Intestinal absorption and cleavag

of β-carotene in rat, hamster an

human mode

Trinette van Vli

Intestinal absorption and cleavage of β-carotene in rat, hamster and human models

Aan mijn ouders

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Vliet, Trinette van

Intestinal absorption and cleavage of β-carotene in rat,
hamster and human models / Trinette van Vliet. - Delft :
Eburon P&L
Proefschrift Universiteit van Amsterdam. - Met lit. opg. -
Met samenvatting in het Nederlands.
ISBN 90-5651-012-6
NUGI 813
Trefw.: beta-caroteen ; absorptie.

Omslag : β-caroteen kristallen. Foto Hoffmann-La Roche, vormgeving: Alex Vermeule
Vormgeving : Trinette van Vliet
Drukwerk : Ponsen & Looijen BV, Wageningen
Distributie : Eburon P&L, Postbus 2867, 2601 CW Delft

The work described in this thesis was carried out at the TNO Nutrition and Food Research Institute in Zeist.
Financial support for the publication of this thesis was provided by: Stichting Dr. Ir. J.H.J. van de Laar, Quest, Hoffmann-La Roche, Roche Nicholas, Nutricia Nederland and TNO Nutrition and Food Research.

Intestinal absorption and cleavage of β-carotene in rat, hamster and human models

No reprints available.

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Universiteit van Amsterdam, op gezag van
de Rector Magnificus, Prof. Dr. P.W.M. de Meijer,
ten overstaan van een door het college van dekanen ingestelde
commissie in het openbaar te verdedigen in de Aula der Universiteit
(Oude Lutherse kerk, ingang Singel 411, hoek Spui)
op vrijdag 13 oktober 1995 om 13.00 uur

door

Trinette van Vliet

Geboren te Dordrecht

Promotor : Prof. dr. W.H.P. Schreurs
Co-promotor : Dr. H. van den Berg

Contents

		Page
1	Introduction	1
2	β-Carotene absorption and cleavage in animals and man. A review *In abbreviated form, European Journal of Clinical Nutrition (in press)*	5
3	Determination of several retinoids, carotenoids and E vitamers by high-performance liquid chromatography. Application to plasma and tissues of rats fed a diet rich in either β-carotene or canthaxanthin *Journal of Chromatography 1991;553:179-186*	51
4	In vitro measurement of β-carotene cleavage activity: methodological considerations and the effect of other carotenoids on β-carotene cleavage *Submitted for publication*	63
5	Uptake but no cleavage of β-carotene by cultures of rat and human intestinal cell lines	81
6	Effect of a low vitamin A or high β-carotene diet on intestinal and liver β-carotene cleavage activity. A pilot study with hamsters	93
7	β-Carotene absorption and cleavage in the rat is affected by the vitamin A content of the diet *Submitted for publication*	101
8	Intestinal β-carotene absorption and cleavage in men: response of β-carotene and retinyl esters in the triglyceride-rich lipoprotein fraction after a single oral dose of β-carotene *American Journal of Clinical Nutrition 1995;62:110-116*	123
9	General discussion	141
Summary		151
Samenvatting		155
Dankwoord		159
Curriculum vitae		161

Chapter 1

Introduction

The importance of β-carotene for humans has long been thought to be limited to its function as provitamin A. This function is of special importance for vegans, not eating animal products, and for people in many parts of the developing world where β-carotene is more readily available in the diet than vitamin A.

A renewed interest for β-carotene in the prevention of (chronic) diseases arose when in 1981 Peto and coworkers[1] hypothesized that dietary carotenoids may reduce human cancer rates. A large number of epidemiological studies have been reported since, indicating that the consumption of foods rich in carotenoids is associated with a reduced risk for certain types of cancer as recently reviewed by van Poppel[2]. The effects of β-carotene in cellular and animal systems have been studied extensively[3]. Whether the active agent is β-carotene, vitamin A or another product formed from β-carotene such as retinoic acid still has to be elucidated. Interest for β-carotene in chronic diseases was extended to cardiovascular disease when Steinberg et al.[4] hypothesized that low-density lipoprotein cholesterol becomes atherogenic upon oxidation, leading to intensified research on the potential protective role of antioxidants such as β-carotene. Inverse associations between β-carotene, intake or plasma levels, and the risk of cardiovascular disease have been found as reviewed by Gey et al.[5]

For a better understanding of the mechanisms of action of β-carotene, information on β-carotene metabolism is needed. In addition, information on factors affecting metabolism may enable the formulation of recommendations for β-carotene intake for populations with a low pre-formed vitamin A intake and possibly also for populations at risk for certain chronic diseases. A large number of studies on β-carotene metabolism have been reported and different aspects of β-carotene metabolism have been reviewed[6-10]. Briefly, the fat-soluble β-carotene is absorbed in the small intestine and subsequently incorporated in chylomicrons or cleaved, mainly into retinyl esters. Both β-carotene and retinyl esters are transported with chylomicrons to the liver. β-Carotene can be

resecreted from the liver with lipoproteins for transport to other tissues. Sever
details of β-carotene metabolism still have to be elucidated.

The work described in this thesis was intended to obtain more insight in
intestinal β-carotene absorption and cleavage. Questions addressed were:

- How to assess intestinal β-carotene uptake and cleavage in rats and humans.
- Which cleavage products are formed from β-carotene in the intestine.
- Does dietary vitamin A or β-carotene intake moderate intestinal absorptio and cleavage of β-carotene.

Chapter 2 provides an overview of current knowledge on carotenoid absorptio and especially cleavage in animal models and humans, with the emphasis β-carotene. To study β-carotene metabolism and possible cleavage products β-carotene, accurate analytical methods for these compounds are neede Chapter 3 describes the high-performance liquid chromatography metho developed for this work. Intestinal cleavage of β-carotene was studied using t dioxygenase assay. Implementation and optimization of this assay is describ in Chapter 4. In addition, the effect of other carotenoids in the assay β-carotene cleavage is reported. We concluded that the assay appears to be appropriate method to measure cleavage activity under different feedi conditions. However, for the purpose of studying *in vivo* cleavage products β-carotene, the use of a system with intact cells was thought more appropria Therefore we evaluated rat as well as human intestinal cell lines for the ability to convert β-carotene; the results of this evaluation are described Chapter 5. The dioxygenase assay was applied in a pilot study with hamste described in Chapter 6, to investigate whether regulation of dioxygena activity by vitamin A or β-carotene intake may occur. To assess the relevance the *in vitro* measured dioxygenase activity for *in vivo* cleavage, in a study wi rats a similar protocol was used, combined with measurement of β-carote absorption and cleavage in lymph-cannulated rats. The results of this study a described in Chapter 7. Since no animal model reflects precisely huma β-carotene metabolism, an approach to assess absorption and intestin cleavage in humans was evaluated (Chapter 8). We measured the β-carote and retinyl ester responses in a triglyceride-rich lipoprotein fraction of plasn after a single oral dose of β-carotene. Finally, in Chapter 9, the results of

studies are discussed in view of the questions addressed, and the overall conclusions and implications for future research are given.

References

1. Peto R, Doll R, Buckley JD and Sporn MB. Can dietary beta-carotene materially reduce human cancer rates? Nature 1981;290:201-208.
2. van Poppel G. Carotenoids and cancer - An update with emphasis on human intervention studies. Eur J Cancer 1993;29A:1335-1344.
3. Krinsky NI. Effects of carotenoids in cellular and animal systems. Am J Clin Nutr 1991;53:S238-S246.
4. Steinberg D, Parthasarathy S, Carew TE, Khoo JC and Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med 1989;320:915-924.
5. Gey KF, Moser UK, Jordan P, Stahelin HB, Eichholzer M and Ludin E. Increased risk of cardiovascular disease at suboptimal plasma concentrations of essential antioxidants - An epidemiological update with special attention to carotene and vitamin-C. Am J Clin Nutr 1993;57:S787-S797.
6. Bauernfeind JC. Carotenoids as colorants and vitamin A precursors. New York: Academic Press, 1981.
7. Ganguly J and Sastry PS. Mechanism of conversion of β-carotene into vitamin A- Central cleavage versus random cleavage. Wld Rev Nutr Diet 1985;45:198-220.
8. Parker RS. Carotenoids in human blood and tissues. J Nutr 1989;119:101-104.
9. Tee ES. Carotenoids and retinoids in human nutrition. Crit Rev Food Sci 1992;31:103-163.
10. Packer L, ed. Carotenoids part B: Metabolism, genetics, and biosynthesis. Methods in Enzymology vol 214, San Diego: Academic Press, 1993.

Chapter 2

β-Carotene absorption and cleavage in animals and man. A review

Trinette van Vliet[1,2] and Henk van den Berg[1]

[1] TNO Nutrition and Food Research Institute, Zeist, The Netherlands; [2] Department of Experimental Zoology, University of Amsterdam, Amsterdam, The Netherlands

In abbreviated form: European Journal of Clinical Nutrition (in press)

Contents

Carotenoids: chemistry and function
Chemistry and occurrence
Biological activities of carotenoids
Functions in photosynthesis
Protective function against photosensitization
Provitamin A function
Antioxidant action
Other actions
Inverse associations with chronic diseases
Carotenoid metabolism
Carotenoid absorption
Carotenoid cleavage in intestinal mucosa
In vivo studies
In vitro studies
Mechanism of cleavage: central or eccentric?
Postabsorptive transport and metabolism of carotenoids
Postabsorptive transport
Tissue distribution
Extra-intestinal cleavage
Methods and models for studying β-carotene absorption and cleavage
Animal models
Primates other than man
Rat
Chicken
Ferret
Preruminant calf
Other species
Evaluation of various species as model for humans
Methods applicable to humans
In vitro
In vivo
Factors affecting carotenoid absorption and cleavage
Food matrix
Meal composition
Inclusion in a meal
Fat
Fat-soluble compounds
Other compounds
Physiological conditions
Age
Gender
Nutrient status
Other factors
Medical drugs
Smoking
Conclusion
References

Carotenoids: chemistry and function

Chemistry and occurrence

Carotenoids represent the most widespread group of naturally occurring pigments in nature. They are of plant origin and animals are not able to synthesize them, but can store them as absorbed or with some alteration of their basic structure[1]. In plants, carotenoids are present in all photosynthetic tissues, but also occur in roots, seeds, flower petals and fruits. Furthermore, carotenoids occur in some algae, fungi and bacteria. The yellow, orange and red colours of flesh, shell and skin of animal species such as salmon and flamingo are due to carotenoids, and the colour of egg yolk is also determined by carotenoid intake. Green, blue and purple colours can be achieved when carotenoids are present as carotenoproteins as in lobster[2]. Nowadays, commercially synthesized carotenoids are widely used as food colours.

The history of research on carotenoids started with the isolation in 1831 of a red pigment from carrots, called *carotene*, and followed with the isolation in 1837 of a yellow pigment from autumn leaves called *xanthophyll*. The generic name *carotenoids* was suggested in 1911 by Tswett, who had discovered the polymorphism of carotenes and xanthophylls and their relationships. The history of carotenoids has been reviewed by Karnaukhov[3].

Most carotenoids can be described by the general formula $C_{40}H_{56}O_n$ where n is 0 - 6. Hydrocarbons ($n = 0$) are termed "carotenes" and oxygenated carotenoids "xanthophylls". The latter group includes oxy (= keto), hydroxy, epoxy and furanoxy derivatives of the carotenes[4]. Carotenoids mainly occur in their all-*trans* form, but can also occur as *cis* isomers. A total number of 563 carotenoids were listed in 1987, without listing *cis-trans* isomers separately[5]. Typical structures of commonly found carotenoids are shown in **Figure 1**.

β-Carotene, α-carotene, β-cryptoxanthin, lycopene and lutein are generally mentioned as the five most common carotenoids in human plasma and tissues. However, Khachick et al.[6] recently reported the presence of phytofluene and ξ-carotene in plasma in concentrations comparable to those of β-carotene and β-cryptoxanthin. Since analytical methods are still improving, other quantitatively important carotenoids may be described in the future.

Important food sources for the different carotenoids may be: carrots, broccoli, dark-green leafy vegetables and tomatoes for β-carotene; carrots and tomatoes for α-carotene; citrus fruits, papaya and mango for β-cryptoxanthin; tomatoes,

guava and watermelon for lycopene; and spinach, mustard greens, broccoli an pumpkin for lutein[7,8]. Phytofluene and ξ-carotene originate from yellow-orang and yellow-red fruits and vegetables[9]. Fruits and vegetables are known t contain varying amounts of *cis* isomers, while isomers can also be formed durin processing. Chandler and Schwartz[10] reported that in fresh fruits an vegetables 0 - 28% of α- and β-carotene was present as mono-*cis* isomer whereas this proportion was 20 - 54% in commercially processed product Carotenoid intake may vary largely, dependent on the amount and type of fruit and vegetables consumed. In the Netherlands, the median intake of β-caroten from food is 0.78 mg per day (mean 1.08)[11], whereas a mean intake of 2.6 mg ha been reported for women in the USA[7].

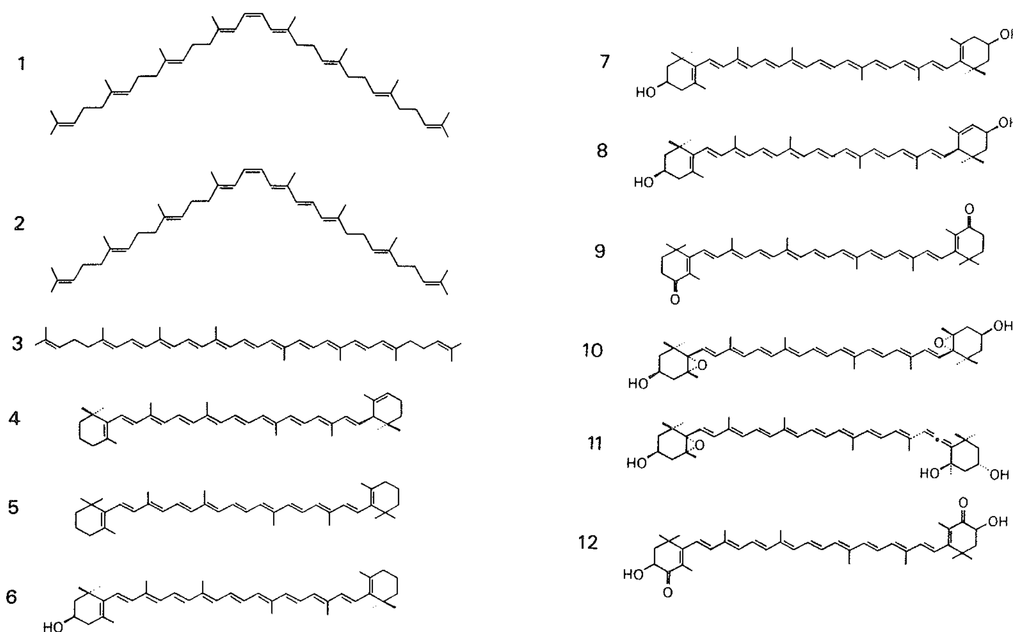


Figure 1. Carotenoids in foods that may also be found in human and animal tissues. 1, phytoene; 2, phytofluene; 3, lycopene; 4, α-carotene; 5, β-carotene; 6, β-cryptoxanthin; 7, zeaxanthin; 8, lutein; 9, canthaxanthin; 10, violaxanthin; 11, neoxanthin; 12, astaxanthin. (source: ref 5).

Biological activities of carotenoids

Bendich and Olson[5] suggested to divide the biological activities of carotenoids into three different categories: functions, actions and associations. Functions of carotenoids are defined as essential roles they play. Their absence leads to impaired physiological capability. Examples are accessory pigments in photosynthesis, protection against photosensitization, and provitamin A. Actions can be defined as responses to their addition, which are not essential for physiological well-being. Examples are colouring of the plumage of birds, and antioxidant and immunoenhancing activities. Associations are correlations between the ingestion of carotenoids and the occurrence of diseases, either causal or not.

In plants and mammals carotenoids show several activities, but whether carotenoids in lower animals have a metabolic activity apart from their action as colorant is not clear[2]. In the next section the most important activities of carotenoids are described.

Functions in photosynthesis

In all photosynthetic systems (green plants, algae and photosynthetic bacteria) carotenoids are present along with chlorophyll. Carotenoids have been shown to function as accessory light-harvesting pigments and as photoprotective agent[12]. Their light-harvesting function is based on the energy transfer from singlet excited carotenoid to the first excited singlet state of the (bacterio)chlorophyll. In this way carotenoids extend the spectral range over which light drives photosynthesis[12]. Their photoprotective function is based on the quenching of triplet (bacterio)chlorophyll. Alternatively, this triplet sensitizer initiates the formation of singlet oxygen, which can also be quenched by carotenoids as described by Krinsky[13].

Protective function against photosensitization

Apart from a photoprotective function in photosynthesis, carotenoids have been reported to protect non-photosynthetic bacteria, and patients with erythropoietic protoporphyria against photosensitization. In this disease the porphyrins produced resemble the porphyrin group of chlorophyll and act as photosensitizers. Treatment with β-carotene increased the tolerance to sun exposure of these patients[14]. β-Carotene was also reported to protect against photosuppression of the immune function[15].

Provitamin A function

In higher animals and man the oldest known function of a number c carotenoids is as provitamin A, which function was discovered in 1930 b Moore[16]. All carotenoids containing at least one unsubstituted β-ionone ring an a polyene side-chain attached are potential precursors of vitamin A, wit β-carotene showing the highest vitamin A activity on a molar basis. Th importance of this function of carotenoids depends on the intake of pre-forme vitamin A, which is only present in animal products, such as liver, eggs, chees butter and eels. In countries where the intake of animal products is very lov almost all of the vitamin A requirement has to be met by carotenoids. Even i industrialized countries, where animal products are widely availabl carotenoids contribute to the vitamin A requirement. The functions of vitamin in humans, extensively described by Ganguly[17], can be broadly grouped int vision, bone growth, overall growth, reproduction and maintenance of epithelia.

To provide a basis for describing the vitamin A activities of carotenoids an retinol on a common basis, the concept of the retinol equivalent (RE) wa introduced. The following relationships among dietary sources of vitamin were established: 1 RE is equal to 1 µg of retinol, 6 µg of β-carotene or 12 µg c other provitamin A carotenoids[18]. These factors are arbitrary values, based o the assumptions that carotenes are absorbed half as well as pre-forme vitamin A, and that the average extent of bioconversion is 33%[19]. Th assumption that the symmetric β-carotene is twice as active as othe carotenoids was supported by a report from Bauernfeind[20], who concluded fror the literature that the provitamin A activity of α-carotene is 50 - 54% and c β-cryptoxanthin is 50 - 60% compared to 100% for β-carotene. *Cis* isomers c provitamin A carotenoids have a lower biopotency than the all-*trans* forms, an vary largely in activity[21]. For 9-*cis*- and 15-*cis*-β-carotene a provitamin A activit of 30 - 65% has been reported[21,22].

The conversion factors are seen as operational equivalencies for practica application in mixed diets, although many factors may affect the efficiency c carotenoid utilization in mixed diets. The bioavailability of β-carotene in oil, fo instance, is expected to be two times higher than in food, and the conversio factor of 6 is expected to apply for β-carotene doses of 1 to 4 mg per mea whereas a factor of 4 has been suggested for lower doses and a factor of 10 fo higher doses[18]. As was clearly explained by Solomons and Bulux[19], th

conversion factor of 6 is questionable and must be re-examined, especially in the context of dietary plants.

The existence of a second system with a different equivalency is confusing. Based on older experiments with animals under optimum conditions of absorption and conversion, 1 international unit (IU) β-carotene was defined as 0.6 μg β-carotene and was presumed to be equivalent to 0.3 μg retinol[23]. The use of this system is not to be recommended.

Antioxidant action

Carotenoids can also serve as antioxidants under conditions other than photosensitization. Carotenoids can quench singlet oxygen formed in other than photosensitized reactions and they can interact with radical species. Comprehensive reviews on the antioxidant actions of carotenoids both *in vitro* and *in vivo* have been published by Krinsky[13,24,25]. Free radicals can originate both endogenously, from normal metabolic reactions and exogenously, for instance as a component of tobacco smoke. Carotenoids can interact with radical species and thus prevent, for example, damage of membranes due to lipid peroxidation, or DNA damage.

Carotenoids differ in their antioxidant capacity, depending on the test system used. Di Mascio et al.[26] reported the highest singlet oxygen quenching ability for lycopene (more than double that of β-carotene) followed by γ-carotene, astaxanthin, canthaxanthin, α-carotene, β-carotene, zeaxanthin, lutein and cryptoxanthin (one fifth as potent as lycopene). The most potent inhibitor of lipid peroxidation in 10T1/2 cells was lutein, followed by lycopene and canthaxanthin, while α- and β-carotene were the least potent ones[27].

The different isomers may also differ in anti-oxidant activity. Conn et al.[28] showed that both 9-*cis*- and 15-*cis*-β-carotene quenched singlet oxygen as efficiently as the all-*trans* isomer. Levin and Mokady[29] reported a higher antioxidant activity for 9-*cis*- than for all-*trans*-β-carotene on the free-radical oxidation of methyl linoleate in solution. However, Lavy et al.[30] showed that all-*trans*-β-carotene prevented *in vitro* lipid peroxidation of lipoproteins more efficiently than the 9-*cis* isomer. The relative efficiency of *in vitro* antioxidant behaviour is probably highly dependent on the system employed.

Other actions

Carotenoids have been reported to induce gap-junctional intercellular communication[27], with β-carotene being the most potent one, followed by canthaxanthin, lutein, lycopene and α-carotene. Carotenoids have also been reported to modulate the activity of drug-metabolizing enzymes[31]. Bendich[32] and Prabhala et al.[33] have reviewed the immune-enhancing effects of β-carotene and discussed the mechanism of enhancement being through retinol, through their antioxidant capacity, or both.

Inverse associations with chronic diseases

Inverse associations have been found between carotenoid intake, or plasma levels, and the risk for cancer at certain sites (recently reviewed by van Poppel[34]), the risk for some cardiovascular events (reviewed by Gey et al.[35] and Manson et al.[36]), and the risk for macular degeneration[37].

