin A status, provitamin A carotenoids and their occurrence, distribution, conversion to vitamin A and their bioavailability is given below. A number of reviews on chemistry, nutritional aspects, molecular biology of vitamin A and provitamin A are available (Bertram 1999; Singh et al., 1998; Castermiller and West, 1998; Rock 1997; Edge, 1997; Wyss, 2001, Wolf, 1995; 2001).

1.2 Chemistry

Structured of important provitamin A carotenoids and the active vitamin forms are given below.

![Diagram of retinoid formation and metabolism](image)

**Fig. 1.1 Overview of the main steps in retinoid formation and metabolism**
1.3 Vitamin A and its biological functions

Vitamin A is directly involved in the process of vision and its deficiency leads to night blindness, xerophthalmia and related ocular defects, which may ultimately result in total blindness (Lugtenburg, 1996). Vitamin A in its various oxidative and isomeric forms is essential for vision (Redmond et al., 2001). The membrane proteins in the rods and cones of our eye absorb light and, via their photoreactions, translate the light information into nerve impulses that results in the sense of vision in our brain (Fein and Szuts, 1982). All visual pigments in man and other vertebrates contain the protonated 11-cis-retinal at the active site of the membrane protein. Recently, Russel (2000) reviewed the role of different doses of vitamin A supplements in vision and related functions, wherein he has used dark adaptation as a tool for identification of sub clinical vitamin A deficiency. In the eye vitamin A has specific, highly complex function in dark vision. Together with the protein opsin, 11-cis-isomer of retinal forms the light sensitive visual pigment rhodopsin located in the rods of retina. Upon the light sensation, visual pigments disintegrate releasing the energy signals that translate into picture. New rhodopsin will then be formed in the visual cycle. Using Drosophila blind mutants which lack ability to synthesise the key enzymes for vitamin A formation have been identified. Mutations in the corresponding genes which lead to blind vitamin A deficient phenotype are evident (von Lintig et al., 2001; Kiefer et al., 2004).

In parallel with the studies on vitamin A deficiency and supplementation programmes, there has been an upsurge of investigative efforts on functions of vitamin A at organ and cellular level. Ross and Hammerling (1994) have reviewed the properties of vitamin A, its role in the integrity of lymphoid organs, lymphocyte proliferation and functions, circulating antibody concentration and antibody responses, mucosal immunity and responses to various types of challenges imposed by microorganisms. They have also reported that vitamin A interferes with responses to measles virus and HIV; it also reduces response to parasitic infection. Semba (1993) and co-workers illustrate the complexity of these responses in a human population by examining T-cell sub types and their differential responses to supplementation. In another study by Ross and Hammerling (1994) 14-hydroxy retroretinol (HRR), a metabolite of vitamin A showed higher specific activity than retinol for lymphocyte regulation, but its exact role is not known. However, retinoids involving retinoic acid probably influence other important effect on immune functions, including differentiation of cells, stimulation of phagocytosis and modulation of
cytokines (Verma, 1991). Another role of vitamin A, often called the ‘anti-infective vitamin’, is its protection against infection. The precise mechanism of this effect is not known but decreased resistance to infection is observed with low vitamin A intake (Gerster, 1997). Moreover abnormal T-cell subpopulations with lower proportions of circulating CD4/CD8 positive cells have been observed in vitamin A deficient children (Semba, 1994). Vitamin A and provitamin A carotenoids especially β-carotene have been found to have an immune stimulating effect (Chandra, 1994; West et al., 1991; Ross and Hammerling, 1994).

Extensive animal studies have shown that vitamin A is required for normal growth and development. Deficiency in animals is expressed as loss of appetite within a few weeks of vitamin A deprivation, followed by rapid loss of weight and stunted growth (Olson, 1991). Lack of vitamin A results in keratinisation of mucus secretory ciliated epithelium, and epithelial cells in the trachea, skin, cornea, testis and salivary gland (Rosenthal et al., 1994). In the eye, corneal lesions such as erosion of epithelial layers, foamy Bitot’s spots, xerophthalmia and finally blindness result from changes in differentiation of surface epithelium. Vitamin A deficiency is constantly associated with anemia characterised by low serum iron and increased iron levels in the liver. Controversies still exist regarding the mechanism by which vitamin A induces cell differentiation. Both 9-cis and all trans retinoic acid interact in the nucleus with ligand-dependent transcription factors to regulate the expression of retinoid-responsive genes (Mangelsdorf et al., 1992). Chytil and Ong (1979) have suggested that retinoic acid derived from retinol binds to one of its nuclear retinoic acid receptors (RARs or RXRs) which regulate gene expression by interacting with retinoic acid response elements (RARE) close to target genes and thus induces a cascade of regulatory events through the activation of specific gene networks. Nuclear retinoic acid receptor belongs to a super family of hormone regulatory proteins including those for steroids and thyroid hormone. At least six genes have been identified coding for nuclear retinoic acid receptors, viz RAR α,β,γ and RXR α,β,γ. Response elements for these transcription factors have been characterised for different group of genes including those for laminin B1, RAR β, cellular retinol-binding protein type I (CRBP), apolipoprotein A-I, oxytocin etc. (Gudas et al., 1994; Mangelsdorf et al., 1994). Studies with preadipocytes and adipocytes indicate that the cellular processes responsible for accumulating all-trans retinoic acid are cell type-specific and studies of the uptake by tissues of bolus doses of all-trans-retinoic acid indicate that these processes
are also tissue specific. Moreover, the uptake of all-trans retinoic acid is responsive to physiologic (e.g. nutritional) state (Kurlandsky et al., 1995).