The protection against cancer may be effectuated through almost all activities of carotenoids mentioned. Several authors have reviewed the experimental evidence for carotenoid inhibition of mutagenicity, malignant transformation, tumour formation etc.[38-40] De Vet[41] discussed the question whether the cancer-preventive effect should be attributed to carotene or retinol. Alternatively, β-carotene may be converted to retinoic acid, a compound which has anticarcinogenic properties[42].

Carotenoids have been suggested to decrease the risk for cardiovascular events by their action as antioxidant, especially by protecting low-density lipoproteins (LDL) against oxidative modification[43,44]. However, *in vitro* studies on LDL oxidation do not support this. Protection of LDL from lipid peroxidation *in vitro* has only been demonstrated when β-carotene was added to LDL *in vitro*[45], and not when *in vivo* enriched LDL from subjects supplemented with β-carotene was used[46,47].

The antioxidant function of carotenoids in the retina are expected to be responsible for the protection against macular degeneration[37].

Carotenoid metabolism

In this section a description is given of β-carotene metabolism from absorption in the intestine via intestinal cleavage to postabsorptive transport and metabolism. Information on metabolism of β-carotene isomers and other carotenoids is added when available. Most knowledge on β-carotene metabolism is based on animal experiments. Whenever available, data on human metabolism are included. An overview of the main steps in β-carotene metabolism is shown in **Figure 2**.

Carotenoid absorption

After consumption of β-carotene-containing foods, β-carotene is released by the action of digestive enzymes and solubilized with bile salts. β-Carotene becomes incorporated in micellar particles, which cross the unstirred water layer, after which β-carotene is absorbed.

β-Carotene absorption is affected by the conditions in the intestinal lumen, such as the pH and the presence of bile salts and fatty acids. Bile salts are essential for β-carotene absorption, also in the presence of other emulsifiers, indicating that they have a function apart from that as emulsifier[48]. Bile may stimulate β-carotene absorption by means of an interaction with the membrane of intestinal mucosal cells[49]. β-Carotene absorption, measured in everted gut sacs of the proximal third of the rat intestine, has been reported to be maximal at the critical micellar concentration of bile salts (8 - 10 mM)[50]. Using intestinal loops, an increase of the pH from 5.3 to 7.4 or 8.3 was found to decrease absorption by 11 and 27%, respectively. Lowering the pH possibly decreases the negative surface charges of both the micellar particles and the luminal cell membrane, decreasing the resistance for diffusion[51]. Fatty acids were found to increase β-carotene absorption in the presence of taurocholate by a factor of 1.06 to 1.64, depending on the fatty acid used[52]. Addition of mainly polyunsaturated fatty acids expands the micelles to mixed micelles, which can contain more carotene. However, expansion of the micelles would result in a slower diffusion toward the absorptive cell membrane and thus in lower absorption. Thus, the mechanism of the increased β-carotene absorption is still unresolved. The conditions in the intestinal lumen may be affected by the meal composition. Effects of meal composition, food matrix and other factors on absorption will be described later.

Absorption is believed to occur by passive diffusion, as was shown for rat using intestinal loops[52] and a small intestinal cell line (hBRIE 380)[53]. Absorptio by passive diffusion is determined by a concentration difference, and thus by th luminal concentration on the one hand, mainly determined by the dose, and th intracellular concentration on the other. The concentration in the enterocyte i determined by the rate of disappearance of carotenoids, either by incorporatio in chylomicrons or by cleavage. The mechanism of intracellular transport c carotenoids and of incorporation in chylomicrons are still unknown. Hollande and Ruble[52] suggest that the fatty acid binding protein (FABP), responsible fc transfer of fatty acids from the lipid cell membrane to the intracellula organelles, also transports carotenoids. This may be possible, since FABP ha been shown to have greater binding affinity for fatty acids with greater numbe of unsaturated sites or with longer chain lengths[54].

After uptake of β-carotene in the intestinal cell, some may be lost due to th fast renewal of the intestinal epithelia, and only its appearance in the lymph i 'proof' of absorption. In rats, after a single oral dose of 100 µg of β-caroten labelled with ^{14}C, 7% of the label (2 - 17%) was recovered in the lymph[5 whereas Lakshman et al.[56], after a gastric infusion of 200 µg over 1 h, reporte an appearance in lymph of only 3.6%. Absorption in humans has been studie giving 4 lymph-cannulated patients an oral dose (0.05 to 1.3 mg) of radioactivel labelled β-carotene[57,58]. Reported absorption (as a percentage of th administered radioactivity recovered in lymph) was 8.7, 14.6, 16.8 and 52.3% respectively. The latter high absorption was considered an exception, possibl caused by a metabolic disorder.

Reported apparent absorption of β-carotene, obtained using balance methoc (i.e. the difference between β-carotene intake and faecal β-carotene content was high and varied from 21 to 100%[59-62]. The level of apparent absorptio depended on the source of β-carotene, but was not in agreement with live vitamin A storage from the same sources[61,62]. Overestimation of absorption usin the balance method may result from gastric or bacterial degradation of th carotenoids. Nageswara Rao and Narasinga Rao[60] found indications for this, i.e extra pigment bands on their columns, not found in any of the carotene source which might have been of bacterial origin.

Using a modification of the balance method, i.e. the total gut wash-ou method in which the gut is washed just before and about 20 hours after carotenoid dose, β-carotene absorption amounted from 17% when given withou

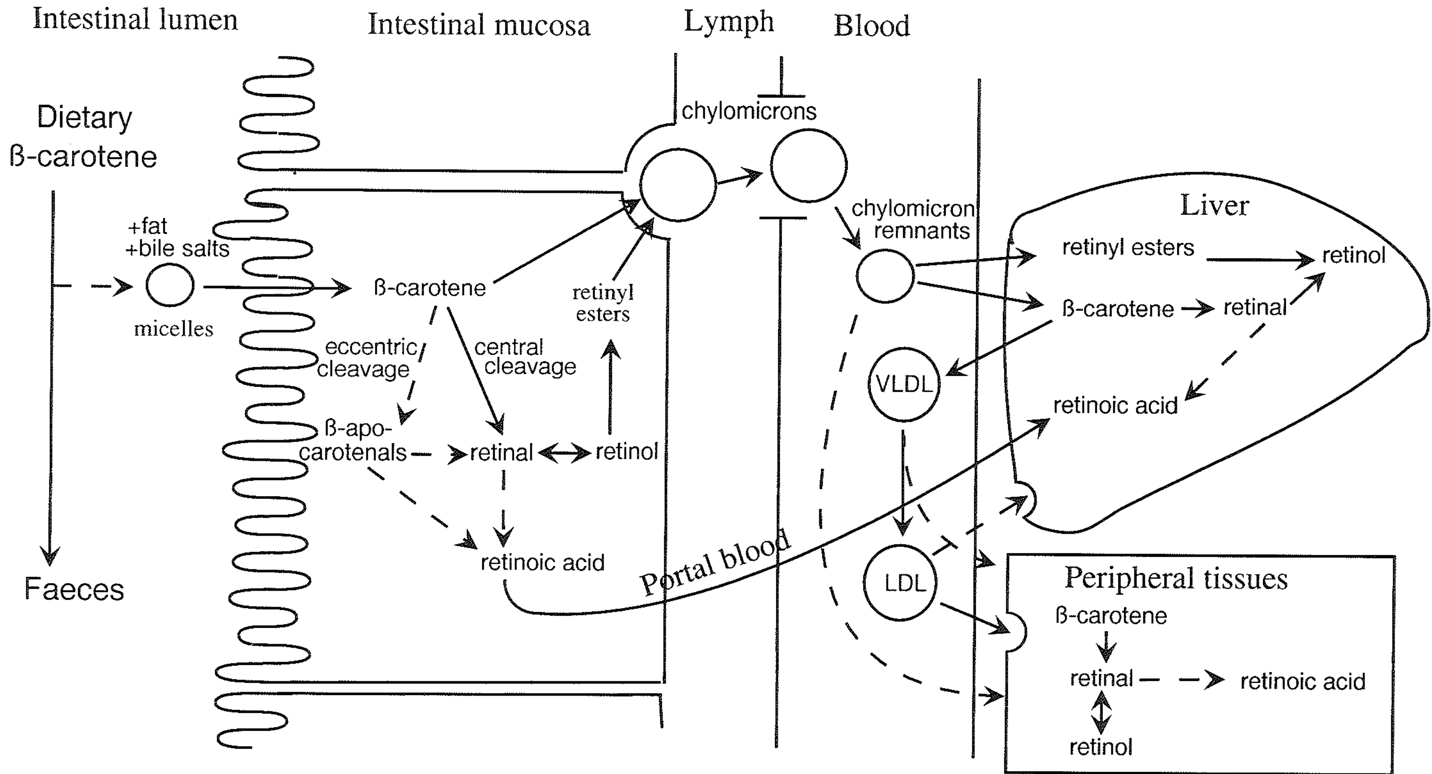


Figure 2. Overview of the main pathways for β-carotene metabolism. ——>, major metabolic pathway; ------>, minor metabolic pathway.

a meal to 29 - 47% when given with a meal[63]. Overestimation of absorption may be less because of the shorter residence time in the gut.

Quantitative data on the absorption of other carotenoids or isomers are lacking. Levin and Mokady[64] reported an increased incorporation of total β-carotene into mixed micelles *in vitro* with increasing levels of the 9-*cis* isomer indicating that in the presence of this isomer increased absorption may occur.

Carotenoid cleavage in intestinal mucosa

After uptake in the enterocyte, β-carotene can be cleaved enzymatically to form retinoids and possibly other products. This section describes the proportion of absorbed β-carotene that is converted and the possible cleavage products formed.

In vivo studies

After Moore[16] had demonstrated, in 1930, that feeding of carotene to rats leads to deposition of vitamin A in the liver, it was widely believed that conversion takes place in the liver. However, in spite of several attempts, cleavage activity could not be demonstrated in the liver. The more efficient conversion of β-carotene when given orally compared to intravenously or intraperitoneally administration eventually made a number of workers conclude that the small intestine must be the main site of conversion. This early work has been reviewed by Glover[65] and Ganguly and Sastry[66].

The chemical structures of β-carotene and vitamin A led Karrer et al.[67] to suggest that the simple addition of two molecules of water at the central double bond of the carotene molecule should give rise to two molecules of vitamin A. Later, two *in vivo* studies proved that molecular oxygen rather than water was needed for the cleavage of β-carotene. Goodman et al.[68], using lymph-cannulated rats and a mixture of β-carotene uniformly labelled with ^{14}C and β-carotene labelled at the central double bond with ^{3}H, demonstrated that during the process of cleavage the hydrogen atoms attached to the central carbon atoms of the β-carotene molecule are not lost. Vartapetyan et al.[69] demonstrated that ^{18}O from molecular oxygen, and not from water, was incorporated into the liver retinyl esters when vitamin A-deficient rats were fed β-carotene. The proposed mechanism for the cleavage of β-carotene at the central double bond is shown in **Figure 3**.

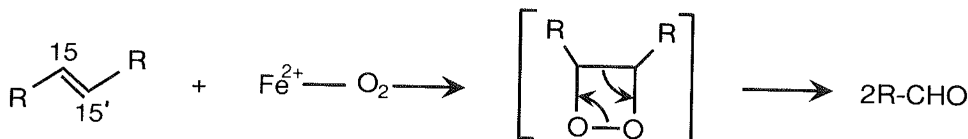


Figure 3. Proposed mechanism for the cleavage of β-carotene at the central double bond. R denotes the C-19 substituent adjacent to the central double bond.

Studies in lymph-cannulated rats showed that about 80% of absorbed β-carotene is converted into retinyl esters[55,56]. However, the former study reported that part of the radioactivity was present in the retinal fraction (4 - 8%), the retinol fraction (3 - 6%), the fraction with more polar compounds and acids (3 - 6%), and only 2% in the β-carotene fraction, while the latter study reported 20% to be present as β-carotene. Using ferrets, Wang et al.[70] reported retinyl esters, retinol and β-carotene to be responsible for 63%, 9% and 10%, respectively, of radioactivity recovered in the lymph after a 4 h perfusion. Remarkably, 90% of absorbed radioactivity was calculated to be absorbed through the portal system, of which 36% as retinyl esters and appreciable amounts of more polar compounds and glucuronides as well as small amounts of β-apocarotenals, retinol and retinoic acid.

In the lymph of the cannulated patients mentioned in the section on absorption, the distribution of label recovered in the various lymph fractions was: retinyl esters 68 - 88%, retinal 2 - 6%, retinol 2 - 9%, and β-carotene 2 - 28%[58]. In the subject with the high absorption rate of 52%, only 10% was found to be present in the retinoid fractions, suggesting a metabolic disorder.

In conclusion, retinyl esters seem the main β-carotene cleavage product *in vivo* and amounts of absorbed β-carotene varying from 2 to 28% are transported intact.

In vitro studies

Intact cell systems

Olson[48] in 1961 was the first to demonstrate unequivocally that radiolabelled β-carotene was converted to retinol. After injection of ^{14}C-β-carotene in intestinal loops of living rats he demonstrated the presence of labelled retinyl esters and small amounts of retinal, retinol and acidic compounds in the intestinal wall.

β-Carotene cleavage has also been studied in intestinal cell lines. Using a rat intestinal epithelial cell line (hBRIE 380), Scita et al.[53] reported conversion of

β-carotene into retinol and retinoic acid of 17% and 5% of the amount absorbe by the cells, respectively. Quick and Ong[71] reported the formation of retiny esters from β-carotene, albeit in very small amounts, in Caco-2 cells (a huma colon carcinoma cell line that spontaneously undergoes enterocyte-lik differentiation in culture).

Bile salts were found to be essential for β-carotene cleavage measured i everted gut sacs of the rat[50], or in intestinal slices[49]. Bile salts probably promot the accessibility of the highly water-insoluble substrate to the water-solubl enzyme and, in addition, may directly affect the cleavage enzyme.

Intestinal mucosal homogenates

In 1965, Goodman and colleagues[72] and Olson and Hayaishi[73] independentl reported that ^{14}C-retinal is formed when ^{14}C-β-carotene is incubated with th 104,000 *g* supernatant of an homogenate of rat intestinal mucosa in th presence of oxygen. A central cleavage mechanism was proposed and later th responsible enzyme was called β-carotene 15,15′-dioxygenase (EC 1.13.11.21 Subsequent communications described partial purification of the enzyme, but because of lability during purification, the pure enzyme has still not bee isolated. The enzyme is known to be cytosolic, requires molecular oxygen and i inhibited by sulphydryl-binding and iron-binding reagents. However, no genera agreement exists as to the reaction products and the mechanism of cleavage.

Table 1 gives an overview of reports on the *in vitro* β-carotene cleavag assay with rat or rabbit intestinal mucosal preparations. Cleavage activities ar not shown in this table since it was impossible to express activity in a unifor manner. Activity may differ among breeds as was shown by Villard and Bates[80] who found a 2.3-fold higher activity in Norwegian hooded rats than in Spragu Dawley rats.

Most studies mentioned in Table 1 report retinal as sole or main product Exceptions were the studies of Hansen and Maret[81], who could not demonstrat any enzymatic cleavage, Napoli and Race[82], who reported the formation o retinol and retinoic acid, and Wang and Tang and colleagues[84,85], who reporte the formation of mainly β-apo-13-carotenone and β-apocarotenals.

Comparing the enzyme preparations used, it can be seen that, except fo Wang and Tang, who used a 800 *g* supernatant, all studies with rats use cytosol or partially purified cytosol as enzyme source and the studies wit rabbits used various supernatant fractions after partial purification. In mos studies an assay pH of 7.7/7.8 is used, as Goodman et al.[74] reported maxima

retinal formation at this pH. Only Napoli and Race[82] and Wang and Tang[84,85] used a lower pH, i.e. 7.0 and 7.35, respectively. Although buffer compositions varied, similar functional additions were made. In all studies a sulphydryl-protecting agent was added, either glutathione, dithiotreitol or cysteine. To solubilize the fat-soluble β-carotene in the aqueous incubation mixture, Goodman et al.[74] reported solubilizers such as sodium dodecyl sulphate, phosphatidylcholine or bile salts to be essential, whereas Olson and Hayaishi[73] added β-carotene in an emulsified form and reported no effect of bile salt addition. Both methods have been used successfully in subsequent studies. The buffer composition and solubilizers used probably only affect cleavage rate.

The method used by Hansen and Maret[81] was comparable with methods reported by others, leaving the absence of cleavage activity unexplained. For some reason retinal may have been formed, but it was unstable, or the enzyme preparation used was inactive. In most studies no special precautions were taken to prevent enzyme destruction during isolation except that all handlings were carried out between 0 and 4°C. Only some studies used a sulphydryl-protecting agent such as cysteine[73] or DTT[83,86], and only Ershov et al.[86] reported the use of a digestive enzyme inhibitor (soybean trypsin inhibitor). A possible explanation for the eccentric cleavage reported only by Wang and Tang and colleagues[84,85] may be the presence of eccentric cleavage enzyme(s) not present in the enzyme preparations used by others. Another possibility is that apocarotenals were formed in the other assays, but were directly converted into retinal, while this second step was very slow in the assay of Wang et al.[84]. This seems unlikely because retinal formation was reported to be linear with time and no lag-time was seen[74]. The lower pH in the study of Wang et al.[84] cannot explain the low retinal formation, since Goodman et al.[74] reported still about 70% of maximum retinal formation at pH 7.35. Finally, the apocarotenals might have been formed by the action of an oxidant in the enzyme preparation. However, addition of antioxidants had no effect on the formation of metabolites[85].

When comparing the results reported by the various groups, we should realize that the presence of other enzymes is of importance as these may convert the initially formed product. Some characteristics of intestinal enzymes reported to convert retinal are given in **Table 2**. Cellular retinol-binding protein type II (CRBP II), an abundant cytosolic protein of the intestine, appears to play an important role in the further metabolism of retinal[87]. After reduction to

Table 1. Overview of reports on the β-carotene-cleavage assay using intestinal preparations of rat or rabbit[a].

Species, m/f Breed	Enzyme preparation[b]	Incubation buffer Buffer[c] pH	 Components[d]	β-carotene Solvent	 Radioactive label, +/-	Products identified (%)[e]	Reference
Rat, m SD	F_{20}^{45} of S104	0.1 KPi 7.7	PPC, GC GSH, α-TP NAD	acetone	+	retinal (100)	72 74 75
Rat, m+f Wistar	S105	0.15 Tris 8.0	- cysteine NAD	20% Tween 40 in acetone+Tris	+	retinal (most) retinol (?)	73
Rat, m Wistar	S105	0.1 KPi 7.7	TC GSH -	acetone	+	retinal (87)[f] retinol (7) retinoic acid (7)	76
Rabbit	F_{25}^{50} of S43	0.1 KPi 7.8	SDS GSH -	acetone	-	retinal (100)	77
Rat, m+f SD	S96	1.0 KPi 7.7	SDS, PPC GSH, α-TP $MgCl_2$, NAD	acetone	+	retinal (100)	78
Rabbit	S20+purification	0.04 Tris 7.8	- GSH $Fe(NH_4)_2(SO_4)_2$	Tween + H_2O	-	retinal (100)	79
Rat, f SD/Norw. hooded	S104	0.1 KPi 7.7	SDS, PPC, GSH, α-TP, $MgCl_2$, NAD	acetone	+	retinal (most) retinol (?)	80

Rat, m+f SD	F_{20}^{45} of S104	0.1 KPi 7.7	PPC, GC, GSH, α-TP NAD	acetone	-	no	81
Rat, m SD	S104	0.02 Hepes 7.0	- DTT NAD, KCl	DMSO	-	retinol (75) retinoic acid (25)	82
Rabbit Rat, m Wistar-Furth	S100+purification	0.1 KPi 7.8	- GSH NAD, $FeSO_4$	Tween + H_2O	+	retinal (100)	83
Rat, m SD	S0.8	0.02 Hepes 7.35	- DTT NAD, KCl	propylene glycol	-	β-apo-13-carotenone (57) β-apocarotenals (39) retinal (2) retinoic acid (1)	84 85
Rabbit	F_{0}^{60} of S22	0.1 KPi 7.8	- GSH / DTT $FeSO_4$	Tween 20 + KPi		retinal (100)	86

[a] Only the standard method described in a study is given.
[b] Fraction of the mucosal homogenate used: S104, 104,000 *g* supernatant; F_{20}^{45}, fraction obtained after ammonium sulphate precipitation between 20 and 45% saturation.
[c] Concentration in mol/L and type of buffer: KPi, potassium phosphate buffer.
[d] Solubilizers: GC, glycocholate; TC, taurocholate; PPC, phosphatidylcholine; SDS, sodium dodecyl sulphate. Antioxidants: GSH, glutathione; DTT, dithiothreitol; α-TP, α-tocopherol. Other: NAD, nicotinamide.
[e] Percentage of products formed in mol.
[f] In the presence of NADH, retinal, retinol and retinoic acid were 36, 9 and 55% of the products formed, respectively.

retinol, CRBP II-bound retinol can be esterified by microsomal lecithin-retinc acyltransferase (LRAT), while unbound retinol can be esterified by microsoma acyl CoA-retinol acyltransferase (ARAT)[90].

Table 2. Proposed intestinal enzymes with retinal as substrate.

Enzyme	Substrate	Optimum pH	K_m (μM)	Cofactor	Product	Reference
Microsomal retinal reductase	retinal-CRBP II retinal	5.0-6.0	0.5 0.8	NAD(P)H	retinol-CRBP II retinol	87
Cytosolic retinal reductase	retinal	6.3	20	NAD(P)H	retinol	88
Cytosolic retinal oxidizing enzyme	retinal	7.7	300	NAD	retinoic acid	89

Only Lakshman and colleagues[83] used an enzyme preparation devoid of retina reductase and oxidase activity, while in all other studies these enzymes migh have been present. Precipitation of the cytosol with ammonium sulphat between 20% and 45% saturation may result in the loss of most accompanyin enzymes; only cytosolic retinal reductase is most likely not lost as it is purifie with a similar method[88].