Vitamin A is essential for the maintenance of proper physiological functions in both males and females. Deficient animal model systems have demonstrated the degeneration of germinal epithelium and impaired spermatogenesis in males. Eskild and Hansson (1994) reviewed role of vitamin A in the reproductive system. Apart from the status of precursor molecule, retinol has a separate role in reproductive system as all-trans-retinoic acid and 9-cis-retinoic acid have modulatory functions during spermatogenesis; however, the mechanism is not known. Wellik and Deluca (1995) established the requirement of retinol rather than retinoic acid for successful completion of pregnancy in rats.

1.4 Vitamin A metabolism and transport

Dramatic changes have occurred in recent years in our understanding of uptake, transport and metabolism of vitamin A; however mechanisms involved in many of these activities are yet to be fully understood. Preformed retinols are provided in the diet as foods of animal origin rich in retinyl esters or as carotenoids, which after ingestion are hydrolysed to retinol in the intestinal lumen. Of the carotenoids, β-carotene has the major provitamin A potential. Retinol absorbed by the enterocyte is subsequently reesterified into retinyl esters. It is assumed that food borne β-carotene is absorbed by the intestinal enterocytes and part of the absorbed β-carotene is metabolized into retinaldehyde being reduced to retinol, which is subsequently esterified into retinyl esters (van Vliet et al., 1996). The rest of the β-carotene is absorbed into the lymph unchanged. Both retinyl esters and β-carotene are incorporated in chylomicrons and secreted into the lymph.

The dietary retinyl esters may be hydrolysed in the intestinal lumen by pancreatic enzymes like pancreatic lipase and triolein hydrolase (Goodman and Blaner, 1984). In vitro experiments using intestinal brush border membranes showed the involvement of two separate enzyme systems sensitive to different bile salts conditions. These findings suggest that intestinal retinyl ester hydrolysis may play crucial role in the utilization of retinyl esters. A small portion of the dietary retinyl ester in chylomicrons is taken up by some extra hepatic tissues, particularly bone marrow, kidney and adipose tissue where retinyl ester may serve as source of retinol for meeting tissue needs (Kurlandsky et al., 1995).
Retinol and retinal produced in the intestinal enterocytes have to be converted into retinyl esters before further mobilization to liver. Cellular retinol binding protein (CRBP) II appears to have a major role in intestinal retinyl ester formation. Kakkad and Ong (1998) reported that the CRBPII-retinal complex serves as a good substrate for the reduction of retinal into retinol and also mediates the esterification of retinol into retinyl esters. CRPB II in the intestine may be required in the metabolic processing of retinal from β-carotene and retinol from dietary retinyl esters. Ong et al. (1987) localized CRBP II in the intestine of adult animals and found similarity with CRBP I (Ong et al., 1994), including molecular weight and specificity to antibodies. CRBP I differs from CRBP II on its binding affinities for retinoids, former binds retinol with much higher affinity than retinal whereas CRBP II binds both retinol and retinal at physiological concentrations.

![Fig.1.2 Absorption, metabolism and transport of carotenoids. CAR, carotenoids; apo-CAR, apo-carotenoids; RAL, retinal; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein (Yeum and Russell, 2002).](image)

Retinyl esters are secreted from the intestine as chylomicrons which are eventually transported to the liver. Most of the chylomicron remnants originating from the intestine are taken up by the liver, especially by liver parenchymal cells (Hendriks, 1996). Hydrolysis of chylomicrons remnants and retinyl ester is the primary step in the further processing in the liver. Various mechanisms involving cholate-dependent hydrolysis and pancreatic cholesterol ester hydrolase activity have been attempted by many workers like Prystowsky et al. (1981); Blaner et al. (1984); Harrison and Gad (1989), wherein they have localized enzyme activity in the microsomal fractions specifically in the liver cell plasma membranes and suggested that the cholate-independent activity may play a role in the hydrolysis of retinyl ester delivered to the liver by chylomicron remnants. There are
reports on the involvement of CRBP on hydrolysis of retinyl ester mediated by apo-CRBP in cholate-independent retinyl ester hydrolase activity (Boerman and Napoli, 1991) and holo-CRBP during retinyl ester formation. Thus retinyl esterification and hydrolysis would appear to be regulated by the balance between apo- and holo-RBP (Ong et al., 1994).

After the hydrolysis of retinyl ester in the liver parenchymal cells, a fraction of the retinol is secreted bound to retinol binding protein RBP, which subsequently complexes with another plasma protein, transthyretin (TTR) (Wei et al., 1995). Remaining retinol in the liver is transferred to and stored in the fat storing stellate cell. When the dietary vitamin A level is low, the stored retinol is mobilized. Plasma retinol is internalized from RBP through a process involving the action of CRBP (Soprano et al., 1994). Inside the cells, the retinol can be oxidized to retinoic acid through enzymatic process (Blaner and Olson, 1994). In addition to these transport processes, other pathways for the delivery of retinoid to target tissue may operate within the body. Retinoic acid available as such through the foodstuff or from the retinol is absorbed via the portal system as retinoic acid bound to albumin (Blaner and Olson, 1994). In plasma, retinoic acid circulates bound to albumin (Blomhoff et al., 1990). However, the mechanism for retinol uptake is still unclear.

1.5 Mechanism of action of retinoids

The mechanism of action of natural vitamin A derivatives in several biological processes such as vision, spermatogenesis, carcinogenesis and development has been known since decades. However, it is only since 1987, when nuclear receptors for retinoic acid (RA) were discovered (Giguere et al., 1987; Petkovich et al., 1987), that our understanding has advanced significantly regarding vitamin A derivatives. The retinoid receptors belonging to the super family of steroid/thyroid hormone receptors characterised by a common structure in which the different functions (trans-activation, DNA binding and ligand binding) are present in discrete modular domains in the protein.