In view of the enzyme characteristics given in Table 2, using cytosol a enzyme source, one might speculate that only retinal not bound to CRBP I might be converted to retinol or retinoic acid. However, Napoli and Race[8] reported the formation of retinol and retinoic acid, but not retinal Alternatively, retinoic acid might have been formed through eccentri cleavage[91], possibly through apocarotenoic acids[92]. The formation of retinol, bu not retinal in the presence of NAD, suggests the presence of an unknow enzymatic activity.

An interesting, but unexplained observation was made by Crain et al.[76], wh showed retinoic acid as the main product in the presence of NADH, wherea retinal was the main product without added NADH.

Most studies have been performed with β-carotene as the substrate, bu other provitamin A carotenoids may be converted as well in the assay. Singl and Cama[79] reported the conversion of α-carotene into equal amounts of retina and α-retinal, and of β-cryptoxanthin in retinal and hydroxy-retinal, wit activities relative to β-carotene of 56 and 3%, respectively.

Recently, the conversion of β-carotene isomers was reported. Nagao and Olson[93] showed the conversion of 9-*cis*-β-carotene by an intestinal preparation of the rat into all-*trans*-retinal (50%), 9-*cis*-retinal (31%) and 13-*cis*-retinal (19%), with a relative activity of only 6.8% compared to all-*trans*-β-carotene. Subsequent conversion of *cis*-retinal to retinol, when first bound to CRBP II, may be limited when the binding affinity of the *cis* isomer is lower, as has been reported for retinol isomers[94].

Wang et al.[95], using human intestinal preparations, reported the conversion of 9-*cis*-β-carotene into equal amounts of all-*trans*- and 9-*cis*-retinoic acid, with a cleavage rate of about half that of conversion of all-*trans*-β-carotene into all-*trans*-retinoic acid.

Mechanism of cleavage: central or eccentric?

The chemical structures of vitamin A and β-carotene would suggest central cleavage of β-carotene into two molecules of vitamin A. However, already in 1937, the finding of Holmes and Corbet[96] that, on a weight basis, vitamin A is twice as active as β-carotene suggested that either central cleavage was not the only pathway or half of the β-carotene was completely degraded, possibly by a different oxidative enzyme.

Zechmeister et al.[97] already suggested that, because of resonance stabilization, the centrally located double bond of a conjugated system should be more stable than the terminal ones, which would imply that terminal attack should be preferred to central fission. Chemical fission experiments carried out with various oxidizing agents showed that β-apocarotenals were indeed the major product (as reviewed by Ganguly and Sastry[66]).

In 1960, Glover[65] discussed various pathways for retinal formation including oxidation from one side of the molecule. Based on the metabolism (mainly *in vivo*) of possible intermediates, he concluded that oxidation from one side was not the main cleavage pathway. However, biological vitamin A activity *in vivo* was found for possible intermediates such as apocarotenals[65], as was also shown by Sharma et al.[92]. Conversion of β-apocarotenals has also been demonstrated in the *in vitro* assay, with relative activities either lower[79] or higher[77] than for β-carotene.

As shown in Table 1, most studies with the dioxygenase assay with β-carotene as the substrate found retinal as main product. However, information on the stoichiometry of the reaction is limited. When cleavage is

eccentric the molar ratio of retinal formed to β-carotene consumed should be 1 whereas central cleavage should result in a ratio of 2 (or at least greate than 1). Olson[98] reported an average molar ratio of 1.2 with a range of 0.9 to 1.8 Goodman and Huang[74], using ^{14}C-β-carotene, stated that most of th reaction-product retinal must have arisen by central cleavage of the substrat β-carotene into two molecules of retinal, but this is only based on th radioactivity recovered in the retinal fraction eluted from the column, withou further purification or identification. Singh and Cama[79] stated that the reactio was strictly stoichiometric and that 2 mol of retinal were formed upo utilization of 1 mol of β-carotene. However, no details of their results are give Liver vitamin A storage after *in vivo* administration of β-carotene depends o too many other factors to draw any conclusion on the stoichiometry of th conversion.

In conclusion, the available information on the stoichiometry is limited an does not allow a definitive conclusion on the type of cleavage, although retina remains to be the main reaction product. The demonstration of apocarotenals i *in vitro*[84,85] and *in vivo* studies[70,99] leaves the possibility of eccentric cleavage a well.

Postabsorptive transport and metabolism of carotenoids

Postabsorptive transport

In the enterocyte β-carotene and most of its cleavage products are incorporate in chylomicrons and transported via the lymph to the blood. In the bloodstrea chylomicrons undergo lipolysis, catalysed by lipoprotein lipase, giving rise t chylomicron remnants, which are cleared from the plasma by the liver.

From the liver, carotenoids can be resecreted with lipoproteins for transpo to other tissues. The kinetics of β-carotene among various lipoprotein fraction after administration of an oral dose of β-carotene in humans were studied b Johnson and Russell[100]. Hepatic resecretion of β-carotene seemed to occur b very-low-density lipoproteins (VLDL), which are transformed to low-densit lipoproteins (LDL) by delipidation. Because of the large difference in residenc time between VLDL and LDL (< 1 h and 2 - 2.5 d[100]), β-carotene in plasma i mainly present in LDL. LDL have been reported to contain 67 - 79% of plasm β-carotene, while 8 - 22% is associated with high-density lipoprotein (HDL)[101-103]. HDL containing β-carotene may directly originate from th

intestine where small amounts of HDL are produced, or originate from the liver[100]. The plasma half-life of β-carotene is about 5 days[104,105].

The plasma response of β-carotene in humans after a single oral dose was found to be largely variable[100,104,106], and Johnson and Russell[100] even suggested the existence of 'responders' and 'non-responders'. After a single oral dose of 120 mg β-carotene no plasma β-carotene response could be demonstrated in 7 of the 11 volunteers.

Plasma responses after single doses of other carotenoids have not been determined in humans because no preparations appropriate for human consumption are available and absorption from natural sources seems rather low[107]. In preruminant calves the plasma responses to 20 mg doses of α-carotene, β-carotene, lycopene and lutein have been compared[108]. Peak times in serum were 12 h for lutein, 16 h for lycopene and 24 h for α- and β-carotene, suggesting a relation with the polarity of the carotenoid. The polarity may be a determinant for the distribution of carotenoids over the various lipoproteins. In human plasma, lycopene, α-carotene and β-cryptoxanthin are mainly present in LDL (73, 58 and 42%, respectively, compared with 67% for β-carotene), whereas lutein is mainly associated with HDL (53%)[102]. Because of its non-polarity β-carotene is expected to be located in the core of lipoproteins and not to exchange easily to other lipoproteins[109], while more polar carotenoids may be located on the surface of lipoproteins, enabling exchange with other particles as has been reported for vitamin E[103].

Apart from the above described main transportation route for β-carotene and its cleavage products, some minor routes may exist. The more polar metabolites, such as retinoic acid, are most likely absorbed through the portal system. Although this pathway was found to be the major route in intestinal perfusion experiments with β-carotene in ferrets (also for retinyl esters)[70], for rats the lymph route has been found to be the most important one[55].

Although chylomicron remnants are removed from the circulation mainly by the liver, Hussain et al.[110] reported the uptake of small amounts by other tissues such as spleen, bone marrow and adipose tissue in rats and guinea pigs, whereas in rabbits and primates (marmoset) even 20 - 50% of chylomicron lipid and retinyl ester was removed by the bone marrow. Recent findings of Blaner et al.[111] that lipoprotein lipase can hydrolyse retinyl esters *in vitro* suggest that *in vivo* small amounts of retinyl esters in chylomicrons may be hydrolysed and not taken up by the liver. Wolf[112] suggests direct uptake by adipose tissue.

Apart from in plasma, carotenoids have also been demonstrated in bloo cells. Norkus et al.[113] reported a positive relationship between platelet an plasma levels of lutein, lycopene, α-carotene, β-carotene and cryptoxanthin. I red blood cells they only found lutein and cryptoxanthin in part, but not all, c the samples. However, in earlier studies the presence of β-carotene in red bloo cells was reported and found to increase upon supplementation[109].

Tissue distribution

Since β-carotene in plasma is mainly present in LDL, uptake in tissues might b mediated via LDL receptors. Tissue distribution of β-carotene in rat[114] is indee in agreement with total LDL clearance in rat tissues[115]. The liver is the mai storage tissue, but high concentrations have also been demonstrated in adrena gland, spleen and ovary. In contrast to rats, humans store large amounts c β-carotene in adipose tissue, while distribution over the other tissues seem comparable with rats[116]. If we assume that reported β-carotene concentration for subcutaneous and abdominal adipose tissue are representative of total bod adipose tissue, then liver and adipose tissue together are responsible fo 85 - 95% of β-carotene storage in humans[116,117]. The concentration of β-caroten in subcutaneous adipose tissue was reported to correlate rather well with th plasma concentration and a similar increase in adipose tissue and plasm β-carotene was found after supplementation with β-carotene for 6 months[11 Generally, the adipose tissue concentration of β-carotene is assumed to be long-term indicator for β-carotene intake whereas the plasma concentratio appears to be the short-term indicator.

Also α-carotene, lycopene, cryptoxanthin and zeaxanthin were demonstrate in high concentrations in the adrenals, as for β-carotene, while testes, live ovary and pancreas also contained relatively high concentrations[116]. Howeve some differences in distribution between the various carotenoids were found storage in fat tissue was found to be lowest for β-carotene and highest fo zeaxanthin, while the opposite was found for the liver. Whether thes differences may be explained by differences in distribution over lipoprotein remains to be established.

Isomers of β-carotene were also demonstrated in human tissues. The 9-*ci* isomer and the 13-*cis* plus15-*cis* isomers accounted each for 10 - 20% of tissu β-carotene, whereas they were either absent or only present in very smal amounts in plasma[119]. No increase in the plasma concentration of the 9-*ci*

isomer was found after either a large single dose of a 9-*cis* rich β-carotene algae preparation[120] or after a 7-day supplementation[121]. After a single oral dose of ^{13}C-9-*cis*-β-carotene (99.4% pure) administered to 2 subjects You et al.[122] measured in plasma labelled all-*trans*-retinol, 9-*cis*- and all-*trans*-β-carotene, with the 9-*cis* isomer accounting for only 9% (subject 1) or 40% (subject 2) of total labelled β-carotene. The authors suggested isomerization to take place in the intestine. This means, however, that isomerization must also occur in the tissues. Alternatively, isomers are preferentially cleared from the plasma and the retinol found may have been formed directly from the 9-*cis* isomer.

Extra-intestinal cleavage

β-Carotene that escaped conversion in the intestine may be cleaved in other tissues. The first indication for extra-intestinal cleavage was the presence of small amounts of vitamin A in tissues after parenteral, intravenous or intramuscular administration of β-carotene in animals from which the intestine or other tissues had been removed[65,66]. Later on, cleavage activity was demonstrated in several tissues using the *in vitro* β-carotene cleavage assay. Olson and Hayashi[73] reported the formation of retinal and retinol in liver preparations. Napoli and Race[82] reported the formation of retinol and retinoic acid, with liver, lung, kidney and testes preparations, like in the intestine, although the proportions of both products varied. Wang et al.[84] demonstrated cleavage activity in liver, lung, kidney and fat tissue of rats, ferrets and monkeys as well as in human adipose tissue.

Extra-intestinal β-carotene cleavage may especially be of relevance to compensate for 'local' vitamin A deficiencies induced, for instance, by carcinogens[123] or by hampered retinol transport as may occur in protein malnutrition[124].

Methods and models for studying β-carotene absorption and cleavage

Traditionally most research of β-carotene metabolism has been carried out animals, mainly by determination of liver vitamin A stores after feedi carotenoids, while human studies have been limited. Although it is realized th β-carotene metabolism in an animal may differ from that in humans, anim models are used because metabolic studies in humans suffer from a lot restrictions from both ethical and technical points of view. In this section t various animal models are discussed and results compared with availal information from human studies. In addition, methods in use in humans a evaluated.

Animal models

Animal species differ markedly in their ability to absorb carotenoids and convert provitamin A carotenoids to retinol. Exclusive carnivores consume diet containing pre-formed retinol and thus do not depend on carotenoids their vitamin A supply. They may even lack the ability to absorb or conv carotenoids as has been reported for the cat[125]. On the other hand, str herbivores are dependent on carotenoids for their vitamin A and are expected absorb and convert provitamin A carotenoids to retinol efficiently. But, ev within this group large differences in efficiency exist. 'Yellow-fat' animals su as humans and cows accumulate carotenoids, whereas 'white-fat' animals, su as rabbits, guinea pigs and sheep, do not readily store carotenoids. Therefo an overview is given of available information on β-carotene metabolism species commonly used for β-carotene research and species recently suggest as a model for β-carotene metabolism in humans.

Primates other than man

Krinsky and colleagues[126] showed that when a single dose of β-carotene label with ^{14}C was fed to rhesus monkeys (*Macaca mulatta*), after 72 h most of t absorbed radioactivity was stored in the liver, 85 - 95% as retinol and reti esters and 2 - 8% as β-carotene. Only very small amounts of radioactivity w detected in the other organs.

Rat

The rat is an efficient β-carotene converter which can accumulate carotenoids to a limited extent, only after being fed high doses. As already described under metabolism, after a single dose of β-carotene 2 - 8% was recovered in the lymph, mainly as retinyl esters with some intact β-carotene (2%)[55]. However, Lakshman et al.[56] reported a higher absorption of intact β-carotene. Accumulation of radioactive label in the liver after a single radioactive labelled dose of β-carotene also showed nearly complete cleavage of β-carotene (up to 6% of the label was present as β-carotene)[126,127].

Even after long-term supplementation with β-carotene no β-carotene or only very small amounts could be demonstrated in liver and plasma, whereas liver vitamin A storage is strongly increased[128-131]. After supplementation for 10 weeks with high doses of β-carotene, tissue distribution could be determined and showed that the liver contained about 90% of body β-carotene[114,126]. As already mentioned, tissue distribution of β-carotene seems similar to distribution in man, except that in man adipose tissue is a very important storage site, whereas in rat β-carotene could not be demonstrated in perirenal fat[114], abdominal fat[132] or adipose tissue (not further specified)[133].

Chicken

Carotenoids, mainly oxycarotenoids, are responsible for the colours of plumage and egg yolk of birds. Chickens have been found to be selective absorbers of carotenoids; they do not absorb cryptoxanthin, while they absorb lutein in the duodenal and upper jejunal region and zeacarotene only in the ileal region[134]. This suggests the existence of a regulatory mechanism for carotenoid absorption in chickens, which may or may not include β-carotene.

The chicken is an efficient β-carotene converter; after feeding β-carotene large increases of vitamin A stores in the liver were seen[135,136], while only small amounts of β-carotene could be demonstrated in the liver[135,136], but not in lungs, kidney or perirenal fat[137], and only in one study in plasma[136]. However, in laying hens on a farm diet (rich in carotenoids) β-carotene was demonstrated in plasma, liver, ovary and body fat[135]. Poor and coworkers[137] suggest that the chick liver has no long-term β-carotene storage role, but is only a site of accumulation since in their study β-carotene accumulation and depletion occurred within 2 days.

Ferret

β-Carotene absorption and cleavage was studied by Wang et al.[70] in intestin
perfused ferrets. During the 4-h perfusion period 0.3% of the radioactive lab
was recovered in the lymph of which 10% was present as intact β-carotene ar
63% as retinyl esters. Based on portal blood samples, a calculated amount
2.7% of the radio label was absorbed through the portal vein, of which 36% wer
retinyl esters. In feeding studies the percentage of β-carotene absorbed inta
seem to be higher, since β-carotene was demonstrated in most tissues after
single dose of 10 - 13 mg β-carotene (10 mg/kg body wt)[138,139]. After suppleme
tation for 3 weeks β-carotene was demonstrated in various tissues, includir
adipose tissue. The liver was the main storage site, while a high concentratio
was found in the adrenals[140]. However, no significant increases in tissue retin
ester levels could be demonstrated after supplementation[140,141]. This may part
be due to relatively high levels of retinyl esters in ferret tissues.

Preruminant calf

The study of carotenoid metabolism in ruminants has a long history, since the
strict herbivores are dependent on carotenoids for their vitamin A provisio
Around 1950, a number of studies with calves showed that feeding the
β-carotene led to a dose-dependent accumulation of both intact β-carotene ar
vitamin A in the liver[142,143], while plasma β-carotene also increased[142-144].

Recently, the preruminant calf (a new-born calf maintained in a monogastr
state by feeding an all-liquid diet containing no rumen micro-organisms) wa
suggested as a model for carotenoid absorption and metabolism in human
Bierer et al.[145] reported serum kinetics of β-carotene after a single oral dose
20 mg to be very similar to that in humans, except that HDL is the maj
lipoprotein fraction in the blood of calves. Tissue analyses after a single do
showed the highest concentrations in liver and adrenals and an intermedia
concentration in the spleen, while β-carotene was also demonstrated in adipo
tissue[146].

Other species

<u>Rodents other than rat</u>

Hamsters fed different levels of β-carotene had only very small amounts
β-carotene in the liver, while rather large increases in liver vitamin A stora
were seen, indicating that they are efficient converters[147]. Both mice and guin

pigs supplemented for 3 months with large amounts of β-carotene were reported to accumulate β-carotene in several tissues, although in largely varying amounts[148]. In the guinea pigs, but not in the mice, liver vitamin A storage was strongly increased[148]. However, Jones et al.[149] did demonstrate an increase of liver vitamin A upon β-carotene feeding in mice.

Rabbit

Although rabbit intestine has often been used in the dioxygenase assay[77,79,83,86], information on *in vivo* β-carotene metabolism in rabbits is scarce. No carotenoids could be detected in plasma of rabbits on a carotenoid-rich diet[143], whereas large amounts of β-carotene had to be fed to significantly increase (2.3-fold) the retinyl ester concentration in the liver[150], indicating that absorption is low in rabbits.

Gerbil

Recently, Pollack and colleagues[151] fed Mongolian gerbils (*Meriones unguiculatus*) a physiological dose (0.15 mg) of β-carotene in a test meal. Serum β-carotene reached a maximum value 4 h after the meal. Before the test dose β-carotene was already demonstrated in liver, spleen, kidney plus adrenal, perirenal fat and lungs. Only the liver content increased significantly after the test dose.

Pig

In general, pigs are mentioned as a good model for digestion and absorption of food in man; the morphology and physiology of the gastro-intestinal systems are much alike[152] and the pig closely approximates the human in distribution and composition of lipoproteins[153]. However, the pig seems to be rather inefficient in absorption and conversion of β-carotene[137].

Evaluation of various species as a model for humans

Several characteristics of β-carotene metabolism in humans and in the animal species mentioned above are summarized in **Table 3**. The main difference among the species is the efficiency of β-carotene conversion; rats and chicks are efficient converters that accumulate β-carotene only under conditions of very high intake, whereas ferrets and preruminant calves, and possibly also monkeys, like humans, absorb appreciable amounts of intact β-carotene. This difference is not necessarily related to intestinal cleavage activity, since Wang et al.[84], using an *in vitro* β-carotene cleavage assay, reported a 5-fold higher rate of product formation in humans and in monkeys (*Saguinus oedipus*) than in ferrets and rats.

Table 3. Overview of available data on some aspects of β-carotene metabolism in different species[a].

Species	Intestinal absorption (% of dose)	Intestinal cleavage to retinyl esters %	Absorption of intact β-carotene %	Main transport lipoprotein for β-carotene (%)	Conversion factor[b,21]	Plasma retinyl esters[c] (μmol/L)
Human	9 - 17[57,58]	60 - 70[57,58]	20 - 30[57,58]	LDL (58 - 67)[101-103]	4 - 6	0 - 0.34[154]
Monkey (Macaca)	na	85 - 95[126]	2 - 8[126]	na	na	0.08 - 0.24[154]
Rat	2 - 8[55,56]	90 - 99[55] 80[56]	2[55] 20[56]	HDL (ca. 50)[d]	2	na
Chick	na	almost complete[135,137]		na	2	na
Ferret	Lymph: 0.3[70] Portal: 2.7	Lymph: 63[70] Portal: 36	Lymph: 10[70] Portal: -	na	na	18.6 - 36.8[140]
Preruminant calf	na	na, not complete [e]	na[e]	HDL (na)[157]	8 - 10	Low[158]

[a] Numbers in superscript are references; LD, low-density lipoprotein; HDL, high-density lipoprotein; na, no information available.
[b] μg β-carotene equivalent to 1 IU vitamin A.
[c] Fasting levels when on a ‘normal’ diet.
[d] Estimated from the percentage in LDL[156] and the distribution of cholesterol over LDL and HDL[153].
[e] Both conversion and intact absorption occur.

The main difference in tissue distribution among the species is that ferrets and calves, like humans, store β-carotene in adipose tissue, while the rat does not. Humans seem to be unique in transporting β-carotene mainly in the LDL fraction. Monkeys are also expected to transport β-carotene mainly in the HDL fraction; HDL is the main transport protein for cholesterol in monkeys[153], and a positive relationship between cholesterol and β-carotene distribution over LDL and HDL has been found in humans[159].

Ferrets seem to differ from humans in their retinoid metabolism; portal absorption may be important in this species and they have high fasting plasma retinyl ester concentrations and relatively high tissues retinyl ester concentrations.

Finally, it may be important to note that chickens do not have intestinal lymphatics while rats have no gall-bladder. However, the possible implications of these traits for β-carotene metabolism are not clear.

In conclusion, no animal model seems to reflect human β-carotene metabolism perfectly. The gerbil and monkeys seem promising models, but more information on their β-carotene metabolism is needed for a proper evaluation. For study of specific aspects of β-carotene metabolism an animal model may be appropriate; which would be the model of choice depends on the aspect to be studied. Efficient converters, for instance, could be used to study intestinal β-carotene conversion, but are not very useful for studying the extra-intestinal metabolism of β-carotene.

For the other carotenoids the situation may be different. Humans seem to be indiscriminate accumulators of carotenes and oxycarotenoids[116], but carotenoid absorption in chickens may be regulated[134].

Methods applicable to humans

In vitro

The *in vitro* β-carotene cleavage assay has been carried out with human intestinal biopsies[78] and with specimens of intestinal and fat tissue obtained during surgery[84]. With the assay enzyme characteristics can be studied in comparison to other species, but it is not possible to obtain samples before and after an intervention, as can be done with animals.

As mentioned in the section on β-carotene cleavage, cell lines, such as the human Caco-2 cell line, can be used[71].

In vivo

Although lymph-cannulation experiments have been carried out in patients[57,5 studies in healthy volunteers are usually restricted to sampling of blood an faeces. Autopsy samples can be used to study tissue distribution of β-caroten under uncontrolled conditions, as was reported by Kaplan et al.[116] and Schmi et al.[160].

Balance method

In the balance method the apparent absorption is estimated as the differen between β-carotene intake and faecal β-carotene excretion. As mentioned in th section on absorption, this method seems to overestimate absorptio Overestimation may be limited in the total gut wash-out method, but th method seems restricted to use in a clinical setting and is rather unpleasant f the subjects.

Plasma responses

Plasma responses after intake of carotenoids can be useful for the study relative availability of carotenoids.

After a single oral dose both β-carotene and retinyl ester plasma respon curves can be measured. However, because of the relatively high baseline leve of carotenoids the sensitivity of the single-dose method is rather low an relatively high doses have to be used. This approach has frequently bee applied, albeit mostly without measuring retinyl esters[104,106,107,161-164]. The meth is not suitable to study effects on absorption of factors that may also affe plasma β-carotene clearance.

Chronic dosing rather than a single dose has also been used[163-166]. T advantage is that a steady state can be reached, but the method seems le appropriate to study cleavage products.

As can be concluded from most of the studies mentioned, between pers variation in plasma responses of β-carotene is very large, both after a sing dose as well as after long-term supplementation, indicating that both metho can best be used for comparisons within persons.

Chylomicrons

Plasma responses do not allow for distinction between newly absorb carotenoids and those of endogenous origin. To overcome this proble chylomicrons representing only the newly absorbed β-carotene and its cleava product can be isolated. This method has been used in the past, although

these studies cleavage products could not be demonstrated[100] or were not measured[167].

Stable isotopes

Another solution to distinguish between absorbed and endogenous β-carotene is the use of stable isotope-labelled β-carotene. Application of stable isotopes allows the use of physiological doses (ca. 1 mg). Methods for the use of stable isotope-labelled β-carotene have been reported by Parker et al.[168] (^{13}C-labelled β-carotene) and by Dueker et al.[169] (β-carotene-d_8).

Factors affecting β-carotene absorption and cleavage

Food matrix

The plasma β-carotene response has been reported to be about 5 times greater when crystalline β-carotene is given than when carrots are given (both with a meal), both as a single dose[107] and as a daily supplement for 6 weeks[166]. Bulux et al.[170] found not even an increase of plasma β-carotene in children supplemented with 6 mg β-carotene given as cooked carrots for 20 days, while plasma β-carotene increased 3-fold in children supplemented with β-carotene beadlet capsules. The low availability from vegetables is possibly explained by their binding to protein in the food. In green leaves β-carotene is expected to be present as pigment-protein complex in the chloroplast of the cell, while in other fruit or vegetable sources it is most often present in lipid droplets in the chromoplast. However, in carrots α- and β-carotene were reported to be protein-bound[171]. Alternatively, the fibre present may decrease availability.

Softening or disruption of plant cell walls by cooking or grinding is expected to increase bioavailability. However, processing may also lead to isomerization or degradation of β-carotene and thus decrease bioavailability. Khachik et al.[172] demonstrated that β-carotene in broccoli, spinach, green beans and tomatoes was stable during various cooking processes (e.g. microwave oven). However, β-carotene in sweet potato leaves was found to be degraded for 25, 53 or 71% during microwave cooking for 2, 4 or 8 min, respectively[173].

In addition, it is important to realize that the carotenoid content of fruits and vegetables may vary largely because of differences in genotype, ripening or storage conditions[1,174-176]. Furthermore, industrial processing may result in losses and isomerization of carotenoids[177].

Meal composition

Inclusion in a meal

Absorption of β-carotene is generally assumed to be higher when given togeth with a meal because a meal stimulates bile secretion. Shiau et al.[63], using t total gut wash-out method, indeed found about 2-fold higher appare absorption of β-carotene when given with a meal, whereas almost no plasm response was seen when β-carotene was given without a meal. Cornwell et al. demonstrated a higher plasma response when β-carotene was given with meal, either with or without fat.

Fat

Fat is important for micelle formation and thus fat stimulates β-carote absorption. Fat metabolism disorders may result in malabsorption of β-caroten Increases in plasma β-carotene after a 5-day supplementation were mu higher in subjects on a high-fat diet than in subjects on a low-fat diet[165].

The type of fat consumed may also influence β-carotene metabolism. Kasp and Ernst[178] reported higher plasma β-carotene concentrations in voluntee who received several doses of β-carotene in maize oil compared to arachidic olive oil, independent of the vitamin E content of the oil. However, in the sa study, liver vitamin A storage in β-carotene supplemented rats was not affect by the type of oil used. Blakely et al.[179] reported an increase in β-carote storage and a small decrease in vitamin A storage in the livers of rats f β-carotene with maize oil compared to mixed fat. Huang and Goodma collected lymph of rats after a single dose of β-carotene and reported a 2-fo higher absorption when β-carotene was given in olive oil than when it was giv in triolein or trilinolein, but conversion to retinyl esters was lower with olive (80 vs. 93%).

Fat-soluble compounds

When β-carotene is consumed together with other fat-soluble compoun competition might occur in the intestinal lumen at the level of fat emulsificati and incorporation in the micelles. Once absorbed in the enterocyte, competiti for the cleavage enzyme or for incorporation in chylomicrons may occur.

An indication for competition with vitamin E was found by Willet et al.[183]. subjects supplemented with β-carotene, simultaneous supplementation wi vitamin E caused a decrease of plasma total carotenoids to about 80% of t

concentration in subjects only supplemented with β-carotene. However, competition at the level of incorporation in lipoproteins cannot be excluded. From studies with rats, reviewed by Arnrich and Arthur[184], it was concluded that the presence of large amounts of vitamin E in the ingesta interferes with the accessibility of β-carotene to the enzyme. The β-carotene cleavage activity is not affected. Low intakes of vitamin E may enhance utilization of β-carotene, possibly by protecting β-carotene against oxidative destruction.

Other carotenoids may also compete with β-carotene. Already in the 1950s lutein was reported to reduce liver vitamin A stores formed from β-carotene in rats, while lycopene did not[185]. Competition might occur at the cleavage level, as Ershov et al.[186] reported an inhibition of β-carotene conversion in the dioxygenase assay by lutein, but also by lycopene. Recently, the plasma responses after single and combined doses of β-carotene and canthaxanthin were compared in two subjects[187]. In both subjects canthaxanthin had no effect on the β-carotene response, although β-carotene was found to reduce the canthaxanthin response. In ferrets, however, a canthaxanthin dose was found to reduce the response of β-carotene[138].

Other compounds

<u>Fibre</u>

The possible effects of dietary fibre on β-carotene utilization have been summarized[180]. In humans pectin was found to reduce the β-carotene plasma response after a single dose of β-carotene with a meal[161].

<u>Nitrite</u>

Nitrite, either present in the diet or formed from nitrate in the stomach, and sulphite have been suggested to degrade β-carotene in the digestive tract before absorption[181,182]. Whether these effects are important in species other than ruminants is not clear.

<u>Vitamin A</u>

The efficiency of β-carotene conversion may decrease in the presence of vitamin A as was demonstrated by Olson[48] in ligated loops of rat intestine.

Physiological conditions

Age

β-carotene cleavage activity is already present in neonates as was demonstrated by Lakshman et al.[188] using the *in vitro* β-carotene cleavage assay. Reported

effects of older age on β-carotene metabolism are controversial. After a sing
oral dose of β-carotene, Maiani and coworkers[189] reported a higher seru
response in old than in young women, while Sugerman and colleagues
reported a higher response in young than in old men. Since Nierenberg et al.
reported no effect of age on the increase of plasma β-carotene levels after o
year of supplementation, the differences in response to a single dose may
caused by differences in clearance rather than absorption.

Gender

Higher serum levels of β-carotene in women than in men can often be explain
by differences in β-carotene intake, body weight or smoking habits. However,
a study of Stryker et al.[191] non-smoking women had higher plasma β-carote
concentrations than non-smoking men with the same intake. Reinersdorf
demonstrated that men need 0.020 mg β-carotene per kg body weight a
women 0.017 mg to maintain the same plasma β-carotene concentration. Th
difference may be explained by the finding of Kübler[193] that women have a low
elimination rate constant for β-carotene than men.

Nutrient status

In rats a low vitamin A status was found to increase the β-carotene cleava
activity measured *in vitro*[80]. Thus, β-carotene might be used more efficiently
case of a low vitamin A status.

A normal protein intake is required for maximum vitamin A storage
tissues after feeding β-carotene[78]. However, conflicting results have be
reported for the effect of dietary protein on β-carotene conversio
Gronowska-Senger and Wolf[78] reported the highest β-carotene cleavage activi
as measured in the dioxygenase assay, in rats fed a 10% protein diet (the le
of highest protein synthesis), with lower activities in animals fed 5, 20 or 40
protein. Kamath and Arnich[194] fed rats a 10% or 40% protein diet and measur
more cleavage products in both intestine and liver after an injection
^{14}C-β-carotene in the intestine in rats fed the 40% protein diet. Both the prote
'status' and the simultaneous presence of protein and β-carotene in the intesti
were responsible for this effect.

Possibly a low zinc status affects β-carotene conversion.
vitamin A-deficient hens[195] and rats[196] liver vitamin A stores after feedi
β-carotene were lower in zinc-deficient animals than in zinc-sufficient anima

The dioxygenase enzyme may be zinc-dependent as was suggested by Sklan[197] for the chick enzyme. Huber and Gershoff[198] reported that retinal reductase was zinc-dependent in the retina but not in the liver.

Other factors

Medical drugs
Since absorption and, possibly, cleavage of β-carotene is affected by the amount of bile acids in the intestinal lumen, bile-acid sequestering agents may affect β-carotene utilization. Indeed, patients treated with colestipol were found to have 30% lower serum carotenoid levels than untreated controls[199].

Smoking
Smokers were reported to have much lower plasma levels of β-carotene than did non-smokers in spite of their only slightly lower[191] or equal[200] carotenoid intake. One year of supplementation with β-carotene also resulted in lower plasma β-carotene concentrations in smokers compared to non-smokers[190]. A number of possible explanations for this difference have been described by Nierenberg et al.[201]. Over-reporting of dietary carotene intake by smokers is not likely since the discrepancy was not found for lycopene. Alternatively, smoking may destroy β-carotene or may alter metabolism in one of the ways suggested by Nierenberg et al.[201].

Conclusion

Although much work has been done on carotenoid metabolism and factors affecting it, it may be clear from this review that still several questions remain. The mechanism of β-carotene absorption in the enterocyte and of further transport to either chylomicrons or a cleavage enzyme remains to be elucidated. Also, the mechanism of β-carotene cleavage and, in relation to this, the stoichiometry of the cleavage reaction needs further study. In addition, studies into specific dietary factors affecting absorption and cleavage remain important.

There is an urgent need for finding appropriate models to answer these questions. For a number of studies a suitable *in vitro* model should be developed. Effects of dietary factors are best studied in humans, since no animal model truly reflects human β-carotene metabolism. However, studies in humans will always be restricted by ethical and technical limitations. Application of

chylomicron response studies and the use of stable isotopes are complex, bu promising. Animal models provide a useful alternative for specific studies c carotene absorption and cleavage to elucidate mechanisms and pathways.

References

1. Simpson KL and Chichester CO. Metabolism and nutritional significance of carotenoids Annu Rev Nutr 1981;1:351-374.
2. Goodwin TW. Metabolism, nutrition and function of carotenoids. Annu Rev Nutr 1986;6 273-297.
3. Karnaukhov VN. Carotenoids: Recent progress, problems and prospects. Comp Biochem Physiol 1990;95B:1-20.
4. Kläui H and Bauernfeind JC. Carotenoids as food color. In: Bauernfeind JC, ed. Carotenoids as colorants and vitamin A precursors. New York: Academic Press, 1981: 38-319.
5. Bendich A and Olson JA. Biological actions of carotenoids. FASEB J 1989;3:1927-1932.
6. Khachik F, Beecher GR, Goli MB, Lusby WR, Smith JC. Separation and identification o carotenoids and their oxidation products in the extracts of human plasma. Anal Chem 1992;64:2111-2122.
7. Chug-Ahuja JK, Holden JM, Forman MR, Mangels AR, Beecher GR and Lanza E. The development and application of a carotenoid database for fruits, vegetables, and selecte multicomponent foods. J Am Diet Assoc 1993;93:318-323.
8. Mangels AR, Holden JM, Beecher GR, Forman MR and Lanza E. Carotenoid content of fruits and vegetables: an evaluation of analytic data. J Am Diet Assoc 1993;93:284-296.
9. Khachik F, Beecher GR and Goli MB. Separation, identification, and quantification of carotenoids in fruits, vegetables and human plasma by high performance liquid chromatography. Pure Appl Chem 1991;63:71-80.
10. Chandler LA and Schwartz SJ. HPLC separation of cis-trans carotene isomers in fresh and processed fruits and vegetables. J Food Sci 1987;52:669-672.
11. Hulshof KFAM, Löwik MRH and Kistemaker C. Antioxidanten: de consumptie onder de loep. In: Stasse-Wolthuis M, Douwes AC, eds. Eten als medicijn. Houten: Bohm Stafleu van Lochem, 1995:63-77.
12. Cogdell RJ and Gardiner AT. Functions of carotenoids in photosynthesis. Methods Enzymol 1993;214:185-193.
13. Krinsky NI. Antioxidant functions of carotenoids. Free Rad Biol Med 1989;7:617-635.
14. Mathews-Roth MM. Beta-carotene: Clinical aspects. Curr Top Nutr Dis 1989;22:17-38.
15. Fuller CJ, Faulkner H, Bendich A, Parker RS and Roe DA. Effect of β-carotene supplementation on photosuppression of delayed-type hypersensitivity in normal young men. Am J Clin Nutr 1992;56:684-690.
16. Moore T. LXXIX. Vitamin A and carotene. V. The absence of the liver oil vitamin A from carotene. VI. The conversion of carotene to vitamin A *in vivo*. Biochem J 1930;24:692-702.
17. Ganguly J. Biochemistry of vitamin A. Boca Raton, Florida: CRC press, 1989.
18. FAO/WHO. Requirements of vitamin A, iron, folate and vitamin B12. FAO Food and Nutrition Series 23. Rome: FAO, 1988.
19. Solomons NW and Bulux J. Plant sources of provitamin-A and human nutriture. Nutr Rev 1993;51:199-204.
20. Bauernfeind JC. Carotenoid vitamin A precursors and analogs in foods and feeds. J Agr Food Chem 1972;20:456-473.

21. Bauernfeind JC, Adams CR and Marusich WL. Carotenes and other vitamin A precursors in animal feed. In: Bauernfeind JC, ed. Carotenoids as colorants and vitamin A precursors. New York: Academic Press, 1981: 563-743.
22. Sweeney JP and Marsh AC. Liver storage of vitamin A in rats fed carotene stereoisomers. J Nutr 1973;103:20-25.
23. Olson JA. Vitamin A, retinoids, and carotenoids. In: Shils ME, Olson JA and Shike M, eds. Modern nutrition in health and disease. 8th ed. Philadelphia, USA: Lea & Febiger, 1994 vol 1:287-307.
24. Krinsky NI. Carotenoid protection against oxidation. Pure Appl Chem 1979;51:649-660.
25. Krinsky NI. Actions of carotenoids in biological systems. Annu Rev Nutr 1993;13:561-587.
26. Di Mascio P, Kaiser S and Sies H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch Biochem Biophys 1989;274:532-538.
27. Zhang L-X, Cooney RV and Bertram JS. Carotenoids enhance gap junctional communication and inhibit lipid peroxidation in C3H/10T1/2 cells: relationship to their cancer chemopreventive action. Carcinogenesis 1991;12:2109-2114.
28. Conn PF, Schalch W and Truscott TG. The singlet oxygen and carotenoid interaction. J Photochem Photobio B 1991;11:41-47.
29. Levin G and Mokady S. Antioxidant activity of 9-cis compared to all-trans β-carotene *in vitro*. Free Radical Bio Med 1994;17:77-82.
30. Lavy A, Ben Amotz A and Aviram M. Preferential inhibition of LDL oxidation by the all-trans isomer of β-carotene in comparison with 9-cis β-carotene. Eur J Clin Chem Clin Biochem 1993;31:83-90.
31. Edes TE, Thornton W and Shah J. β-Carotene and aryl hydrocarbon hydroxylase in the rat: An effect of β-carotene independent of vitamin A activity. J Nutr 1989;119:796-799.
32. Bendich A. β-Carotene and the immune response. Proc Nutr Soc 1991;50:263-274.
33. Prabhala RH, Garewal HS, Meyskens FL and Watson RR. Immunomodulation in humans caused by beta-carotene and vitamin A. Nutr Res 1990;10:1473-1486.
34. van Poppel G. Carotenoids and cancer - An update with emphasis on human intervention studies. Eur J Cancer 1993;29A:1335-1344.
35. Gey KF, Moser UK, Jordan P, Stahelin HB, Eichholzer M and Ludin E. Increased risk of cardiovascular disease at suboptimal plasma concentrations of essential antioxidants - An epidemiological update with special attention to carotene and vitamin-C. Am J Clin Nutr 1993;57:S787-S797.
36. Manson JE, Gaziano JM, Jonas MA and Hennekens CH. Antioxidants and cardiovascular disease: a review. J Am Coll Nutr 1993;4:426-432.
37. Seddon JM, Ajani UA, Sperduto RD, et al eye disease case-control study group. Dietary carotenoids, vitamins A, C and E, and advanced age-related macular degeneration. J Am Med Assoc 1994;272:1413-1420.
38. Gerster H. Anticarcinogenic effect of common carotenoids. Int J Vitam Nutr Res 1993;63:93-121.
39. Krinsky NI. Effects of carotenoids in cellular and animal systems. Am J Clin Nutr 1991;53:S238-S246.
40. Rousseau EJ, Davison AJ, Dunn B. Protection by beta-carotene and related compounds against oxygen-mediated cytotoxicity and genotoxicity - Implications for carcinogenesis and anticarcinogenesis. Free Radical Bio Med 1992;13:407-433.
41. de Vet H. The puzzling role of vitamin A in cancer prevention (review). Anticancer Res 1989;9:145-152.
42. Moon RC and Itri LM. Retinoids and cancer. In: Sporn MB, Roberts AB and Goodman DS, eds. The Retinoids. London: Academic Press, 1984:327-372.

43. Halliwell B. Current status review: Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. Br J Exp Pathol 1989;70:737-757.
44. Gerster H. Potential role of beta-carotene in the prevention of cardiovascular disease. Int J Vitam Nutr Res 1991;61:277-291.
45. Jialal I, Norkus EP, Cristol L and Grundy SM. β-Carotene inhibits the oxidative modification of low-density lipoprotein. Biochim Biophys Acta 1991;1086:134-138.
46. Princen HMG, van Poppel G, Vogelezang C, Buytenhek R and Kok FJ. Supplementation with vitamin-E but not beta-carotene *in vivo* protects low density lipoprotein from lipid peroxidation *in vitro* - effect of cigarette smoking. Arterioscler Thromb 1992;12:554-562.
47. Reaven PD, Ferguson E, Navab M and Powell FL. Susceptibility of human LDL to oxidative modification - Effects of variations in beta-carotene concentration and oxygen tension. Arterioscler Thromb 1994;14:1162-1169.
48. Olson JA. The conversion of radioactive β-carotene into vitamin A by the rat intestine *in vivo*. J Biol Chem 1961;236:349-356.
49. Olson JA. The effect of bile and bile salts on the uptake and cleavage of β-carotene into retinol ester (vitamin A ester) by intestinal slices. J Lipid Res 1964;5:402-408.
50. El-Gorab MI, Underwood BA and Loerch JD. The roles of bile salts in the uptake of β-carotene and retinol by rat everted gut sacs. Biochim Biophys Acta 1975;401:265-277.
51. Hollander D. Intestinal absorption of vitamins A, E, D, and K. J Lab Clin Med 1981;97:449-462.
52. Hollander D and Ruble PE. β-Carotene intestinal absorption: bile, fatty acid, pH, and flow rate effects on transport. Am J Physiol 1978;235:E686-E691.
53. Scita G, Aponte GW and Wolf G. Uptake and cleavage of β-carotene by cultures of rat small intestinal cells and human lung fibroblasts. J Nutr Biochem 1992;3:118-123.
54. Ockner RK, Manning JA, Poppenhausen RB and Ho WKL. A binding protein for fatty acids in cytosol of intestinal mucosa, liver, myocardium, and other tissues. Science 1972;177:56-58.
55. Huang HS and Goodman DS. Vitamin A and carotenoids. I. Intestinal absorption and metabolism of ^{14}C-labeled vitamin A alcohol and β-carotene in the rat. J Biol Chem 1965;240:2839-2844.
56. Lakshman MR, Asher KA, Attlesey MG, Satchithanandam S, Mychkovsky I and Coutlakis PJ. Absorption, storage, and distribution of β-carotene in normal and beta-carotene-fed rats: roles of parenchymal and stellate cells. J Lipid Res 1989;30:1545-1550.
57. Goodman DS, Blomstrand R, Werner B, Huang HS and Shiratori T. The intestinal absorption and metabolism of vitamin A and β-carotene in man. J Clin Invest 1966;45:1615-1623.
58. Blomstrand R and Werner B. Studies on the intestinal absorption of radioactive β-carotene and vitamin A in man. Conversion of β-carotene into vitamin A. Scand J Clin Lab Invest 1967;19:339-345.
59. Hussein L and El-Tohamy M. Vitamin A potency of carrot and spinach carotenes in human metabolic studies. Int J Vitam Nutr Res 1990;60:229-235.
60. Nageswara Rao C and Narasinga Rao BS. Absorption of dietary carotenes in human subjects. Am J Clin Nutr 1970;234:1:105-109.
61. Guggenheim K. The biological value of carotene from various sources and the effect of vitamin E on the utilization of carotene and of vitamin A. Biochem J 1944;38:260-264.
62. Sweeney JP and Marsh AC. Liver storage of vitamin A by rats fed carrots in various forms. J Nutr 1974;104:1115-1120.