The gene for RARs and RXRs generally consists of eight exons which have been localized and characterised (Zelent et al., 1991; Mangelsdorf et al., 1992). These different genes encoding sub types for RAR’s and RXR’s exist in man and in rodents; they are α, β and γ. RAR α, -β and -γ are on human chromosomes 17q 21, 3p 24 and 12q 13 respectively (Mattei et al., 1991) which are also closely related to human malignancies. A number of target genes with response elements for RAR/RXR has been
identified and these findings offer exciting prospects for utilization of these retinoids as anti carcinogenic agents (van der Saag, 1996).

1.6 Vitamin A deficiency

Vitamin A deficiency has been recognized as a root cause of nutritional blindness and related complications. In recent years it has been demonstrated that the risk of morbidity and mortality from infectious diseases is compounded by poor vitamin A nutriture (Dutra-de-Oliveira et al., 1998). It is known that both too low a vitamin A intake, leading to a deficient or marginal vitamin A status and too high an intake resulting in vitamin A induced toxicity are associated with health risks. It is estimated that about 124 million children worldwide are deficient in vitamin A (Humphrey et al., 1992) and that improved nutrition could prevent at least one million deaths annually among children (West et al., 1989).

Vitamin A is found in the plasma as retinol, 5-10% of which is present as retinyl esters. Experts have defined plasma or serum retinol level less than 0.35 \( \mu \text{mol/L} \) as the cut-off point for vitamin A deficiency (WHO/UNICEF, 1994). However plasma retinol levels in the range of 0.70-1.22 \( \mu \text{mol/L} \) can be found to co-exist with lowered hepatic vitamin A stores and plasma vitamin A starts falling only after the hepatic vitamin A stores have dropped to less than 0.70 \( \mu \text{mol/g} \) of tissue (Manesme et al., 1987 and Olson, 1984). In the absence of dietary vitamin A, children grow poorly and develop signs of deficiency. Xerophthalmia and night blindness are most common and plasma concentrations of vitamin A also decrease markedly (Olson, 1990 and Sommer, 1982).

According to Olson (1994), five states of vitamin A nutrition exists: deficient, marginal, satisfactory, excessive and toxic. Deficient and toxic states are characterised by a fairly specific set of clinical signs. In the satisfactory state, there is no clinical or physiological defect, whereas both marginal and excessive status, which showing no clinical signs, place individuals at greater risk of either deficiency or toxicity, respectively. Differentiating between the marginal and satisfactory and excessive state are somewhat subjective. Under marginal category, a total liver concentration of 0.07 \( \mu \text{mol/g} \) wet weight of liver seems to provide both adequate vitamin A for all physiological processes and a body reserve adequate to meet deficiency during low dietary intake for approximately 3 months periods. However, it is difficult to fix a cut off point between the satisfactory and excessive states. In marginal vitamin A deficiency, keratinized tissue can undergo
squamous metaplasia, for instance in mucous membranes lining the respiratory and genitourinary tract (Chytil, 1983).

More severe stages of deficiency are characterised by loss of appetite, decreased resistance to infection, especially pneumonia, olfactory and auditory dysfunction, impaired fertility, embryonal malformation or foetal death. Among the best known signs are night blindness due to diminished synthesis of rhodopsin and various stages of xerophthalmia such as conjunctival xerosis (dryness), Bitot’s spots, corneal xerosis, corneal ulceration and necrosis, keratomalacia, blindness and finally death (Underwood, 1993).

1.7 Methods for assessment of intake, status and safety

Both low intake and high intake of vitamin A are associated with health risk. For diagnosis of deficient vitamin A status, a number of valid biochemical and functional tests and criteria for interpretation are available. Nutritional, biochemical, functional and clinical tests are being used for the assessment of vitamin A status and classification of individuals. The specificity and accuracy of these tests differ with respect to their applicability and has been used by many workers in this field. Application of these methods depends on the vitamin A intake/status in the population or individual under study as well as on the aim of the experiment (Olson, 1992). Various aspects of these methods have been described by Underwood (1994); Maiani et al. (1993); WHO/UNICEF (1994) etc., they have concluded that clear relation between measures of vitamin A status and morbidity and mortality exists in the areas where vitamin A deficiency remains as health problem especially in developing countries in Africa, and south East Asia.

Due to considerable individual variation in the requirement and daily intake, dietary assessment is a rather ‘rough’ indicator of vitamin A status (Nelson et al., 1974). Individuals with lower intake, i.e. below the recommended daily allowance (RDA) have a higher chance of having depleted stores or impaired function. According to Olson (1987) the minimum daily intake levels for vitamin A for adults are set at 500-600 retinol equivalents (RE) and safe levels of intake for adults are generally higher (800-1000 RE); these recommended intake levels are based on estimation and assumptions with respect to the appropriate body reserve, catabolic rate and availability for tissue storage. In general, vitamin A situation is complicated due to the contributions of provitamin A carotenoids in the diets; the carotenoid factor used, takes into account differences in biological activity and bioavailability of carotenoids from vegetables and fruits (van den Berg, 1996).
1.7.1 Assessment of body stores

Other than liver, which represents the largest stores of vitamin A, smaller amounts of vitamin A are also present in tissues like kidneys, lung, colon and skin (Nirenberg and Nann, 1992). In liver, retinyl palmitate is the prominent ester form (57-83%) and significant amount of carotenoids, mainly β-carotene, α-carotene, lutein and lycopene are also present. Due to the difficulty in taking these samples non-invasively, indirect methods like relative dose response (RDR) test (Loerch et al., 1979) have been developed in rat models as a measure of liver vitamin A reserve. The basic principle of this test is that apo-retinol binding protein (apo-RBP) accumulates in the liver when liver vitamin A content decreases; giving an oral dose of retinol to a person with depleted liver stores, results in an ‘immediate’ release of RBP-bound retinol (holo-RBP) with a maximum increase in serum retinol after 5 hrs. The relative increase between 0 hr. (pre-dose) and 5 hr. after dosing is an index of liver depletion. When liver stores are adequate (> 0.07 μmol/g) no increase in serum retinol is observed. An RDR >20% is considered to reflect inadequate vitamin A status. A modified RDR (MRDR) was developed using 3, 4- dihydro retinol (Tanumihardjo et al., 1990) as the test dose and requires only one blood sample. Non-availability of this retinol derivative is noted as one of the disadvantages and there are objections, about the reliability in vitamin A replete populations and the state of protein-energy malnutrition. It needs further validation because of its poor reproducibility.