63. Shiau A, Mobarhan S, Stacewiczsapuntzakis M, Benya R, Liao YL, Ford, C, Bowen P, Friedman H and Frommel TO. Assessment of the intestinal retention of beta-carotene in humans. J Am Coll Nutr 1994;13:369-375.
64. Levin G and Mokady S. Incorporation of all-trans or 9-cis-beta-carotene into mixed micelles *in vitro*. Lipids 1995;30:177-179.
65. Glover J. The conversion of β-carotene into vitamin A. Vitam Horm 1960;18:371-386.
66. Ganguly J and Sastry PS. Mechanism of conversion of β-carotene into vitamin A- Central cleavage versus random cleavage. Wld Rev Nutr Diet 1985;45:198-220.
67. Karrer P, Morf R, Schöpp K. Zur Kenntnis des Vitamins-A aus Fischtranen II. Helv Chim Acta 1931;14:1431-1436.
68. Goodman DS, Huang HS and Shiratori T. Mechanism of the biosynthesis of vitamin A from β-carotene. J Biol Chem 1966;241:1929-1932.
69. Vartapetyan BB, Dmitrovski AA, Alkhazov DG, Lemberg IKh, Girshin AB, Gusinskii GM, Starikova NA, Erofeeva NN and Bogdanova IP. A new approach to the study of the mechanism of biosynthesis of vitamin A from carotene by activation of oxygen O^{18} as a result of a nuclear reaction O^{18} $(\alpha, n\gamma)$ Ne^{21} by means of α-particles accelerated in a cyclotron. Translated from Biokhimiya 1966;31:881-886.
70. Wang X-D, Krinsky NI, Marini RP, Tang G, Yu J, Hurley R, Fox JG and Russell RM. Intestinal uptake and lymphatic absorption of β-carotene in ferrets: a model for human β-carotene metabolism. Am J Physiol 1992;263:G480-G486.
71. Quick TC and Ong DE. Vitamin A metabolism in the human intestinal Caco-2 cell line. Biochemistry 1990;29:11116-11123.
72. Goodman DS and Huang HS. Biosynthesis of vitamin A with rat intestinal enzymes. Science 1965;149:879-880.
73. Olson JA and Hayaishi O. The enzymatic cleavage of β-carotene into vitamin A by soluble enzymes of rat liver and intestine. Proc Natl Acad Sci USA 1965;54:1364-1370.
74. Goodman DS, Huang HS, Kanai M and Shiratori T. The enzymatic conversion of all-trans β–carotene into retinal. J Biol Chem 1967;242:3543-3554.
75. Goodman DS and Olson JA. The conversion of all-trans β-carotene into retinal. Methods Enzymol 1969;15:462-475.
76. Crain FD, Lotspeich FJ and Krause RF. Biosynthesis of retinoic acid by intestinal enzymes of the rat. J Lipid Res 1967;8:249-254.
77. Lakshmanan MR, Pope JL and Olson JA. The specificity of a partially purified carotenoid cleavage enzyme of rabbit intestine. Biochem Biophys Res Com 1968;33:347-352.
78. Gronowska-Senger A and Wolf G. Effect of dietary protein on the enzyme from rat and human intestine which converts β-carotene to retinal. J Nutr 1970;100:300-308.
79. Singh H and Cama HR. Enzymatic cleavage of carotenoids. Biochim Biophys Acta 1974;370:49-61.
80. Villard L and Bates CJ. Carotene dioxygenase (EC 1.13.11.21) activity in rat intestine: effects of vitamin A deficiency and of pregnancy. Br J Nutr 1986;56:115-122.
81. Hansen S and Maret W. Retinal is not formed *in vitro* by enzymatic central cleavage of β-carotene. Biochemistry 1988;27:200-206.
82. Napoli JL and Race KR. Biogenesis of retinoic acid from β-carotene: Differences between the metabolism of β-carotene and retinal. J Biol Chem 1988;263:17372-17377.
83. Lakshman MR, Mychkovsky I and Attlesey M. Enzymatic conversion of all-*trans*-β-carotene by a cytosolic enzyme from rabbit and rat intestinal mucosa. Proc Natl Acad Sci USA 1989;86:9124-9128.
84. Wang X-D, Tang G-W, Fox JG, Krinsky NI and Russell RM. Enzymatic conversion of β-carotene into β-apocarotenals and retinoids by human, monkey, ferret, and rat tissues. Arch Biochem Biophys 1991;285:8-16.

85. Tang G, Wang X-D, Russell RM and Krinsky NI. Characterization of β-apo-13-carotenone and β-apo-14'-carotenal as enzymatic products of the excentric cleavage of β-carotene. Biochemistry 1991;30:9829-9834.
86. Ershov YV, Dmitrovskii AA and Bykhovskii VY. Properties of beta-carotene 15,15'-dioxygenase stabilized by lutein and dithiothreitol during isolation. Biochemistry - Russia 1993;58:263-269.
87. Kakkad BP and Ong DE. Reduction of retinaldehyde bound to cellular retinol-binding protein (type II) by microsomes from rat small intestine. J Biol Chem 1988;263:12916-12919.
88. Fidge NH and Goodman DS. The enzymatic reduction of retinal to retinol in rat intestine. J Biol Chem 1968;243:4372-4379.
89. Moffa DJ, Lotspeich FL and Krause RF. Preparation and properties of retinal-oxidizing enzyme from rat intestinal mucosa. J Biol Chem 1970;245:439-447.
90. Ong DE, Kakkad B, MacDonald PN. Acyl-CoA-independent esterification of retinol bound to cellular retinol-binding protein (type II) by microsomes from rat small intestine. J Biol Chem 1987;262:2729-2736.
91. Wang X-D, Krinsky NI, Tang G and Russell RM. Retinoic acid can be produced from excentric cleavage of β-carotene in human intestinal mucosa. Arch Biochem Biophys 1992;293:298-304.
92. Sharma RV, Mathur SN and Ganguly J. Studies on the relative biopotencies and intestinal absorption of different apo-β-carotenoids in rats and chickens. Biochem J 1976;158:377-383.
93. Nagao A and Olson JA. Enzymatic formation of 9-cis, 13-cis, and all-trans retinals from isomers of beta-carotene. FASEB J 1994;8:968-973.
94. Dew SE and Ong DE. Specificity of the retinol transporter of the rat small intestine brush border. Biochemistry 1994;33:12340-12345.
95. Wang XD, Krinsky NI, Benotti PN and Russell RM. Biosynthesis of 9-*cis*-retinoic acid from 9-*cis*-beta-carotene in human intestinal mucosa *in vitro*. Arch Biochem Biophys 1994;313:150-155.
96. Holmes HN and Corbet RE. The isolation of crystalline vitamin A. J Am Chem Soc 1937;59:2042-2047.
97. Zechmeister L, Le Rosen AL, Schroeder WA, Polgár A and Pauling L. Special characteristics and configuration of some stereoisomeric carotenoids including prolycopene and pro-γ-carotene. J Am Chem Soc 1943;65:1940-1951.
98. Olson JA. The alpha and the omega of vitamin A metabolism. Am J Clin Nutr 1969;22:953-962.
99. Sharma RV, Mathur SN, Dimitrovskii AA, Das RC and Ganguly J. Studies on the metabolism of β-carotene and apo-β-carotenoids in rats and chickens. Biochim Biophys Acta 1977;486:183-194.
100. Johnson EJ and Russell RM. Distribution of orally administered β-carotene among lipoproteins in healthy men. Am J Clin Nutr 1992;56:128-135.
101. Mathews-Roth MM and Gulbrandson CL. Transport of beta-carotene in serum of individuals with carotenemia. Clin Chem 1974;20:1578-1579.
102. Reddy PP, Clevidence BA, Berlin E, Taylor PR, Bieri JG and Smith JC.Plasma carotenoid and vitamin E profile of lipoprotein fractions of men fed a controlled typical U.S. diet. FASEB J 1989;3:A955 (Abstr).
103. Traber MG, Diamond SR, Lane JC, Brody RI and Kayden HJ. beta-Carotene transport in human lipoproteins. Comparisons with alpha-tocopherol. Lipids 1994;29:665-669.
104. Dimitrov NV, Boone CW, Hay MB, Whetter P, Pins M, Kelloff GJ and Malone W. Plasma beta-carotene levels-kinetic patterns during administration of various doses of β-carotene. J Nutr Growth Cancer 1986;3:227-237.

105. Kübler W, Reinersdorff D von. The dose dependency of plasma carotenoids. Biokinetic studies in men. In: Schlemmer U, ed. Proceedings of the international conference bioavalability '93 - Nutritional, chemical and food processing implications of nutrient availability. Berichte der Bundesforschungsanstalt für Ernährung BFE-R--93-01, 1993;Part 2:331-335.
106. Henderson CT, Mobarhan S, Bowen P, Stacewicz-Sapuntzakis M, Langenberg P, Kiani R, Lucchesi D and Sugerman S. Normal serum response to oral beta-carotene in humans. J Am Coll Nutr 1989;8:625-635.
107. Brown ED, Micozzi MS, Craft NE, Bieri JG, Beecher G, Edwards BK, Rose A, Taylor PR and Smith JC,jr. Plasma carotenoids in normal men after a single ingestion of vegetables or purified β-carotene. Am J Clin Nutr 1989;49:1258-1265.
108. Bierer TL, Merchen NR and Erdman JW. Comparative absorption and lipoprotein transport of five common carotenoids in the preruminant calf. FASEB J 1994;8:A422 (Abstr).
109. Bjornson LK, Kayden HJ, Miller E and Moshell AN. The transport of α-tocopherol and β-carotene in human blood. J Lipid Res 1976;17:343-352.
110. Hussain MM, Mahley RW, Boyles JK, Lindquist PA, Brecht WJ, Innerarity TL. Chylomicron metabolism. Chylomicron uptake by bone marrow in different animal species. J Biol Chem 1989;264:17931-17938.
111. Blaner WS, Obunike JC, Kurlandsky SB, Alhaideri M, Piantedosi R, Deckelbaum RJ and Goldberg IJ. Lipoprotein lipase hydrolysis of retinyl ester - Possible implications for retinoid uptake by cells. J Biol Chem 1994;269:16559-16565.
112. Wolf G. Uptake of retinoids by adipose tissue. Nutr Rev 1994;52:356-358.
113. Norkus EP, Bhagavan HN and Nait PP. Relationship between individual carotenoids in plasma platelets and red blood cells (RBC) of adult subjects. FASEB J 1990;4:A1174 (Abstr).
114. Shapiro SS, Mott DJ and Machlin LJ. Kinetic characteristics of β-carotene uptake and depletion in rat tissue. J Nutr 1984;114:1924-1933.
115. Spady DK, Turley SD, Dietschy JM. Receptor independent low density lipoprotein transport in rat *in vivo*. J Clin Invest 1985;76:1113-1122.
116. Kaplan LA, Lau JM and Stein EA. Carotenoid composition, concentrations, and relationships in various human organs. Clin Physiol Biochem 1990;8:1-10.
117. Parker RS. Carotenoid and tocopherol composition of human adipose tissue. Am J Clin Nutr 1988;47:33-36.
118. Kardinaal AFM, van 't Veer P, Brants HAM, van den Berg H, van Schoonhoven J and Hermus RJJ. Relations between antioxidant vitamins in adipose tissue, plasma and diet. Am J Epidemiol 1995;141:440-450.
119. Stahl W, Schwarz W, Sundquist AR and Sies H. *Cis-trans* isomers of lycopene and β-carotene in human serum and tissues. Arch Biochem Biophys 1992;294:173-177.
120. Stahl W, Schwarz W, Sies, H. Human serum concentrations of all-trans beta-carotene and alpha-carotene but not 9-cis beta-carotene increase upon ingestion of a natural isomer mixture obtained from Dunaliella-Salina (Betatene). J Nutr 1993;123:847-851.
121. Jensen CD, Howes TW, Spiller GA, Pattison TS, Whittam JH and Scala J. Observations on the effects of ingesting cis- and trans-beta-carotene isomers on human serum concentrations. Nutr Rep Int 1987;35:413-422.
122. You C-S, Parker RS, Goodman K, Brenna JT and Canfield W. Metabolism of [13C]9-cis β-carotene in humans. FASEB J 1994;8:A422 (Abstr).
123. Edes TE, Gysbers DG, Buckley CS and Thornton WH. Exposure to the carcinogen benzopyrene depletes tissue vitamin A: β-carotene prevents depletion. Nutr Cancer 1991;15:159-166.

124. Carlier C, Coste J, Etchepare M, Periquet B and Amedeemanesme O. A Randomised controlled trial to test equivalence between retinyl palmitate and beta-carotene for vitamin-A deficiency. Br Med J 1993;307:1106-1110.
125. Gershoff SN, Andrus SB, Hegsted DM and Lentini EA. Vitamin A deficiency in cats. Lab Invest 1957;6:227-240.
126. Krinsky NI, Mathews-Roth MM, Welankiwar S, Sehgal PK, Lausen NCG and Russett M The metabolism of [^{14}C]β-carotene and the presence of other carotenoids in rats and monkeys. J Nutr 1990;120:81-87.
127. Kamath SK and Arnrich L. Effect of dietary protein on the intestinal biosynthesis of retinol from ^{14}C-β-carotene in rats. J Nutr 1973;103:202-206.
128. Alam BS and Alam SQ. The effect of different levels of dietary β-carotene on DMBA-induced salivary gland tumors. Nutr Cancer 1987;9:93-101.
129. Alam BS, Alam SQ, Bendich A and Shapiro SS. Effect of dietary lipids on hepatic and plasma β-carotene and vitamin A levels in rats fed β-carotene. Nutr Cancer 1989;12:57-60.
130. Blakely SR, Grundel E, Jenkins MY and Mitchell GV. Alterations in β-carotene and vitamin E status in rats fed β-carotene and excess vitamin A. Nutr Res 1990;10:1035-1044.
131. Bendich A and Shapiro SS. Effect of β-carotene and canthaxanthin on the immune response of the rat. J Nutr 1986;116:2254-2262.
132. Ben-Amotz A, Mokady S, Edelstein S and Avron M. Bioavailability of a natural mixture as compared with synthetic all-*trans*-β-carotene in rats and chicks. J Nutr 1989;119:1013-1019.
133. Serbinova E, Choo M and Packer L. Distribution and antioxidant activity of a palm oil carotene fraction in rats. Biochem Int 1992;28:881-886.
134. Tyczkowski JK and Hamilton PB. Evidence for differential absorption of zeacarotene, cryptoxanthin, and lutein in young broiler chickens. Poultry Sci 1986;65:1137-1140.
135. Ganguly J, Mehl JW and Deuel HJ. Studies on carotenoid metabolism. XII. The effect of dietary carotenoids on the carotenoid distribution in the tissues of chickens. J Nutr 1953;50:59-72.
136. Mayne ST and Parker RS. Subcellular distribution of dietary β-carotene in chick liver. Lipids 1986;21:164-169.
137. Poor CL, Miller SD, Fahey GC Jr, Easter RA and Erdman JW Jr. Animal models for carotenoid utilization studies: evaluation of the chick and the pig. Nutr Rep Int 1987;36:229-234.
138. White WS, Peck KM, Bierer TL, Gugger ET and Erdman JW. Interactions of oral beta-carotene and canthaxanthin in ferrets. J Nutr 1993;123:1405-1413.
139. Gugger ET, Bierer TL, Henze TM, White WS and Erdman JW. β-Carotene uptake and tissue distribution in ferrets (*Mustela putorius furo*). J Nutr 1992;122:115-119.
140. Ribaya-Mercado JD, Fox JG, Rosenblad WD, Blanco MC and Russell RM. beta-Carotene retinol and retinyl ester concentrations in serum and selected tissues of ferrets fed β-carotene. J Nutr 1992;122:1898-1903.
141. Ribaya-Mercado JD, Holmgren SC, Fox JG and Russell RM. Dietary β-carotene absorption and metabolism in ferrets and rats. J Nutr 1989;119:665-668.
142. Rousseau JE, Eaton HD, Teichman R, Helmboldt CF, Jungherr EL, Bacon EL and Beal G. Relative value of carotene from alfalfa and vitamin A from a dry carrier fed at medium to high levels to Holstein Calves. ?1956;?:1565-1573.
143. Kon SK, McGillivray WA and Thompson SY. Metabolism of carotene and vitamin A give by mouth or vein in oily solution or aqueous dispersion to calves, rabbits and rats. Br J Nutr 1955;9:244-267.

144. Davis CL, Elliott RF and Lassiter CA. The effect of lecithin, Choline, and methionine on the vitamin A and carotene plasma levels and liver stores of young dairy calves. J Diary Sci 1956;39:440-447.
145. Bierer TL, Merchen NR, nelson DR and Erdman JW. Transport of newly-absorbed beta-carotene by the preruminant calf. Ann N Y Acad Sci 1993;691:226-229.
146. Schmitz HH, Poor CL, Gugger ET and Erdman JW. Analysis of carotenoids in human and animal tissues. Methods Enzymol 1993;214:102-116.
147. Beems RB, van Beek L, Rutten AAJJL and Speek AJ. Subchronic(106-day) toxicology and nutrition studies with vitamin A and β-carotene in Syrian hamsters. Nutr Rep Int 1987;35:765-770.
148. Mathews-Roth MM, Hummel D and Crean C. The carotenoid content of various organs of animals administered large amounts of beta-carotene. Nutr Rep Int 1977;16:419-423.
149. Jones CS, Sly L, Chen LC, Ben T, Brughcollins M, Lichti U and Deluca LM. Retinol and beta-carotene concentrations in skin, papillomas and carcinomas, liver, and serum of mice fed retinoic acid or beta-carotene to suppress skin tumor formation. Nutr Cancer 1994;21:83-93.
150. Folman Y, Russell RM, Tang GW and Wolf G. Rabbits fed on β-carotene have higher serum levels of all-*trans*-retinoic acid than those receiving no β-carotene. Br J Nutr 1989;62:195-201.
151. Pollack J, Campbell JM, Potter SM and Erdman JW. Mongolian gerbils (*Meriones unguiculatus*) absorb beta-carotene intact from test meal. J Nutr 1994;124:869-873.
152. Miller ER and Ullrey DE. The pig as a model for human nutrition. Annu Rev Nutr 1987;7:361-382.
153. Havel RJ, Eder HA and Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 1955;34:1345-1353.
154. Bankson DD, Russell RM and Sadowski JA. Determination of retinyl esters and retinol in serum or plasma by normal-phase liquid chromatography: Method and applications. Clin Chem 1986;32:35-40.
155. Eckhoff C, Bailey JR, Collins MD, Slikker W and Nau H. Influence of dose and pharmaceutical formulation of vitamin A on plasma levels of retinyl esters and retinol and metabolic generation of retinoic acid compounds and β-glucuronides in the cynomolgus monkey. Toxicol Appl Pharm 1991;111:116-127.
156. Blakely SR, Slaughter L, Adkins J and Knight EV. Effects of β-carotene and retinyl palmitate on corn oil-induced superoxide dismutase and catalase in rats. J Nutr 1988;118:152-158.
157. Schweigert FJ, Rambeck WA and Zucker H. Transport of β-carotene by the serum lipoproteins in cattle. J Anim Physiol Anim Nutr 1987;57:162-167.
158. McGillivray WA. Wld Rev Nutr Diet 1960;2;131-155.
159. Manago M, Tamai H, Ogihara T and Mino M. Distribution of circulating beta-carotene in human plasma lipoproteins. J Nutr Sci Vitaminol 1992;38:405-414.
160. Schmitz HH, Poor CL, Wellman RB and Erdman JW. Concentrations of selected carotenoids and vitamin A in human liver, kidney and lung tissue. J Nutr 1991;121:1613-1621.
161. Rock CL and Swendseid ME. Plasma β-carotene response in humans after meals supplemented with dietary pectin. Am J Clin Nutr 1992;55:96-99.
162. Sugerman SB, Mobarhan S, Bowen PE, Stacewicz-Sapuntzakis M, Langenberg P, Henderson C, Kiani R, Friedman H and Lucchesi D. Serum time curve characteristics of a fixed dose of β-carotene in young and old man. J Am Coll Nutr 1991;10:297-307.
163. Prince MR and Frisoli JK. Beta-Carotene accumulation in serum and skin. Am J Clin Nutr 1993;57:175-181.

164. Mobarhan S, Bowen P, Andersen B, Evans M, Stacewicz-Sapuntzakis M, Sugerman S, Simms P, Lucchesi D and Friedman H. Effects of β-carotene repletion on β-carotene absorption, lipid peroxidation, and neutrophil superoxide formation in young men. Nutr Cancer 1990;14:195-206.
165. Dimitrov NV, Meyer C, Ullrey DE, Chenoweth W, Michelakis A, Malone W, Boone C and Fink G. Bioavailability of β-carotene in humans. Am J Clin Nutr 1988;48:298-304.
166. Micozzi MS, Brown ED, Edwards BK, Bieri JG, Taylor PR, Khachik F, Beecher GR and Smith JC. Plasma carotenoid response to chronic intake of selected foods and beta-carotene supplements in men. Am J Clin Nutr 1992;55:1120-1125.
167. Cornwell DG, Kruger FA and Robinson HB. Studies on the absorption of beta-carotene and the distribution of total carotenoid in human serum lipoproteins after oral administration. J Lipid Res 1962;3:65-70.
168. Parker RS, Swanson JE, Marmor B, Goodman KJ, Spielman AB, Brenna JT, Viereck SM and Canfield WK. Study of β-carotene metabolism in humans using ^{13}C-β-carotene and high precision isotope ratio mass spectrometry. Ann N Y Acad Sci 1993;691:86-95.
169. Dueker SR, Jones AD, Smith GM and Clifford AJ. Stable isotope methods for the study of beta-carotene-d(8) metabolism in humans utilizing tandem mass spectrometry and high-performance liquid chromatography. Anal Chem 1994;66:4177-4185.
170. Bulux J, Deserrano JQ, Giuliano A, Perez R, Lopez CY, Rivera C, Solomons NW and Canfield LM. Plasma response of children to short-term chronic beta-carotene supplementation. Am J Clin Nutr 1994;59:1369-1375.
171. Dietz-Bryant J, McCord JD, Knight-Unlu L and Erdman JW. Isolation and partial characterization of α- and β-carotene-containing carotenoprotein from carrot (*Daucus carota* L.) root chromoplasts. J Agric Food Chem 1992;40:545-549.
172. Khachik F, Goli MB, Beecher GR, Holden J, Lusby WR, Tenorio MD, Barrera MR. Effect of food preparation on qualitative and quantitative distribution of major carotenoid constituents of tomatoes and several green vegetables. J Agric Food Chem 1992;40:390-398.
173. Chen BH and Chen YY. Stability of chlorophylls and carotenoids in sweet potato leaves during microwave cooking. J Agric Food Chem 1993;41:1315-1320.
174. Levy A, Harel S, Palevitch D, Akiri B, Menagem E and Kanner J. Carotenoid pigments and β-carotene in paprika fruits (*Capsicum Spp*) with different genotypes. J Agric Food Chem 1995;43:362-367.
175. Thomas P. Effect of post harvest temperature on quality, carotenoids and ascorbic acid of alphonso mangoes on ripening. J Food Sci 1975;40:704-706.
176. Liu YK and Luh BS. Effect of harvest maturity on carotenoids in pastes made from VF-145-7879 tomatoes. J Food Sci 1977;42:216-220.
177. Chandler LA and Schwartz SJ. Isomerization and losses of trans-β-carotene in sweet potatoes as affected by processing treatments. J Agric Food Chem 1988;36:129-133.
178. Kasper H and Ernst B. Der Einfluss des Nahrungsfettes auf die Vitamin-A- und Carotinresorption. Internat Z Vit Forschung 1969;39:23-29.
179. Blakely SR, Dodge WR, Grundel E, Mitchell GV and Jenkins MY. Corn oil enhances the hepatic accumulation of β-carotene and canthaxanthin in rats. FASEB J 1992;6:A1646 (Abstr).
180. Dietary fiber reduces β-carotene utilization. Nutr Rev 1987;45:350-352.
181. Phillips WEJ. Effect of dietary nitrite on the liver storage of vitamin A in the rat. Can J Biochem 1966;44:1-7.
182. Wedzicha BL and Lamikanra O. Sulphite mediated destruction of β-carotene: The partial characterisation of reaction products. Food Chem 1983;10:275-283.

183. Willet WC, Stampfer MJ, Underwood BA, Taylor JO, Hennekens CH. Vitamins A,E, and carotene: effects of supplementation on their plasma levels. Am J Clin Nutr 1983;38:559-566.
184. Arnrich L and Arthur VA. Interactions of fat-soluble vitamins in hypervitaminoses. Ann N Y Acad Sci 1980;355:109-118.
185. High E, Day HG. Fate of lycopene in the rat and its effects on the utilization of carotene and vitamin A. J Nutr 1952;48:369-376.
186. Ershov YV, Bykhovsky VY and Dmitrovskii AA. Stabilization and competitive inhibition of beta-carotene-15,15'-dioxygenase by carotenoids. Biochem Mol Biol Int 1994;34:755-763.
187. White WS, Stacewicz-Sapuntzakis M, Erdman JW and Bowen PE. Pharmacokinetics of beta-carotene and canthaxanthin after ingestion of individual and combined doses by human subjects. J Am Coll Nutr 1994;13:665-671.
188. Lakshman MR, Johnson LH, Okoh C, Attlesey M, Mychkovsky I and Bhagavan HN. Conversion of all trans beta-carotene to retinal by an enzyme from the intestinal mucosa of human neonates. J Nutr Biochem 1993;4:659-663.
189. Maiani G, Mobarhan S, Ceccanti M, Ranaldi L, Gettner S, Bowen P, Friedman H, De Lorenzo A and Ferro-Luzzi A. Beta-carotene serum response in young and elderly females. Eur J Clin Nutr 1989;43:749-761.
190. Nierenberg DW, Stukel TA, Baron JA, Dain BJ, Greenberg ER and the skin cancer prevention study group. Determinants of increase in plasma concentration of β-carotene after chronic oral supplementation. Am J Clin Nutr 1991;53:1443-1449.
191. Stryker WS, Kaplan LA, Stein EA, Stampfer MJ, Sober A and Willet WC. The relation of diet, cigarette smoking, and alcohol consumption to plasma beta-carotene and alpha-tocopherol levels. Am J Epidem 1988;127:283-296.
192. Reinersdorf D von and Kübler WG. Biokinetische Untersuchungen Über die Bioverfügbarkeit und Kumulation von Carotenoiden beim Erwachsenen. Ernährungs-Umschau 1991;38:90 (Abstr).
193. Kübler W. Pharmacokinetic implications of single and repeated dosage. Int J Vitam Nutr Res 1989; suppl 30: 25-34.
194. Stoecker B and Arnrich L. Patterns of protein feeding and the biosynthesis of vitamin A from carotene in rats. J Nutr 1973;103:1112-1118.
195. Honory K. The effect of zinc on the assimilation of beta-carotene in poultry. Bull Vet Inst Pulawy 1978;22:77-80.
196. Takruri HRH and Thurnham DI. Effect of zinc deficiency on the conversion of β-carotene to retinol as indicated by liver stores. Proc Nutr Soc 1982;41:53A.
197. Sklan D. Carotene-cleavage activity in chick intestinal mucosa cytosol: association with a high-molecular-weight lipid-protein aggregate fraction and partial characterization of the activity. Br J Nutr 1983;50:417-425.
198. Huber AM and Gershoff SN. Effects of zinc deficiency on the oxidation of retinol and ethanol in rats. J Nutr 1975;105:1486-1490.
199. Probstfield JL, Lin T-L, Peters J and Hunninghake DB. Carotenoids and vitamin A: The effect of hypocholesterolemic agents on serum levels. Metabolism 1985;34:88-91.
200. Pamuk ER, Byers T, Coates RJ, Vann JW, Sowell AL, Gunter EW and Glass D. Effect of smoking on serum nutrient concentrations in African-American women. Am J Clin Nutr 1994;59:891-895.
201. Nierenberg DW, Stukel TA, Baron JA, Dain BJ, Greenberg ER and the skin cancer prevention study group. Determinants of plasma levels of beta-carotene and retinol. Am J Epidemiol 1989;130:511-521.

Chapter 3

Determination of several retinoids, carotenoids and E vitamers by high-performance liquid chromatography
Application to plasma and tissues of rats fed a diet rich in either β-carotene or canthaxanthin

Trinette van Vliet[1,2], Frank van Schaik[1], Jan van Schoonhoven[1] and Jaap Schrijver[1]

[1] TNO Nutrition and Food Research Institute, Zeist, The Netherlands; [2] Department of Experimental Zoology, University of Amsterdam, Amsterdam, The Netherlands

Journal of Chromatography 1991;553:179-186

Abstract

A method, using two different systems, is described for the high-performance liquid chromatographic analysis of retinol, retinal, retinoic acid, retinyl acetate, retinyl palmitate, α-, β- and γ-carotene, β-apo-6′-, β-apo-8′-, β-apo-10′- and β-apo-12′-carotenal, ethyl β-apo-8′-carotenoate, α-tocopherol and α-tocopheryl acetate. The first system consists of a laboratory-packed Hypersil-ODS 3-μm column and a mobile phase of acetonitrile - methylene chloride - methanol - water (70:10:15:5, v/v/v/v). The second system consists of a laboratory-packed Nucleosil C_{18} 3-μm column and a mobile phase of acetonitrile - 0.1 mol/L ammonium acetate (80:20, v/v). The detection limits in standard solutions were 10 ng/ml for retinoids and carotenoids and 60 ng/ml for the E vitamers. Analysis of the tissues and plasma of rats, after 2 weeks on a diet supplemented with either β-carotene or canthaxanthin (both 2 mg/g), led to the conclusion that the rats were able both to transport and store β-carotene and canthaxanthin and to convert β-carotene to retinol. Incubation of cytosol preparations from the mucosa of the small intestine of rat with 1 μg of β-carotene resulted in the formation of 10 - 20 ng of retinal within 1 h.

Introduction

Vitamin A and β-carotene may be important agents in the prevention c
cancer[1-3]. After absorption in the small intestine, most β-carotene is believed t
be converted to vitamin A, while a small part may be transported as β-carotene
It is not clear whether β-carotene is protective after conversion to vitamin A, c
by itself, for instance as an antioxidant[5].

The primary interest is in the absorption of β-carotene in the intestine an
its possible cleavage following absorption. Two cleavage theories exist: the firs
assumes a central cleavage by β-carotene 15,15′-dioxygenase, resulting in tw
molecules of retinal, whereas the second assumes a random cleavage, resultin
in retinal, β-apocarotenals and other products.

To validate these two theories it should suffice to measure the amount c
retinal formed from β-carotene. However, the experience is that, unde
experimental conditions *in vitro*, the amount of retinal formed is too small t
confirm the first theory[6]. To confirm the second theory, products other tha
retinal (and not originating from retinal) should be demonstrated.

It is therefore important to measure the possible products resulting fro
random β-carotene cleavage. Several high-performance liquid chromatograph
(HPLC) procedures have already been described for the determination of retino
retinoic acid, retinyl esters and/or carotenoids[7-9]. Furr[10] described a method fc
the analysis of retinal, among other retinoids, using a gradient programme
Hansen and Maret[11] measured retinoids, carotenoids and β-apocarotenals usin
three different HPLC systems.

In this paper a method is described for the HPLC analysis (using tw
different systems) of retinol, retinal, retinoic acid, retinyl acetate, retiny
palmitate, α-, β- and γ-carotene, β-apo-6′-, β-apo-8′-, β-apo-10′- and β-apo-12′
carotenal, ethyl β-apo-8′-carotenoate, α-tocopherol and α-tocopheryl acetate. I
addition, two applications of the method are described. The first is the analysi
of tissues obtained from female rats fed a diet enriched with either β-carotene o
canthaxanthin. The second is the analysis of samples of an *in vitro* cleavag
assay with intestinal cytosol preparations, described as the β-caroten
15,15′-dioxygenase assay by Goodman et al.[12].

Experimental

Chemicals

β-Apo-6′-carotenal was a gift from BASF (Arnhem, The Netherlands). Retinyl stearate, γ-carotene, β-apo-10′-carotenal and β-apo-12′-carotenal were gifts from Hoffmann-La Roche (Mijdrecht, The Netherlands). β-Carotene was obtained from Merck (Darmstadt, Germany). β-Apo-8′-carotenal, ethyl β-apo-8′-carotenoate and canthaxanthin were obtained from Fluka (Buchs, Switzerland). Other reference compounds were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC-grade) from Westburg (Leusden, The Netherlands) was used. All other chemicals were of analytical-reagent grade.

Chromatography

HPLC analyses were performed using a system incorporating a Gynkotek 300 C constant-flow pump (Kipp Analytica, Delft, The Netherlands), an ISS-100 automatic injector with cool tray (Perkin-Elmer, Gouda, The Netherlands) and two programmable 783 absorbance detectors (Applied Biosystems, Rotterdam, The Netherlands). Two stainless-steel Hyperchrome HPLC columns (125 mm × 4.6 mm ID) were packed in the laboratory with Hypersil ODS 3 µm (Shandon Southern Products, Astmoor, UK) or Nucleosil 120-3 C_{18} (Machery-Nagel, Düren, Germany) by the balanced-density slurry technique on a column-packing installation designed at the TNO Toxicology and Nutrition Institute, using a Haskel DSTV-150 pump (Ammann Technik, Stuttgart, Germany). The slurry and packing solvents were isopropanol and methanol, respectively. Elution profiles were displayed on a Kipp BD 41 recorder (Kipp Analytica).

The mobile phase used for the Hypersil column consisted of acetonitrile - methylene chloride - methanol - water (70:10:15:5, v/v/v/v). A flow programme of 0.5 - 2.0 mL/min was used (0 - 13 min at 0.5 mL/min; 14 - 24 min at 1.0 mL/min; 25 - 46 min at 1.5 mL/min; 47 - 57 min at 2.0 mL/min; 58 - 60 min at 0.5 mL/min). The Nucleosil column was eluted at a flow-rate of 1.0 mL/min with acetonitrile - 0.1 mol/L ammonium acetate (80:20, v/v).

Detection after separation on the Hypersil column was carried out using one detector set at 350 nm and a second detector switching after 16 min from 445 to 292 nm and returning 9 min later to 445 nm for the last part of the HPLC run.

Procedures

Standard solutions were prepared in methanol. The preparation of standar solutions and the extraction of plasma and tissue samples were carried ou under subdued light. The actual concentrations of the standards wer determined by measuring the absorbance of diluted stock solutions using Ultrospec K spectrophotometer (LKB, Cambridge, UK) and calculating th concentrations based on published spectral data[13-15].

Livers were dismembrated in liquid nitrogen using a Mikro-dismembrator I (Braun, Melsungen, Germany). All other tissues were homogenised with a Ultra-Turrax homogeniser (Wilten Woltil, de Bilt, The Netherlands). Sample were homogenised in brown-coloured test-tubes with 3 mL of doubly distille water containing EDTA (10 mmol/L), ascorbic acid (1 mg/mL) and acetic aci (1%, v/v). Plasma samples of 100 µl were mixed with 100 µl of 0.9% (w/v) NaC To precipitate proteins, 2 mL (0.2 mL for plasma samples) of methano (containing 1 mg of butylated hydroxytoluene (BHT) per mL added as a antioxidant) were added and the mixture was vortexed for 30 s. After 10 mir 4 mL (0.4 mL for plasma) of chloroform (containing 1 mg/mL BHT) were adde and the sealed tubes were vortexed for 4 min. After centrifugation th chloroform layer was separated and evaporated under nitrogen. The residu was dissolved in methanol, transferred into brown HPLC injection vials an placed in the HPLC tray of the injector, which was cooled down to about +4°C t increase stability, especially of the aldehyde forms of the compounds of interest.

For the incubation mixtures of the dioxygenase assay the same procedur was followed except that ethanol was used to precipitate the proteins an hexane was used for extraction. The residues were dissolved in water-fre eluent.

Results and discussion

Characteristics of the high-performance liquid chromatographic methods

With the Hypersil column the HPLC method described here yielded a complet resolution of retinol, retinyl acetate, retinyl palmitate, α-, β- and γ-carotene β-apo-6′-, β-apo-8′-, β-apo-10′- and β-apo-12′-carotenal, ethyl β-apo-8′-caro tenoate, α-tocopherol and α-tocopheryl acetate. The method using the Nucleosi column gave a complete resolution of retinoic acid, retinal, retinol and retiny acetate. It was not possible to separate all the compounds in a single run usin the Hypersil column; retinoic acid eluted in the void volume and no baselin

separation could be obtained for retinol, retinal and retinyl acetate. Separation could be improved by increasing the polarity of the eluent. However, this extends the elution time of β-carotene by several hours. As the detector was used in a very sensitive setting, gradient elution was not useful because of a rising baseline. Flow programming shortened the run to 45 min.

Using the Hypersil column two spectrophotometric detectors were needed as the time between the elution of retinyl acetate and β-apo-12′-carotenal and the time between the elution of the β-carotene and retinyl palmitate were too short for reliable wavelength switching. This is why the carotenoids and tocopherols were measured with one detector switching from 445 to 292 nm and back to 445 nm (see **Figure 1** for an elution profile) whereas retinol, retinyl acetate and retinyl palmitate were determined using a second detector set at 350 nm (**Figure 2**). **Figure 3** shows the results of the separation of a standard retinoid mixture on the Nucleosil column.

The use of BHT as an antioxidant in the extraction procedure resulted in a BHT peak on the Nucleosil column with a retention time of 6 min, which did not disturb the measurements. However, on the Hypersil column BHT eluted after 11 min, and hence interfered with the peaks of β-apo-10′-carotenal and β-apo-8′-carotenal. In a first trial without BHT no negative effects were found, therefore in further studies BHT was not used if the presence of β-apocarotenals was expected in a sample.

To calculate recoveries, liver samples to which known amounts (comparable to endogenous levels) of β-carotene, retinoic acid, retinol and retinal had been added were analysed in duplicate together with untreated samples. Recoveries, (mean ± SD) were 102.5 ± 0.7% for β-carotene, 102.5 ± 10.6% for retinoic acid, 95.5 ± 4.9% for retinol and 95.5 ± 6.4% for retinal.

Assuming that the signal-to-noise ratio should be at least 3, and using an injection volume of 50 µl, the detection limits of the method correspond to a 10 ng/mL standard solution for retinoids and carotenoids and 60 ng/mL for α-tocopherol and α-tocopheryl acetate.

Using the method described it is possible to measure more compounds than, to the authors' knowledge, are described in the literature, using not more than two systems.

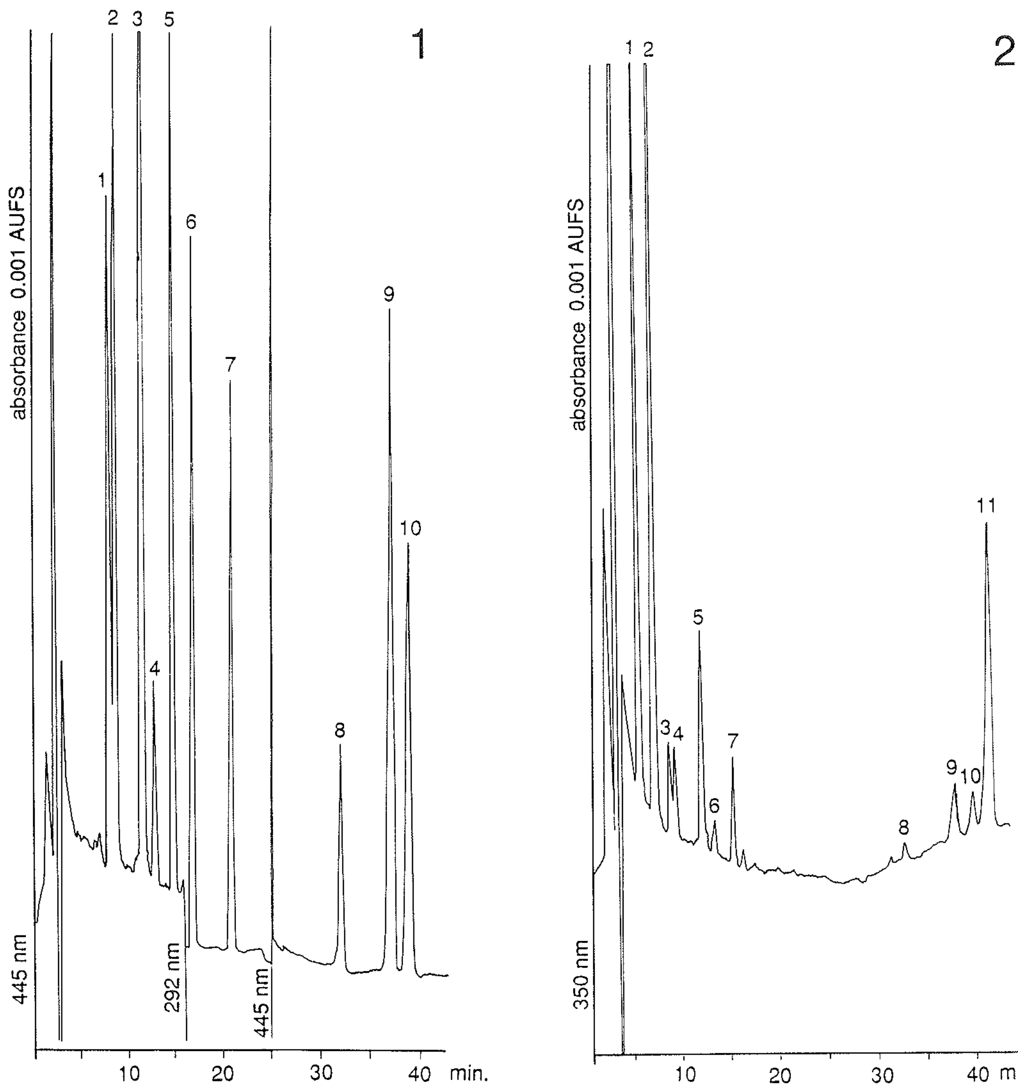


Figure 1. HPLC elution profile of a mixture of reference compounds after injection onto the Hypersil column and detection at a wavelength switching from 445 to 292 nm and back to 445 nm. Peaks: 1, β-apo-12′-carotenal; 2, β-apo-10′-carotenal; 3, β-apo-8′-carotenal; 4, β-apo-6′-carotenal; 5, ethyl β-apo-8′-carotenoate; 6, α-tocopherol; 7, α-tocopheryl acetate; 8, γ-carotene; 9, α-carotene; 10, β-carotene.

Figure 2. HPLC elution profile of a mixture of reference compounds after injection onto the Hypersil column and detection at a wavelength of 350 nm. Peaks: 1, retinol; 2, retiny acetate; 3, β-apo-12′-carotenal; 4, β-apo-10′-carotenal; 5, β-apo-8′-carotenal; 6, β-apo-6′-carotenal; 7, ethyl β-apo-8′-carotenoate; 8, γ-carotene; 9, α-carotene; 10, β-carotene; 11, retinyl palmitate.

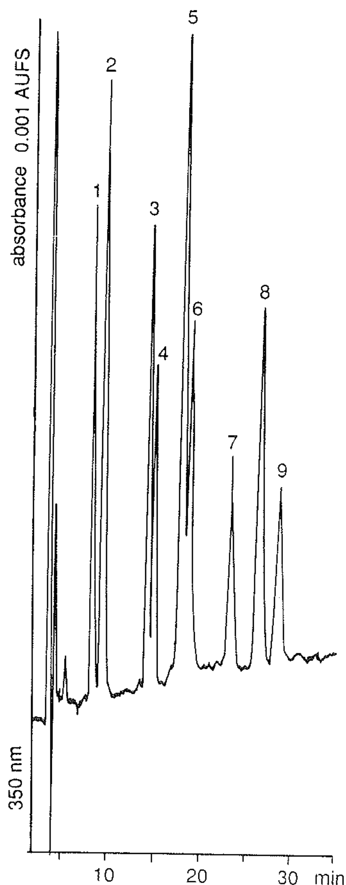


Figure 3. HPLC elution profile of a mixture of reference compounds after injection onto the Nucleosil column and detection at a wavelength of 350 nm. Peaks: 1, 13-cis-retinoic acid; 2, all-trans-retinoic acid; 3, 13-cis-retinol; 4, all-trans-retinol; 5, 9- and 13-cis-retinal; 6, all-trans-retinal; 7, etretinate; 8, 13-cis-retinyl actetate; 9, all-trans-retinyl acetate.