Indirect approach, involves use of isotopic dilution methods using labeled tracers like tetra-deuterated retinyl acetate, Furr et al. (1989) demonstrated that isotope dilution is a valid, relatively harmless procedure to measure total body reserves in human subjects in marginal and satisfactory intake range. The deuterated-retinol-dilution (DRD) technique is an indirect quantitative method for estimating total body stores of vitamin A, and this consists of administering a known dose of deuterium-labeled vitamin A orally and measuring the plasma isotopic ratio of (2H4) retinol to unlabeled retinol after the dose has mixed fully with endogenous vitamin A body stores (Haskell et al., 1999). Buccal mucosal cells, which are easily obtained have also been used as markers for tissue storage and noted large inter individual variability (Peng et al., 1994).

Blood vitamin A level is most frequently used as the biochemical measure of retinol status of individuals. In general, serum retinol level in well-nourished populations were found to be related to serum cholesterol and triglyceride contents, body fat and
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gender (Herbeth et al., 1991). The reproducibility of serum retinol content was studied in detail (Morrow et al., 1990; Olson, 1992; Underwood, 1994) in different populations.

Retinol-binding protein (RBP) levels have been suggested as alternatives to serum retinol especially in population surveys and when applied under field conditions. On marginal intakes, the ratio of apo-RBP to total RBP might be a useful measure (Underwood, 1994): under conditions of adequate supply, RBP is almost completely saturated with retinol, while the percentage of ‘free’ RBP (apo-RBP) increase with decreasing vitamin A status. In rats, the holo-RBP levels gave the highest correlation with liver stores of vitamin A and were found to be a better indicator of marginal vitamin A status than serum retinol (Burri et al., 1992). However the impaired RBP levels due to infections or renal failures sometimes showed low accuracy (Burri et al., 1990) and application of this technique needs further validation.

Breast milk vitamin A level can also be used as another measure to evaluate vitamin A status, where the provitamin A β-carotene, is abundantly present in well nourished women (Canfield et al., 1994) and vitamin A content has been shown to be a relatively sensitive indicator of maternal vitamin A status, particularly in intervention studies (Stolzfus et al., 1993).

Functional assessment like conjunctival impression cytology (CIC), measuring the number of goblet cells as well as size and shape of the conjunctival epithelial cells (i.e., conjunctival integrity) has been reported to be a relatively simple, less invasive test for early diagnosis of sub clinical vitamin A deficiency (Natadisastra et al., 1987). However, in several other studies no relationship between vitamin A status and measures of CIC were noted indicating that this test identifies other marginal (sub) groups than found with biochemical test (Fiteau et al., 1994). There is increasing evidence that vitamin A plays an important role in the immune system and that vitamin A deficiency interferes with the response to parasitic infections (Erdman et al. 1993; Solomons, 1993). It is still unclear whether specific immune tests have the potential to be used as functional markers of vitamin A status (van den Berg, 1996).

1.7.2 Safety

Concerns have been expressed about the vitamin A supplementation with examples of various deleterious effects like teratogenicity and even death. There is no doubt that retinol and its esters are toxic when given to human beings in excessive doses (Bendich
and Langseth, 1989) although single very high doses (200000 IU) have been shown to successfully alleviate xerophthalmia in children and adults. Vitamin A interventions have also reduced to a certain extent child mortality and morbidity (Underwood, 1994). In addition to the beneficial effects of high doses on xerophthalmia, repeated high doses of retinyl palmitate have been reported to lead to remission of symptoms of acute myelogenous leukemia in children (Lie et al., 1998). Reviews on toxicity of vitamin A (Bendich and Langseth, 1989; Hathcoek et al., 1990) and teratogenicity (Teelmann, 1989; Amstrong et al., 1994) have been published. Toxicity usually results from abusively high intakes of vitamin A supplements and rarely, from the consumption of liver from animals or fish. Chronic intakes in adults above 30,000 RE can produce symptoms of hyper vitaminosis including loss of appetite, dry itchy skin, hair loss, weakness, headache, bone thickening, enlarged liver and spleen, nausea and vomiting and blurred vision (Bendich and Langseth, 1989). Vitamin A toxicity was described in patients taking large doses of vitamin A and in patients with type I hyperlipidemias and alcoholic liver disease; and also carotenoids which can cause toxicity in animal models by indicating lung cancer seen in two epidemiologic studies of the effects of high dose of β-carotene supplementation (Russel, 2000).

1.8 Provitamin A carotenoids

A century and a half after Wackenroder’s isolation of first carotene, 563 structurally distinct carotenoids and their glycosides and isomers, had been chemically characterised (Straub, 1987; Britton, 1991) and over 600 different carotenoids are reported to be produced by microorganisms and plants (Schmidt-Dannert et al., 2000). Carotenoids are the most abundant and wide spread group of pigments in nature, which are of plant origin. Carotenoids are required for normal differentiation and function as light absorbing pigments in photosynthesis. Almost all carotenoids contain at least one unsubstituted β-ionone ring with a polyene side chain attached to it. They play a crucial role in electron transport cycles in photosynthesis and also protect plants from damaging action of singlet oxygen and other radicals (Kamauckov, 1990). The oldest known function of carotenoids in animals and man is as provitamin A, of which β-carotene has the highest vitamin A activity on a molar basis (van Vliet, 1996). In addition to their provitamin A activity, carotenoids may have several other important biological functions in animals and man. Carotenoids can protect human beings against photosensitization, as was found
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in bacteria and algae (Mathew-Roth, 1989). Epidemiological studies have indicated that
the consumption of food rich in carotenoids is associated with a reduced risk for certain
types of cancer and cardiovascular diseases (van Poppel, 1993; Gay et al., 1993;
Mathew et al., 1995). The antioxidant role of carotenoids has been studied (Ong et al.,
1988) in many models including human beings and in vitro studies suggest immuno
enhancing effect (Bendich, 1989), intercellular communication (Zhang et al., 1991) and
protection of tissues from ultra violet (UV) light related damage (Mathew-Roth, 1984
and Fuller et al., 1992). Kaplan et al. (1990) demonstrated the presence of five most
common carotenoids in the diet of man and in the blood, viz., β-carotene,
α-carotene and β-cryptoxanthin and non-provitamin A carotenoids, lutein and lycopene.