Application to tissue samples

Two groups, each of five female Wistar rats (post-weaning) were fed regular laboratory feed (containing 2 µg/g retinol) supplemented with either 2 mg/g β-carotene or 2 mg/g canthaxanthin (no provitamin A activity). β-Carotene and canthaxanthin were added as beadlets (a gift from Hoffmann-La Roche, Basel, Switzerland). The rats had free access to food and water and consumed around 10 g of food per day.

After 2 weeks the rats were sacrificed. The animals were anaesthetised with diethyl ether, blood was collected by cardiac puncture and whole body perfusion with 0.9% (w/v) NaCl was carried out before the tissues were collected. The Tissues were immediately frozen in liquid nitrogen and stored at –80°C. Plasma was separated from the blood and also stored at –80°C.

Retinyl stearate and canthaxanthin were measured using the Hypers column. Retinyl stearate was detected at 350 nm and eluted about 10 min afte retinyl palmitate. Canthaxanthin was detected at 445 nm and eluted at th same time as β-apo-10′-carotenal. As canthaxanthin and β-apo-10′-carotena were not expected to be present together in the samples, this was no rea problem. In fact, by using more water in the eluent, canthaxanthin can b separated from the apocarotenals.

Figures 4 and **5** show the chromatographic traces of a lung sample analyse on the Hypersil column. In this example the detection was carried out with on detector set at 445 nm and the second switching from 350 to 292 nm, the returning to 350 nm. The Nucleosil column was used to determine retinoic acid

Table 1 shows the results of the plasma and tissue analyses. Variabl amounts of β-carotene and canthaxanthin were stored in subcutaneous fat i which 1 - 105 μg/g β-carotene and 2 - 113 μg/g canthaxanthin were found.

Apocarotenals could not be confirmed in any sample. In liver and lun samples some indications for apocarotenals were found, but these did not excee the detection limits.

From Table 1 it can be concluded that the rats were able to conve β-carotene to retinol, as the rats fed β-carotene showed higher levels of retin in liver, lung and mamma than rats fed canthaxanthin. Furthermor β-carotene was found in tissues, so the rats were also able to transpo unconverted β-carotene to the liver, lung and mamma. Canthaxanthin was als transported to these organs.

Table 1. Retinoid and carotenoid concentrations in plasma and tissues of rats[1].

Compound	Plasma (μmol/l)		Liver (μg/g)		Lung (μg/g)		Mamma (μg/g)	
	1	2	1	2	1	2	1	2
Retinol	0.62	0.63	7.8	1.3	3.61	0.67	0.38	0.09
Retinyl palmitate	-	-	395	48	18.9	5.6	0.63	-
Retinyl stearate	-	-	61	8.5	8.15	2.75	-	-
Retinoic acid	-	-	0.09	-	0.03	-	0.05	0.01
β-Carotene	0.33	-	51	0.35	2.10	-	0.09	-
α-Carotene	-	-	1.19	0.08	-	-	-	-
Canthaxanthin	-	0.73	-	294	-	6.44	-	2.38
α-Tocopherol	24.4	37.8	61.6	53.0	28.7	37.3	36.7	22.6

[1] Rats had been fed a diet containing either 0.2% β-carotene (group 1) or 0.2% canthaxanthin (group 2) for 2 weeks.

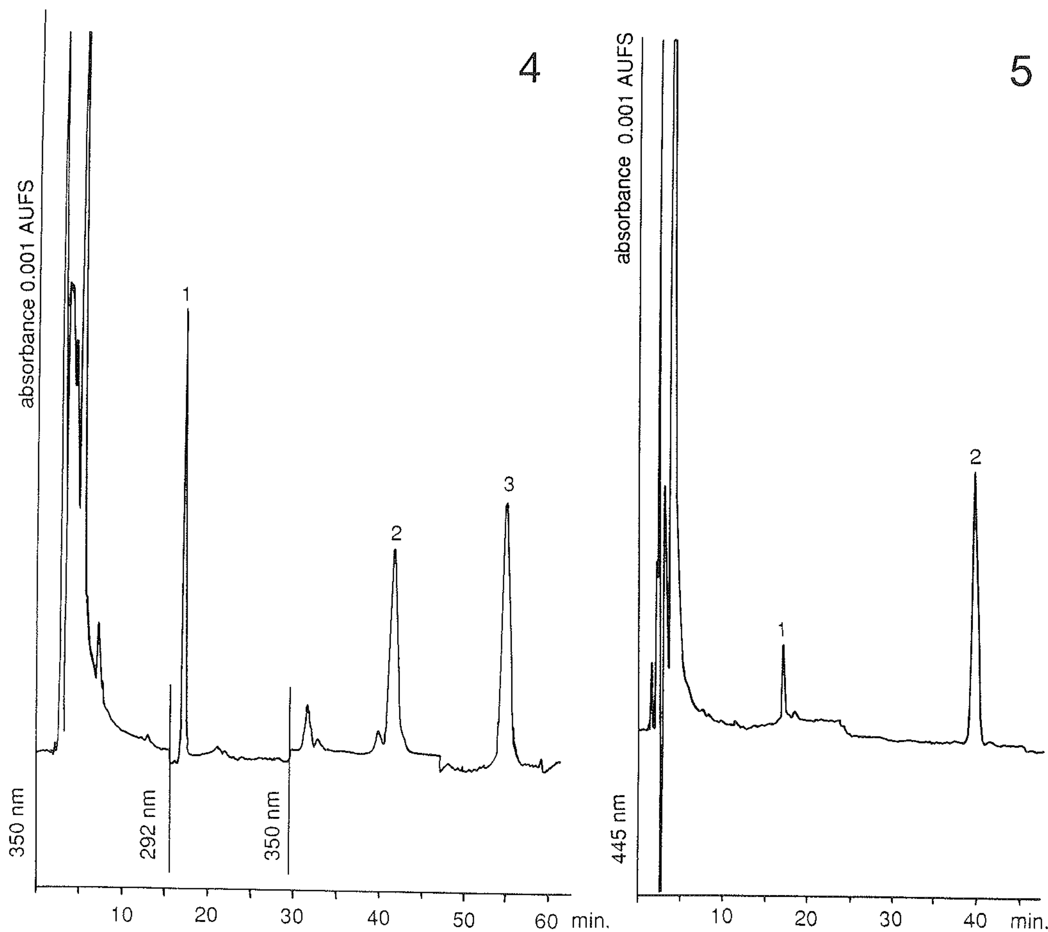


Figure 4. *HPLC elution profile of a lung sample after injection onto the Hypersil column and detection at a wavelength switching from 350 to 292 nm and back to 350 nm. Peaks: 1, α-tocopherol; 2, retinyl palmitate; 3, retinyl stearate.*

Figure 5. *HPLC elution profile of a lung sample after injection onto the Hypersil column and detection at a wavelength of 445 nm. Peaks: 1, α-tocopherol; 2, β-carotene.*

Application to β-carotene 15,15'-dioxygenase assay samples

The proximal 60 cm of the small intestine was removed from male or female Wistar rats and flushed with ice-cold 0.9% NaCl. The mucosa was scraped off in 0.1 mol/L potassium phosphate buffer and cytosol was isolated by differential centrifugation. Incubation was carried out as described by Goodman et al.[12] with 200 µl of cytosol (4 mg of protein), 0.125 g/l α-tocopherol and 1 µg of β-carotene in 0.1 mol/L potassium phosphate buffer, pH 7.7, containing 15 mmol/L

nicotinamide, 2 mmol/L $MgCl_2$, 5 mmol/L glutathione, 1.7 mmol/L sodiu dodecyl sulphate and 0.2 g/l L-α-phosphatidylcholine. After 1 h incubation wi 1 μg β-carotene, 10 - 20 ng of retinal were formed. To optimise the measureme of retinal, the wavelength can be switched to 380 nm. No other products cou be demonstrated, although only about 80% of the added β-carotene was le More experiments are in progress to optimise the assay and to find out wheth other products are formed and whether the β-carotene is partially lost duri the procedure.

From the results obtained, no definite conclusion can be drawn as to t correct β-carotene cleavage theory. To study the β-carotene cleavage furthe experiments with intestinal cell lines are currently being undertaken and *vivo* experiments with rats are being planned.

References

1. Moon RC. Comparative aspects of carotenoids and retinoids as chemopreventive agents f cancer. J Nutr 1989;119:127-134.
2. Peto R, Doll R, Buckley JD and Sporn MB. Can dietary beta-carotene materially reduce human cancer rates? Nature 1981;290:201-208.
3. Hill DL and Grubbs CL. Retinoids as chemopreventive and anticancer agents in intact animals (review). Anticancer Research 1982;2:111-124.
4. Goodman DS, Blomstrand R, Werner B, Huang HS and Shiratori T. The intestinal absorption and metabolism of vitamin A and β-carotene in man. J Clin Invest 1966;45:1615-1623.
5. Temple NJ and Basu TK. Protective effect of β-carotene against colon tumors in mice. JNCI 1987;78:1211-1214.
6. Ganguly J and Sastry PS. Mechanism of conversion of β-carotene into vitamin A- Centr cleavage versus random cleavage. Wld Rev Nutr Diet 1985;45:198-220.
7. McCrehan WA and Schönberger E. Determination of retinol, α-tocopherol, and β-caroter in serum by liquid chromatography with absorbance and electrochemical detection. Clin Chem 1987;33:1585-1592.
8. Aaran R-K and Nikkari T. HPLC method for the simultaneous determination of beta-carotene, retinol and alpha-tocopherol in serum. J Pharm Biomed Anal 1988;6:853-857.
9. Thurnham DI, Smith E and Flora PS. Concurrent liquid-chromatographic assay of retin α-tocopherol, β-carotene, α-carotene, lycopene, and β-cryptoxanthin in plasma, with tocopherol acetate as internal standard. Clin Chem 1988;34:377-381.
10. Furr HC. Gradient reversed-phase high-performance liquid chromatographic separation naturally occurring retinoids. J Chromatogr 1984;309:299-307.
11. Hansen S and Maret W. Retinal is not formed in vitro by enzymatic central cleavage of β-carotene. Biochemistry 1988;27:200-206.
12. Goodman DS, Huang HS, Kanai M and Shiratori T. The enzymatic conversion of all-tra β-carotene into retinal. J Biol Chem 1967;242:3543-3554.
13. Dawson RMC, Elliott DC, Elliott WH and Jones KM. Data for Biochemical Research. 3r ed. Oxford: Clarendon Press, 1986:240.

14. Fragner J. Vitamine, Chemie und Biochemie. Jena: Gustav Fischer Verlag, 1964, Band I: 185.
15. Isler O and Schudel P. Synthese und Markierung von Carotinen und Carotinoiden. In: Lang K, ed. Wissenschaftliche Veröffentlichungen der Deutschen Gesellschaft für Ernährung. 9. Carotine und Carotinoide. Darmstadt: Dietrich Steinkopff Verlag, 1963:54-104.

Chapter 4

In vitro measurement of β-carotene cleavage activity: methodological considerations and the effect of other carotenoids on β-carotene cleavage

Trinette van Vliet[1,2], Frank van Schaik[1], Wil H.P. Schreurs[1,2] and Henk van den Berg[1]

[1] TNO Nutrition and Food Research Institute, Zeist, The Netherlands; [2] Department of Experimental Zoology, University of Amsterdam, Amsterdam, The Netherlands

Submitted for publication

Abstract

In view of controversies about assessment of the β-carotene cleavage activity, methodological aspects and problems of the dioxygenase assay are described. Using rat and hamster intestinal preparations the method was optimized on retinal formation, the only cleavage product we could demonstrate. It appeared that the cell fraction with the highest cleavage activity was the 9,000 *g* supernatant (S-9). Maximal retinal formation was obtained with Sodium dodecyl sulphate, taurocholate and egg lecithin in the buffer and 3 µg β-carotene dissolved in acetone. Ethanol, THF/DMSO (1:1) or propylene glycol as solvent for β-carotene reduced retinal formation to 55, 24, and 19%, respectively. Retinal formation increased proportionally with the amount of protein S-9 used and was linear up to 40 - 60 minutes of incubation. Incubation with α-carotene or β-cryptoxanthin resulted in a retinal formation of 29 and 55% of the amount formed from β-carotene. Addition of 9 µg of lutein to an incubation with 3 µg β-carotene reduced retinal formation, while lycopene had no effect. In conclusion, the β-carotene cleavage assay with S-9 as enzyme source described in this report, seems a useful tool to study (dietary) determinants of β-carotene cleavage activity, but for other purposes adaptation of the method is required.

Introduction

The oldest known function of β-carotene in humans is as source of vitamin which function is especially important in those parts of the world where t intake of animal food products is relatively low. In addition, intake of foods ri in carotenoids is reported to be associated with a reduced risk for certain typ of cancer[1]. Whether the active agent is β-carotene, vitamin A or another produ formed from β-carotene, such as retinoic acid, has still to be elucidated.

The first step of β-carotene metabolism is the intestinal cleavage to retir by the enzyme β-carotene 15,15′-dioxygenase (EC 1.13.11.21), first described 1965 by Olson and Hayaishi[2] and Goodman and Huang[3]. Although the enzyı has been known for almost 30 years now, it has only partially been purified a characterized and several controversies about the enzyme have arisen since first description. Important issues relate to the type of reaction products as w as the stoichiometry of the reaction. In most studies retinal has been identifi as the main or even only reaction product[2-11]. However, the molar ratio of retir formed to β-carotene consumed was either not determined or ranged from 0.9 1.8[11] and the specificity for the 15,15′ double bond of β-carotene has not be established. The possibility of eccentric cleavage was shown by Wang et al who reported the formation of apocarotenals (using a quite different metho Retinoic acid has also been identified as a reaction product of β-carotene eith through eccentric cleavage[13] or through an unidentified pathway[14,15].

If the functional relation between β-carotene and its products is to be studi properly, the availability of an adequate enzyme assay becomes essential. Tl paper deals with the methodology of the β-carotene cleavage assay, bett known as the dioxygenase assay. In addition we compare cleavage activi towards α-carotene, β-cryptoxanthin, lutein and lycopene alone or together wi β-carotene in the assay.

Materials and methods

Chemicals

Tritium-labelled β-carotene (15,15′), β-apo-10′-carotenal, β-apo-12′-caroter and β-cryptoxanthin were gifts from Roche (Mijdrecht, The Netherland β-apo-8′-carotenal was purchased from Fluka (Buchs, Switzerland). β-Caroter $MgCl_2$, THF (pro analysis) and DMSO (pro synthesis) were purchased fro Merck (Darmstadt, Germany). Retinal, retinol, retinoic acid, retinyl aceta

α-tocopherol, α-tocopheryl acetate, α-carotene, lycopene, lutein, NAD, NADH, sodium dodecyl sulphate (SDS), glutathione, taurocholate, glycocholate, and L-α-phosphatidylcholine (type III-E, egg yolk) were obtained from Sigma (St. Louis, MO). Propylene glycol was purchased from BDH Chemicals (Poole, UK) and acetonitrile from Westburg (Leusden, The Netherlands). All other organic solvents used were of analytical-reagent or HPLC grade.

Preparation of solutions

Tritium-labelled β-carotene was purified just before use on an ODS-Hypersil column with acetonitrile - methanol - methylene chloride (700:150:50, v/v/v) as mobile phase. After three passages over the column a purity of 70 - 75% was reached with a specific activity of 19.9 μCi per mg β-carotene. Samples were counted in an Isocap 100 scintillation counter (Searl, Chicago Nuclear) using Lipo-Luma (Lumac-LSC, Olen, Belgium) as scintillation liquid.

A solution of β-carotene in propylene glycol was prepared by first dissolving the β-carotene in a small volume of methylene chloride (0.6 mL methylene chloride in 50 mL propylene glycol). A solution of β-carotene was prepared in 50 mmol/L potassium phosphate buffer containing 69 mmol/L triolein and 6.3 mmol/L glycocholate by sonication in a ultrasone bath. Preparation of all β-carotene solutions and all sample handlings were performed in yellow light.

Preparation of intestinal homogenates

After an overnight fast the small intestine of male Golden hamsters or the proximal 60 cm of the small intestine of male or female Wistar rats were removed and flushed with 0.9% NaCl (0 - 4°C). The intestines were either packed in aluminium foil, frozen in liquid nitrogen and stored at –80°C for later processing, or directly processed as follows. The intestine was cut lengthwise, the mucosa was scraped off with a glass slide and homogenized with a Teflon pestle in about 1 mL buffer per 30 cm intestine. A potassium phosphate buffer, 100 mmol/L, pH 7.7, containing 4 mmol/L $MgCl_2$ and 30 mmol/L NAD was used. The homogenate was centrifuged at 9,000 *g* for 20 min, yielding a 9,000 *g* supernatant (S-9). The supernatant was centrifuged, using a Beckmann ultracentrifuge with 50.2 TL rotor, at 105,000 *g* for 70 min, yielding a supernatant of cytosol and a pellet containing microsomes, light mitochondria and lysosomes. All handlings were carried out on ice and refrigerated centrifuges (4°C) were used. Enzyme preparations obtained were frozen in

liquid nitrogen and stored at –80°C. Intestinal S-9 could be stored at –80°C f
at least 10 months without substantial loss of its original retinal-formir
activity. Protein was determined using BioRad protein assay (Münche
Germany) with BSA type V (Sigma, St Louis, MO) as standard.

Final assay protocol

The incubation mixture consisted of 2.0 mL of 100 mmol/L potassium phospha
buffer, pH 7.7, containing 15 mmol/L nicotinamide, 2 mmol/L $MgCl_2$, 5 mmol
glutathione, 1.7 mmol/L SDS, 6 mmol/L taurocholate and 0.2 g
L-α-phosphatidylcholine. After addition of 25 µL α-tocopherol solution
ethanol (final concentration 0.125 g/L) and 3 µg of β-carotene in 50 µL aceton
the reaction was started by the addition of 3.0 mg of S-9 protein. Incubation wa
carried out in reaction vials in a shaking water bath at 37°C in the dark for 1
Control vials were run in all experiments; one without added β-carotene, ar
one without added or with a heat inactivated enzyme preparation.

Extraction and HPLC of carotenoids and retinoids

To stop the reaction and to precipitate proteins, the incubate was mixed wi
2 mL of ethanol containing retinyl acetate as internal standard for retino
analyses and α-tocopheryl acetate as internal standard for carotenoid analyse
After 10 min, 4 mL of hexane was added and the sealed tubes were vortexed f
4 min. After centrifugation the hexane layer was separated and evaporate
under nitrogen at room temperature. The residues were dissolved in eluent (f
retinoid analyses) or water-free eluent (for carotenoid analyses) and transferre
into brown HPLC injection vials. Using hexane complete extraction wa
obtained for retinol and retinal, while retinoic acid was extracted for 80%.

HPLC analyses were performed as described in detail[16] with sligl
modifications. Briefly, 125 mm × 4.6 mm I.D. columns packed in the laborator
were used. The retinoids were separated on a Nucleosil 120-3 C_{18} colum
(Machery-Nagel, Düren, Germany) with acetonitrile - 100 mmol/L ammoniu
acetate (75:25, v/v) as mobile phase. The ammonium acetate was acidified
pH 4.65 to get a sharp peak for retinal. Carotenoids were separated on
Hypersil ODS 3 µm column (Shandon Southern Products, Astmoor, UK) with
mobile phase consisting of acetonitrile - methanol - methylene chloride - wat
(70:15:10:5, v/v/v/v). Normal flow-rate was 1.0 mL/min, but when specifical
searching for apocarotenals a flow programme of 0.5 - 2.0 mL/min was use

Detection was carried out with programmable absorbance detectors. Retinal was measured at 380 nm and other retinoids at 325 nm, while carotenoids were monitored at 445 nm and α-tocopheryl acetate at 292 nm.

Experiments and results

Optimization of assay conditions

We implemented the assay as described by Goodman et al.[17], with cytosol from rat intestine as the enzyme source. The optimal concentration we found for SDS (500 mg/L) and glutathione (5.0 mmol/L) were the concentrations used by Goodman et al.[17]. Distribution of activity along the small intestine was studied by comparing activity in cytosol obtained from mucosal scrapings of the proximal, middle and distal 30 cm of the small intestine of 10 rats. In the distal part no activity could be demonstrated, while activity in the proximal part was about 65% of the activity in the middle part.

To compare activity in different subcellular fractions, intestinal mucosal scrapings of 6 hamsters were homogenized with 8 mL buffer and part of the total homogenate was used for the preparation of S-9, cytosol and the 105,000 *g* pellet. Incubations with 120 µL total homogenate or equivalent amounts of the other cell fractions were carried out in triplicate. The results are presented in **Table 1**. Dioxygenase activity was only found in the soluble cell fractions with no activity in the pellet fraction. Highest activity was found in the S-9 fraction, both absolute and when expressed on a protein basis. We therefore decided to use S-9 in our final assay.

Table 1. Dioxygenase activity in several subcellular fractions[1].

	Equivalent amounts[2]	Per mg protein
Total homogenate	63 ± 6	15 ± 2
S-9 (9,000 *g* supernatant)	72 ± 3	24 ± 1
Cytosol (105,000 *g* supernatant)	26 ± 4	16 ± 2
105,000 *g* pellet[3]	nd[4]	nd[5]

[1] ng retinal formed during 1 h incubation, mean ± SD of triplicates.
[2] Incubation with 120 µL total homogenate or equivalent amounts of S-9, cytosol or 105,000 *g* pellet.
[3] Contains microsomes, light mitochondria and lysosomes.
[4] Not detectable (< 6 ng retinal).
[5] Not detectable (< 1.5 ng retinal per mg protein).