1.9 Biosynthesis of carotenoids

The crucial role of carotenoids and their metabolites in photo oxidative protection,
photosynthesis, animal nutrition, vision and cellular differentiation make them an important
and complex class of biological pigments. The first unified hypothesis for the biosynthesis
of carotenoids in higher plants was proposed by Porter and Lincoln (1950), and later
revised by Porter and Anderson (1962). Subsequently on the advent of new technologies
like use of inhibitors of carotenogenesis, of microbial mutant strains, and more recently,
attempts to isolate the enzymes and their encoding genes, have all advanced our
understanding of the biosynthesis of these pigments. Numerous general and comprehensive
review articles on carotenoid biosynthesis have been published in recent years (Spurgoen

Advances in the molecular biology technique during the last few years have
enriched our understanding of the genetics and molecular biology of carotenoid
biosynthesis in bacteria (Amstrong, 1994; Ohnuma et al., 1994; Chen and Poulter,
1994), fungi (Armstrong, 1994; Schmidhauser et al., 1994; Ehrenshaft and Paub, 1994),
cyanobacteria (Lang et al., 1994) and higher plants (Bartley et al., 1994; Badillo et al.,
1995). Molecular characterization of all genes for carotenoid biosynthesis in Rhodobacter
capsulatus, a photosynthetic bacterium and from several species of Erwinia, non-
photosynthetic bacteria (Armstrong, 1994) have been worked out. A number of
carotenogenic genes have been cloned from microorganism and plants and expressed in
E. coli, thereby allowing a recombinant biosynthesis of different acyclic and cyclic
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carotenoids (Misawa and Shimada, 1998; Hirschberg, 1998). In addition to the prospects of bioengineering, the accumulation of useful and unusual carotenoids and the expression of carotenogenic genes in *E. coli* represent unique opportunities for the cloning of new genes by heterologous complementation (Sun *et al.*, 1996) for the functional testing of gene products (Li *et al.*, 1996) and for the elucidation of the pathways (Kajiwara *et al.*, 1997; Ruther *et al.*, 1997).

![Carotenoid biosynthesis diagram](image)

Phytoene, the first C₄₀ intermediate in the carotenoid pathway is synthesized by a soluble enzymatic complex localized in plastid stroma (Camara, 1980) through a multistep process. The early steps, for which individual enzymes were recently isolated and purified, are the isomerisation of isopentenyl diphosphate into dimethyl allyl diphosphate followed by prenylations forming successively geranyl diphosphate, farnesyl diphosphate and geranylgeranyl diphosphate. These reactions are common to all terpenoids, including other plastid terpenoids (Chlorophylls, plastoquinones, tocopherols, phylloquinones and polyterpenes). The next two steps, in which prephytoene diphosphate and phytoene are formed, are catalysed by the first carotenoid-specific enzymes in the pathway, “geranylgeranyl pyrophosphate geranylgeranyl transferase” and phytoene synthase (Dogbo *et al.*, 1988).
In general, phytoenes serve as the classical precursor for other carotenoids. Most organisms, particularly higher plants, algae and fungi, synthesise 15,15'-cis-phytoene whereas some microbes produce mixture of isomers that can include all-trans- and 9-cis-phytoene (Bramley and Mackenzie, 1988; Britton, 1988; Ben-Amotz et al., 1988). A series of desaturation and cyclisation reactions convert phytoene into cyclic carotenes, such as β-carotene. Introduction of successive conjugated double bonds during this process lengthens the chromophore, producing coloured carotenoids beginning with ζ-carotene. Other cyclic carotenes, xanthophylls and lycopene generally exist in all-trans forms suggesting that at least one isomerisation step must occur (Goodwin, 1980; Bramley and Mackenzie, 1988; Britton, 1988). The interconversion of cis to trans configuration does not, however appear to require a distinct isomerase activity (Fraser et al., 1992). As far as β- and α-carotene are concerned, lycopene cyclisation process is very important and has been demonstrated in several systems e.g. plastids of fresh bean and tomato (Kushawaha et al., 1970; Hill et al., 1971), spinach chloroplasts (Kushawaha et al., 1969) and cell extracts of flavobacterium. But it is not clear about the number of enzymes involved in the cyclisation of lycopene into β- or ε-ring containing carotenes. Genetic analysis of Erwinia shows that only one gene product is required for the two-step reaction via γ-carotene (Sandmann et al., 1990; Schnurr et al., 1991) and a dimer of the carR gene product catalyses the same sequence in Phycomyces (Candau et al., 1991). Two distinct enzymes CrtY type and CrtL type lycopene cyclase with difference in protein sequence were identified from Erwinia sp. and Synechococcus sp. respectively, both of which produce β-carotene by two successive β-cyclisations (Misawa et al., 1990; Hundle et al., 1994). CrtI type and CrtP type lycopene cyclase are also found in cyanobacteria and no information is available about the presence of distinct lycopene cyclase that introduces the ε-ring into α-carotene, a precursor of the highly abundant xanthophyll and lutein in higher plants (Amstrong and Hearst, 1996).

Recently recombinant DNA technology was used to improve nutritional value of rice (Oryza sativa), a major staple food in the developing countries, by transforming with the entire β-carotene biosynthetic pathways into rice endosperm resulting in the 'Golden rice', Ye et al. (2000) succeeded in the transformations to introduce genes for phytoene synthase originating from daffodil, a bacterial phytoene desaturase (crtI) originating from Erwinia uredovora so as to express the β-carotene production in the endosperm up to a level of 2 µg/g provitamin A (corresponding to 100µg retinol equivalents
at a daily intake of 300g rice per day). They have noted the presence of other carotenoids like, zeaxanthin and lutein also, in the grain.

1.10 Absorption of β-carotene and its conversion to vitamin A

After consumption of the food, carotenoids are released from their matrix by digestive enzymes and solubilized by the bile salts. Hollander and Ruble (1978) reported that the carotenoids are absorbed passively from the micellar particles. Carotenoid digestion and absorption may be strongly affected by dietary factors (de Pee and West, 1996). The type of food matrix is an important factor determining the release of carotenoids. Experiments with the rat model using everted gut sacs of the intestine showed that bile salts are essential for the β-carotene absorption. The pH in the lumen may also regulate absorption by altering the surface charges of micellar particles and luminal membranes (Hollander, 1981). Other carotenoids such as lutein may be dehydrated enzymatically in the strongly acidic media in the digestive system forming 2',3' anhydro lutein (Khachik et al., 1992). Passive absorption is determined by concentration gradient over the intestinal membrane. However the complete mechanism of transport of carotenoids is still unknown.

Animals differ with respect to their ability to absorb carotenoids, for example chickens do not absorb cryptoxanthin, while they absorb lutein. Cats can absorb β-carotene, but it is not converted to vitamin A (Schweigert et al., 2002) while Mongolian gerbils utilised provitamin A (Sulaeman et al., 2002). Man appeared to absorb carotenoids in a relatively non-specific fashion (Goodman et al., 1966; Blomstrand and Werner, 1967). Absorption pattern is also regulated by the nature of isomer; cis-isomers differ from all-trans form. Stahl et al. (1992) reported that in human beings, 20-40% of tissue β-carotene is present as cis-isomers. Jenson et al. (1987) reported that, in plasma, cis isomers were not detected after supplementation of carotene preparations containing 60% 9-cis-β-carotene; and cis-isomers of lycopene have been seen to be better absorbed than the all-trans form (Stahl and Sies, 1992).

Conversion of absorbed β-carotene to retinol by the enzyme 15,15'-dioxygenase was studied in detail (Olson and Hayashi, 1965; Goodman and Hueng, 1965). The main cleavage product of β-carotene in man has been reported to be retinyl esters, accounting for 68-88% of the isotopic label recovered from the lymph (Goodman et al., 1966; Blomstrand and Werner, 1967). Retinal formed is presumably bound to cellular retinol
binding protein type II (CRBP II) and is eventually reduced to retinol by microsomal reductase (Kakkad and Ong, 1988). Cleavage activity in man has been reported to be present already at birth, as measured in vitro (Lekshman et al., 1993). Bile salts were found to be essential for β-carotene cleavage as they promote the accessibility of the highly water insoluble substrate to the water-soluble enzyme or directly affect the dioxygenase enzyme. Vitamin A intake in rats was also found to increase the cleavage activity as evidenced from the in vitro and in vivo studies, whereas a high intake of either vitamin A or β-carotene was found to decrease cleavage activity (van Vliet et al., 1996 a). Isomeric discrimination was also reported as 9-cis isomer of β-carotene is converted to retinal less efficiently than the all-trans forms.

In vitro experiments with dioxygenase (Nagao and Olson, 1994) showed 50-54% provitamin A activity for α-carotene, 50-60% for β-cryptoxanthin (Bauernfeind et al., 1981). In addition to these observations, lutein lowered retinal formation from β-carotene while lycopene had no effect (van Vliet et al., 1996 b). Since the intestine is the primary passage site for β-carotene, it seems to be the most important site for cleavage; this has also been demonstrated in vitro in other tissues such as liver, lung, kidney (Wang et al., 1991). Prominent extra intestinal cleavage activity was reported in the liver of hamsters, which was found to be higher than that of intestine under in vitro conditions (van Vliet et al., 1992).

1.10.1 Enzymatic cleavage of β-carotene

Bioavailability of the ingested carotenoids and the conversion of the carotenoid to vitamin A are considered to be two important factors, which determine vitamin A status (Olson, 2000). The conversion of β-carotene to vitamin A(retinol) was discovered by Moore in 1930. In 1960s, Glover proposed the excentric cleavage mechanism starting at the 8'-double bond, supported by in vivo isolation of β-apo 8'-, 10' - and 12' - apocarotenals. An alternative mechanism was put forwarded by Wang et al. (1991), who isolated apocarotenals from in vitro incubation with intestinal tissue from human, monkey, ferret and rat, resultant of excentric cleavage starting at the end of the double bond, and it was accepted as an alternative mechanism. The enzyme, β-carotene-15, 15'-dioxygenase (EC 1.13.11.21) is found in the intestine and liver cytosol (Goodman and Olson, 1969) and turnover rate of substrate varies in the different reports (Nagao and Olson, 1994; van Vliet et al., 1992). In further studies, Wang et al. (1992)
demonstrated that retinoic acid was the principal metabolite derived from the apocarotenals in their *in vitro* intestinal system. Analysis of stoichiometry of 2 mol retinal produced per molecule of [15, 15'-14C]β-carotene cleaved by guinea pig intestinal mucosal cytosol *in vitro*, provided evidence for existence of central cleavage creating a cleavage ratio of 1:2 β-carotene: retinal (Devery and Milborrow, 1994). Wolf (1995) in his review concluded that as experiments involving enzymatic activity are highly unstable, no group has succeeded in more than three-fold purification. It will be necessary to achieve stabilisation of both enzyme activities by some means.

![Possible mechanisms for cleavage of all-trans-β-carotene](image)

The controversy of enzymatic cleavage of β-carotene appears to be resolved recently. (Barua and Olson, 2000; Wyss et al., 2000; Wyss et al., 2001; von Lintig et al., 2000). The conversion has been securely established to be by central cleavage (Figure 1.5) and the retinal is produced through the action of the enzyme β-carotene-15-15'-dioxygenase. This was in agreement with findings of Barua and Olson (2000). They fed a vitamin A-deficient and vitamin A-sufficient diet for five weeks, and on feeding a single oral dose of β-carotene (5.6 μmol) subsequently, only a small amount of apocarotenals in the intestine of deficient rat was detected indicating that the
cleavage of dietary β-carotene to retinal is the predominant mechanism whereby retinoids are formed in vivo whereas apo-carotenals resulting from excentric cleavage play a minor role (Wolf, 2001).

![Diagram of β-carotene cleavage](image)

**β-carotene**

Fig. 1.5 Recent concept of β-carotene cleavage (von Lintig and Wyss, 2001)

Wyss et al. (2000) purified the recombinant dioxygenase enzyme from chicken duodenum and recombinant β-carotene-15-15'-monoxygenase (Lindqvist and Anderson, 2002). They have constructed full-length cDNA expression library; from the peptide sequence expressed in *E coli*, it was found to have a molecular mass of 60.3 kDa with 526 amino acids. Further, gene was transfected into cultured baby hamster kidney cells that were over expressed β-carotene dioxygenase, and confirmed earlier findings of Nagao et al. (1996), that the enzyme was cytosolic and not membrane bound in mammalian systems. It requires ferrous ion (During et al., 2001) and ascorbate; its Km value is 5mM. Nagao et al. (2000) reported that some dietary antioxidants, such as tocopherol (Yeum et al., 2000), derived from food sources modulated the conversion of β-carotene to vitamin A in intestinal cells and inhibition of this enzyme activity by dietary flavanoids. In a new breakthrough, Lintig and Vogt, reported the presence of vp14, a gene coding for a plant carotenoid dioxygenase, that has sequence homology with dioxygenase, present in insects, birds and mammals, which cleaves one molecule of β-carotene centrally to produce two molecules of retinal in vitro and in vivo. Further they concluded that the enzyme dioxygenase catalyses the oxidative cleavage of conjugated double bonds of carotenoids in bacteria, plants and animals.
1.11 Role of carotenoids in human health

In addition to the provitamin A activity, protection against free-radical damage is the major physiological function of carotenoids. There are many indicators of oxidative stress induced free-radical damage in man due to the carotenoid-deficient diet (Dixon et al., 1994). β-carotene is one of the carotenoid that has been most extensively examined on its antioxidant effect. Rapid conversion to vitamin A limits the antioxidant activity of β-carotene, since vitamin A does not display a prominent antioxidant effect. The extraordinary singlet-oxygen scavenging ability of β-carotene has gained much attention. Protection against low-density lipoprotein oxidation seems to be involved in the effect of carotenoids on atherosclerosis (Frei, 1995); Burton and Ingold (1984) have reported peroxyl-radical-scavenging properties of β-carotene. The immuno-enhancing and anticarcinogenic actions of the carotenoids could be associated with functions unrelated to their ability to form vitamin A (Bendich, 1989). Several groups have described β-carotene-dependent free radical chain breaking effects (Krinsky, 1989; Liebler, 1993). Various products formed from the reaction of β-carotene with oxidants have been described, such as carbonyl derivatives and epoxides (Canfield et al., 1992). Bast et al. (1991) showed that there is a strong interaction between the various endogenous anti oxidants in providing complete protection against oxidative stress and also interaction between vitamin A, glutathione and α-tocopherole has been extensively studied. However, very little is known about the metabolism of β-carotene, the most potent precursor of vitamin A contained in significant amounts in fruits, vegetables and in certain algae like *Dunaliella* (Tamai et al., 1995) and *Spirulina* (Becker, 1994). A species of *Dunaliella, D. salina* has the unique capacity to accumulate naturally occurring β-carotene and 9-cis-β-carotene. With respect to the bioavailability of naturally occurring β-carotene, a few investigations have shown that all-trans-β-carotene is predominant in human plasma (Krinsky et al., 1990; Stahl et al., 1993). Only small amount of geometrical isomers of all-trans-β-carotene is found, even if a large amount of β-carotene isomer derived from algae has been taken orally (Jansen et al., 1987; Stahl et al., 1993; Tamai et al., 1993). Little is known about the absorption of 9-cis-isomer and its conversion to the other stereoisomers during ingestion or whether it is directly stored in specific tissues, but not in plasma (Tamai et al., 1995).

Plasma β-carotene concentration is known as a reliable marker of dietary intake (Willett et al., 1985; Gerber et al., 1988) and is shown to be subject to seasonal variation.
(Rautulahti et al., 1993). But there are reports that single determination of plasma \( \beta \)-carotene is not informative enough to be meaningful with regard to the \( \beta \)-carotene status in the body (Tangney et al., 1987). In a recent study Hickenbottom et al. (2002) analysed variability in conversion of \( \beta \)-carotene to vitamin A in men and found that vitamin A activity of \( \beta \)-carotene, even when measured under controlled conditions, can surprisingly be low and variable.

### 1.12 Bioavailability of carotenoids

Bioavailability can be defined as the fraction of available provitamin A carotenoids that is converted to the active form of retinol (van Lieshout et al., 2001). Provitamin A carotenoids, such as \( \beta \)-carotene are the major source of vitamin A in the diet of a large proportion of world’s population. But bioavailability of the carotenoids in many vegetable and fruits, and the conversion of carotenoids to retinol are lower (de Pee et al., 1995; de Pee et al., 1998) than reported previously. There are various factors that influence the efficacy of absorption. These are (a) species of carotenoids, (b) molecular structure, (c) total amount of carotenoid present in the ingested food, (d) the matrix in which the carotenoids is incorporated, (e) various effectors of absorption, (f) the nutrient status of the host, (g) genetic factors and interactions (de Pee et al., 1996; Castenmiller et al., 1998). The efficiency of absorption of carotenoids was estimated in human beings with use of oral-fecal balance technique, and estimates of blood plasma responses after single dose of carotenoids. Radioactive isotopes also have been less extensively used in human involving \(^{14}\text{C}\) \( \beta \)-carotene (Goodman et al., 1966). Bioavailability studies using stable isotopically labeled \( \beta \)-carotene is possible, Perker et al. (1993) and Tang et al. (1999) used intrinsically labeled \(^{13}\text{C}\) \( \beta \)-carotene from Spinach for estimation of \( \beta \)-carotene status by isotopic reference method. Although much work has been done on carotenoid metabolism, it is clear that several unanswered questions remain and our current knowledge about the bioavailability of provitamin A carotenoids in food is insufficient, fragmentary and difficult to interpret (Olson, 1999). Reported ranges of carotenoid bioavailability (\% dose absorbed) range from 1-99, and variability is generally high, both within and between treatments (Perker et al., 1999). Lin et al. (2000) reported variability in absorption and conversion in humans.
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1.13 Transport and tissue distribution of carotenoids

In the enterocytes, β-carotene and most of its cleavage products are incorporated in chylomicrons and are transported via the lymph to the blood. In the blood stream chylomicrons undergo lipolysis, by lipoprotein lipase, forming chylomicron remnants, which are cleared from the plasma by the liver. From the liver, carotenoids can be resecreted with lipoprotein for transport to other tissues (van Vliet, 1996). β-carotene in plasma is mainly present in LDL (Johnson and Russel, 1992). Other carotenoids like α-carotene and lycopene are also mainly associated with LDL, whereas β-cryptoxanthin is equally distributed over LDL and HDL and lutein is associated mainly with HDL (Reddy et al., 1989). The apolar carotenoids α- and β-carotene are believed to be incorporated in the centre of lipoprotein molecule, whereas the more polar lutein and β-cryptoxanthin may be located on the surface (Traber et al., 1994). In addition to the lymph routes, there exists other routes, like portal systems present in ferrets (Wang et al., 1992). The main storage tissue of β-carotene is liver accounting for about 80%; in addition to this, high concentration have been demonstrated in adrenal gland, spleen, ovary and in adipose tissue (Kaplan et al., 1990).

Due to ethical reasons, use of human beings as model systems for studying various aspects is restricted. Thus many workers have used animal model systems. However animals differ markedly from humans in their ability to absorb carotenoids and subsequent conversion of provitamin A carotenoids to retinol. Strict carnivores often depend on preformed retinol and they lack the ability to absorb or convert carotenoids, as has been reported for cats (Bauernfeind et al., 1981) whereas variability in carotenoid absorption and its conversion exist in strict herbivorous animals which depend on carotenoid for
their vitamin A source. Rats and chicken are very efficient in converting carotenoids and absorb intact β-carotene only at high dose level. Human beings are rather unique in transporting β-carotene mainly in the LDL fraction.

Knowing vitamin A/provitamin A status is an important part of any intervention studies involving different populations. Presently no reliable animal models are available for β-carotene bioavailability studies; it is important to develop methods, which can be directly applied in human beings. Methods like evaluation of carotenoid balances are complicated (Shiau et al., 1994) and cannot be used in non-clinical setting and it is rather unpleasant for the subject. Plasma responses after a single dose or after supplementation are the most frequently used techniques where relative availability of carotenoids can be assessed, but this method seems less appropriate to study cleavage products. After a single oral dose, plasma response of both β-carotene and retinyl ester can be measured. Due to the relatively high baseline levels of carotenoids, the sensitivity of the single-dose method is rather low and relatively high doses have to be used. Duecker et al. (1994) suggests use of stable isotopically labeled carotenoids such as $^{13}$C-labeled β-carotene. The use of stable isotopes is promising (Tang et al., 2000), although intrinsic labeling, i.e. incorporation of the isotope in natural sources, seems complicated (van Vliet et al., 1996).

1.14 Objectives of the present study

As indicated before, carotenoids are the major source of vitamin A. Fruits and vegetables are rich in provitamin A carotenoids. Dietary intervention promoting the consumption of micronutrients rich food appears to be a sustainable public health measure for promoting vitamin A status in target population. But a number of factors such as availability of provitamin A in the diet, their conversion to vitamin A, rate of absorption, transport and dietary level of the nutrients such as fat can affect the bioavailability of vitamin A. Vitamin A status is assessed at different levels; however, routine measurement of plasma vitamin A may not be a good indicator as vitamin A level in the plasma is often ‘buffered’ and the best indicator is the liver level of vitamin A. No non-invasive technique is available to study the bioavailability of provitamin A/vitamin A in human beings. Isotope dilution studies using stable isotopically labeled β-carotene as precursor molecules has been recommended in the intervention trials involving human beings. But such stable isotopically labeled β-carotene produced chemically are expensive. Therefore
investigations were carried out to (a) produce intrinsically labeled deuterated carotenoids using a carotenoid rich blue green algae, *Spirulina platensis* in culture in a bioreactor, (b) evaluate the influence of macronutrients particularly fat on the bioavailability of carotenoids of *Spirulina* in different experimental animals and (c) study the possible use of exfoliated colonic epithelial cells as a non-invasive tool to assess the bioavailability of carotenoids in humans, and the results are presented in the following chapters.