We further optimized the assay on formation of retinal, the only product w could demonstrate. Results described below were obtained with hamster S- prepared as a large pool from the intestines of 60 hamsters. We found low intestinal cleavage activities in hamsters than in rats, but in both species w demonstrated retinal as enzymatic cleavage product, while no retinol, retino acid or apocarotenals could be demonstrated, and optimal assay conditions we found to be similar.

Under the final assay conditions (as described under the Materials an methods) a maximum activity was obtained with a substrate concentration 3 μg β-carotene in 2.175 mL (**Figure 1**). Retinal formation was linear up 40 - 60 min and gradually increased with the amount of protein S-9 use (**Figure 2**). We chose to use 3 mg of enzyme protein for each triplicate + contr incubation since 12 mg was the minimum amount available per anima Incubations were carried out for 60 min; at this time point retinal formatic was found to be linear with the amount of protein S-9 present (Figure 2, insert

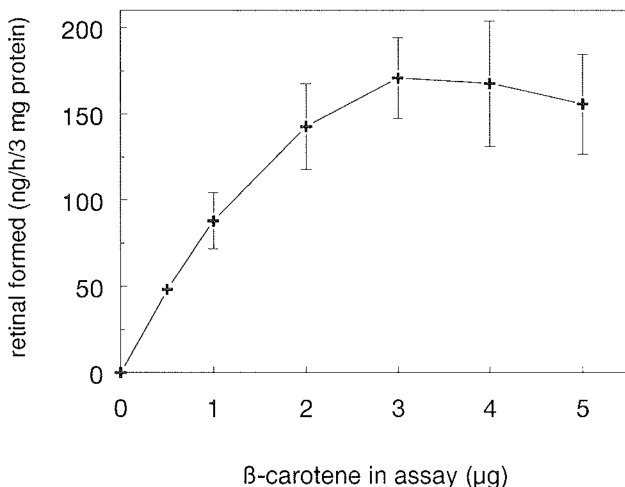


Figure 1. Amount of retinal formed in the dioxygenase assay after incubation for 1 h with 3 mg protein S-9 and different amounts of β-carotene. Mean with SD of triplicate incubations.

As the assay is carried out in an aqueous medium and β-carotene is a relative apolar compound, solubilization of the substrate is of extreme importance. W used different solvents for β-carotene and compared cleavage activity with th measured using β-carotene dissolved in ethanol set at 100%. β-Carotene

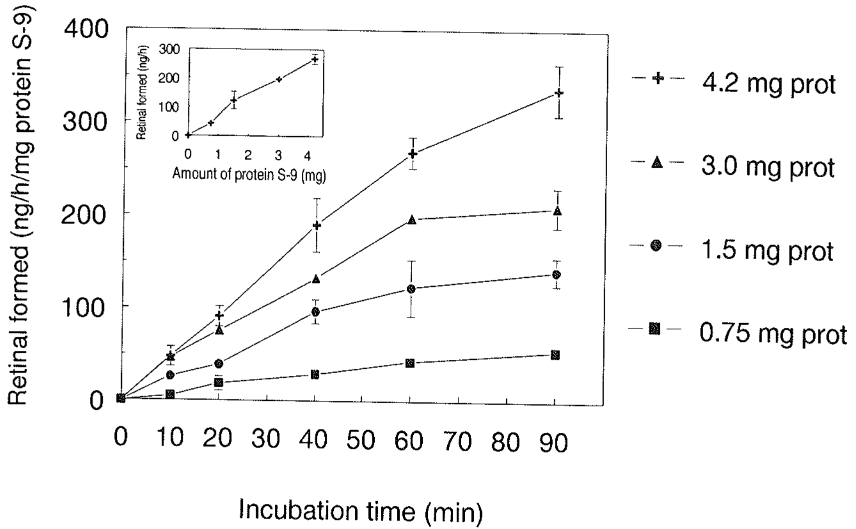


Figure 2. Amount of retinal formed in the dioxygenase assay after incubation for 10 - 90 min with 3 μg β-carotene and varying amounts of protein S-9. Mean with SD of triplicate incubations. Insert: Amount of retinal formed after incubation for 1 h with increasing amounts of protein S-9.

THF/DMSO (1:1) or propylene glycol decreased the formation of retinal to respectively 43% and 34%, whereas acetone increased the retinal formation to 182%. No retinal could be detected after addition of a β-carotene-triolein-glycocholate solution.

Apart from the solvent used for β-carotene, the addition of solubilizing agents to the incubation mixture seems important. In the absence of bile salts, SDS was found to be essential to measure activity, independent of the other components of the buffer and the solvent used for β-carotene. In the presence of SDS, bile salts and phosphatidylcholine enhanced retinal formation. The optimal concentration of taurocholate or glycocholate in the buffer was 6 mmol/L, resulting in a 50% increase in activity. For phosphatidylcholine the optimal concentration was 0.2 g/L, resulting in a 60% increase in activity. Thus, in our final method we used SDS, taurocholate (6 mmol/L) and phosphatidylcholine (0.2 g/L) in the incubation mixture and added β-carotene dissolved in acetone.

Cleavage products

Using the assay method described above, with either cytosol or S-9 as enzyme source, retinal was the only enzymatic cleavage product we found. Retinoic acid

was not demonstrated (detection limit 10 ng) and although small amounts
retinol were present in part of the enzyme preparations, no enzymatic formati
of retinol could be demonstrated (detection limit 10 ng). A chromatogram of
mixture of reference compounds is shown in **Figure 3**, together with a typic
chromatogram of reaction products extracted from the incubation mixture, a
a similar picture after incubation without β-carotene. Apocarotenals could al
not be demonstrated (detection limit 20 ng). The enzyme preparations gave ri
to small disturbing peaks, but no difference in peak pattern could be se
between incubations with and without β-carotene.

To make sure that no products other than the already searched for we
formed in our assay, incubations with tritium-labelled β-carotene we
performed. After 1 h incubation with 1.1 μg ^{3}H-β-carotene, 4.7% of the label w
found in the retinal fraction, while only the background activity of ca 1% of t
label was present in this fraction after incubation without enzyme (**Figure**
Because after incubation with and without enzyme activity was distribut
similar (except for the retinal peak), no unknown products seemed to be forme
Although the sensitivity of this assay was rather low (detection limit for oth
products was 25 ng), the result confirms that retinal is the main product and
only formed during incubation with the enzyme preparation.

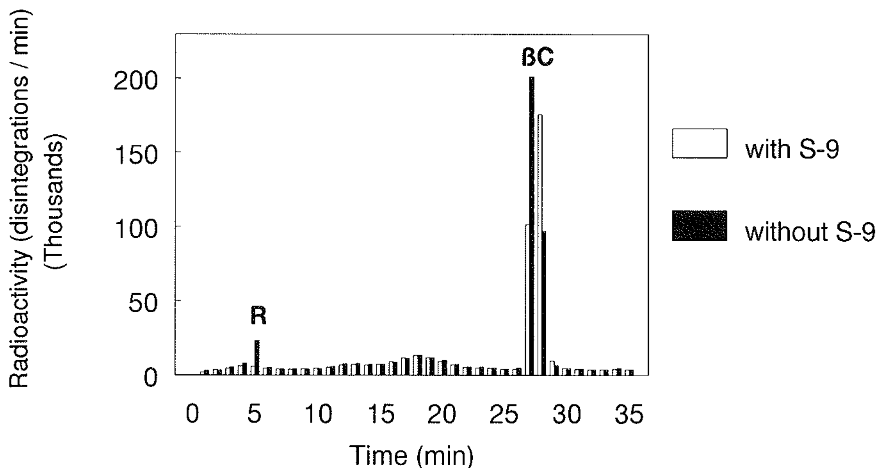


Figure 4. Radioactivity in column fractions after injection on a Hypersil column with acetonitril - methanol - methylene chloride (700:150:50, v/v/v) as mobile phase of extracts from incubation with ^{3}H-β-carotene with or without S-9; R, retinal; βC, β-carotene.

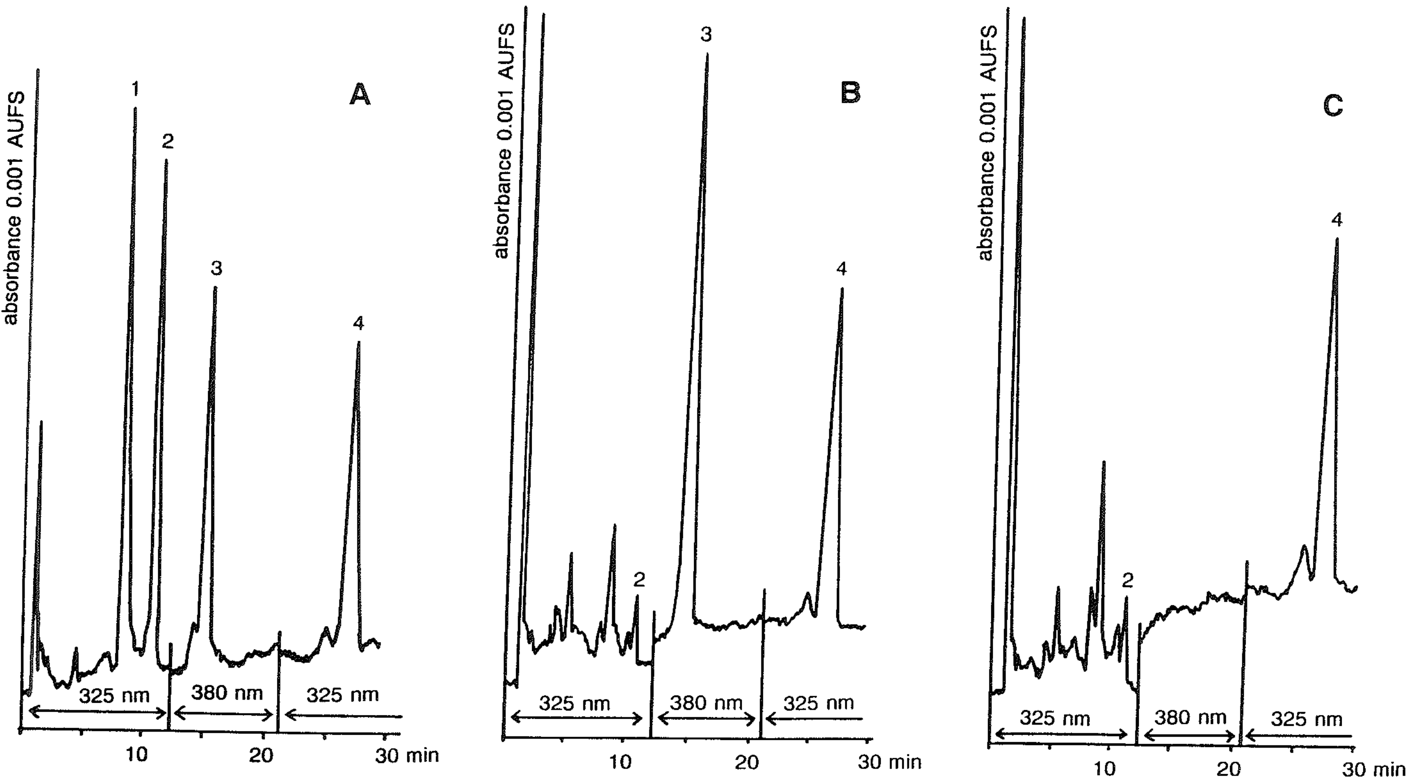


Figure 3. *HPLC chromatograms from injections onto the Nucleosil column with the detector switching from 325 to 380 nm and back to 325 nm. Peaks: 1, all-trans-retinoic acid; 2, all-trans-retinol; 3, all-trans-retinal; 4, all-trans-retinyl acetate (internal standard). A: A mixture of reference compounds. B: An extract from an incubation with S-9 as enzyme source and β-carotene. C: An extract from an incubation with heat-inactivated S-9 and β-carotene.*

To evaluate whether incubation conditions might determine the type
reaction product we carried out the assay described by Wang and colleagues
albeit with S-9 as enzyme source. Using their conditions, however, no retin
(detection limit 2.5 ng) or apocarotenals (detection limit 20 ng) could
detected. To assess whether the type of reaction product could be affected
inhibition or stimulation of the reduction of retinal to retinol, either NAD
NADH was added to the incubation, but this did not affect the results.

Effect of other carotenoids

To assess the activity of the enzyme towards other carotenoids and the potenti
interference of these carotenoids with β-carotene cleavage, incubations we
carried out with 3 µg β-carotene, α-carotene, β-cryptoxanthin, lycopene or lute
alone, and with 3 µg β-carotene combined with either 3 or 9 µg of one of t
other carotenoids, all in triplicate. In one experiment β-carotene, α-carotene a
lycopene were used and in a second experiment β-carotene, β-cryptoxanthin a
lutein were used. **Table 2** shows the amount of retinal formed after incubati
with the different substrates. Lycopene and lutein were not converted by t
enzyme, while incubation with α-carotene resulted in the formation of 24
retinal and a compound eluting just before retinal, probably α-retinal (in
similar amount assuming similar extinction coefficients). Incubation wi
β-cryptoxanthin resulted in retinal as the sole product (49 ng). Retin
formation from β-carotene was slightly lower after addition of 3 µg
α-carotene, β-cryptoxanthin or lutein, while a substantial reduction was fou
after addition of 9 µg α-carotene and, especially, lutein.

Discussion

Measuring *in vitro* dioxygenase activity seems a useful tool to study (dietar
determinants of β-carotene cleavage activity. Remarkably, this enzyme, i.
enzymatic pathway, has not yet been fully characterized although the enzym
and a procedure for activity measurement were already reported in 1965[2
Several controversies have arisen over the years and still exist, especially on t
type of reaction products. When implementing the assay in our laboratory
studied some methodological aspects. Rat and hamster intestinal mucosa we
used as the source of the enzyme in our studies and since most reported studi
used rat intestinal preparations we will focus in this discussion mainly
studies using the rat intestinal enzyme.

Table 2. Amount of retinal formed (ng) after incubation with different carotenoids or combinations for 1 h[1].

Carotenoid	Substrate		
	Carotenoid 3 µg	Carotenoid 3 µg + β-carotene 3 µg	Carotenoid 9 µg + β-carotene 3 µg
β-Carotene	87 ± 7 (100)[2]		
α-Carotene	24 ± 8 (29)	72 ± 14 (85)	57 ± 15 (68)
β-Cryptoxanthin	49 ± 17 (55)	73 ± 14 (82)	72 ± 13 (81)
Lutein	nd[3]	73 ± 15 (82)	40 ± 4 (45)
Lycopene	nd	79 ± 13 (93)	89 ± 10 (106)

1 Values are means ± SD of triplicates (% of the amount formed after incubation with 3 µg β-carotene alone in the same experiment).
2 Mean of two experiments.
3 nd, not detectable (< 6 ng).

Although dioxygenase is a cytosolic enzyme, we found the highest cleavage activity with S-9 as enzyme source. The 105,000 *g* pellet fraction did not contain cleavage activity, but did stimulate β-carotene conversion, as was already reported by Goodman et al.[17].

Solubilization of the apolar β-carotene into an aqueous assay medium seems a very important aspect. After addition of β-carotene in an organic solvent, β-carotene will probably migrate to micelles formed in the incubation mixture with SDS, bile salts, egg lecithin, or any combination. Saturation of the micelles with β-carotene may partly explain the plateau in retinal formation occurring with increasing substrate concentration. The optimum substrate concentration we found is in agreement with the results of Goodman et al.[17], who reported only a very slight increase in activity with addition of more than 3 µg β-carotene. We estimated the apparent K_m from a Lineweaver-Burk plot of our results to be 1.8×10^{-6} M, while Goodman et al.[17] reported a value of 3.3×10^{-6} M.

Although in our assay SDS was essential, cleavage activity has been reported in the absence of detergents or lipids in the incubation buffer but with β-carotene added in Tween[2,4], DMSO[15] or propylene glycol[12]. Apparently, the combination of detergents together with the solvent used for β-carotene determines the cleavage activity measured.

Under our experimental conditions we found retinal as enzymatic cleavage product of β-carotene, while other reported cleavage products such as retinol, apocarotenals and retinoic acid, could not be demonstrated. *In vivo*, intestinally formed retinal will be bound to cellular retinol binding protein type II (CRBP II)

and reduced by a microsomal retinal reductase to retinol[18]. CRBP II bou
retinol is subsequently esterified and incorporated in chylomicrons f
transport. Alternatively, free retinal can be reduced either by the microsom
retinal reductase or by a cytosolic retinal reductase described by Fidge a
Goodman[19]. The pH optimum for the microsomal reductase is between 5.0 a
6.0 and for the cytosolic reductase 6.3, and both are NAD(P)H-dependent[18,
Although *in vivo* intestinal formation of retinoic acid from β-carotene has be
reported[20], it is unclear whether retinoic acid is formed through retinal
through apocarotenals as was demonstrated *in vitro*[13]. A soluble enzyme for t
conversion of retinal into retinoic acid was found in rat intestinal mucosa[21]. T
enzyme had a pH optimum of 7.7 and an apparent K_m of 0.3 mmol/L and w
stimulated by NAD^+ and inhibited by NADH[21]. Whether free or CRBP II bou
retinal is converted by this enzyme is unknown.

Thus the metabolites formed using an *in vitro* assay will largely depend
the enzyme fraction and incubation conditions used, such as pH and presence
cofactors. Indeed, most differences in reported metabolites go together wi
methodological differences. Apocarotenals were only demonstrated by Wang a
Tang and colleagues[12,22], who used a crude enzyme preparation (S-0.8) and a p
of 7.35. The main products were β-apo-13-carotenone and apocarotenals, wh
the small amounts of retinal and retinoic acid formed seemed to be seconda
products from apocarotenals, since these products could only be demonstrat
after 15 min of incubation. Since we did not find apocarotenals or any unkno
enzymatically formed peak under the incubation conditions described by Wa
et al.[12], another enzyme(s) seems to be present. Alternatively, the eccent
cleavage enzyme(s) are unstable during storage at –80°C. Analytical sensitiv
may, of course, also affect the results. We cannot exclude that apocarotena
were also formed under our assay conditions, but in amounts below o
detection limit (ca. 20 ng).

In almost all studies using cytosol preparations and an assay pH of 7.7 to 8
retinal has been reported as the sole or main product[2-6,17,23]. One exception is t
study of Hansen and Maret[24] in which no enzymatic conversion of β-carote
could be demonstrated, possibly due to inactivity of the isolated enzyme
instability of retinal formed. At pH 7.7 to 8.0 activity of retinal reductase
probably negligible. This might also explain why the omission of NAD
addition of NADH in our assay did not affect product formation. Napoli a

Race[15], using a pH of 7.0, did find retinol, however the lack of effect of addition or omission of NAD and NADH cannot be explained.

The conditions resulting in the formation of retinoic acid are less clear. Both Napoli and Race[15] and Crain et al.[14] have reported the formation of retinoic acid. However, the formation reported by the latter group in the presence of NADH is in contrast with the results of Moffa et al.[21] and suggests the existence of another retinoic acid forming pathway. Retinoic acid formation through β-apocarotenals and β-apocarotenoic acids has been suggested[12,25], however, also for this pathway, stimulation by NADH cannot be explained. In our assay retinal might have been CRBP II bound, while the retinoic acid forming enzyme possibly uses only free retinal as substrate. Alternatively, the retinoic acid forming enzyme is unstable during storage at –80°C as we used.

In view of the above described results it will be clear that the products formed in the β-carotene cleavage assay mainly depend on the conditions used, which depend on the purpose of the study. Since more than one enzyme is involved in β-carotene metabolism, to evaluate *in vivo* overall products of β-carotene metabolism a complete system, or at least an intact cell system has to be used.

All carotenoids containing one unsubstituted β-ionone moiety are potential precursors of retinal. The most common dietary carotenoids, α-carotene and β-cryptoxanthin with provitamin A activity and lutein and lycopene without provitamin A activity, were tested in the assay, both alone and together with β-carotene. Retinal formed from α-carotene and β-cryptoxanthin was only 29% and 55%, respectively, of the amount formed from equal amounts of β-carotene. However, we did find a 'second peak' in the chromatogram after incubation with α-carotene, probably α-retinal as was reported by Singh and Cama[7]. Their study showed a total product formation compared to β-carotene of 56% for α-carotene as we found, but only 3% for β-cryptoxanthin. Reported provitamin A activities for α-carotene and β-cryptoxanthin measured in bioassays were 50 - 54% and 50 - 60%, respectively, compared to β-carotene[27].

Lutein and lycopene are not precursors of retinal, but the results of the combined incubations suggest that lutein, but not lycopene, may interact with the enzyme. In Figure 1 we showed that retinal formation was maximal with 3 μg β-carotene. In this experiment we did not run controls with 6 μg and 12 μg β-carotene as substrate to correct for a dilution effect. However, the reduction of

retinal formation after addition of 9 μg lutein is much larger than after additio
of lycopene, suggesting that lutein has an effect on β-carotene conversior
In vivo studies have also shown that liver vitamin A storage from β-carotene i
reduced by lutein[28], but not by lycopene[29,30]. While these studies could nc
discriminate between an effect on absorption per se or at the level of β-caroten
cleavage, our results suggest that β-carotene conversion is affected. Mor
recently, Ershov et al.[31], using the dioxygenase assay, reported 50 - 70
reduction of β-carotene conversion after addition of either lutein or lycopene i
amounts equal to β-carotene.

The *in vivo* consequence of the interactions we found will depend on th
substrate concentration *in vivo* and the proportions in which the carotenoid
occur in the diet. Since fruits and vegetables contain more β-carotene tha
α-carotene and much more β-carotene than β-cryptoxanthin[32], α-carotene an
β-cryptoxanthin are not expected to interfere with β-carotene conversion int
retinal and consequently vitamin A. However, major contributors to lutei
intake such as spinach and broccoli contain more lutein than β-carotene, whil
carrots, an important source of β-carotene, do not contain lutein at all[32,33]. Thu
the lutein/β-carotene ratio in the diet will depend on the composition of the die
but can well be above 1, and hence lutein might seriously affect th
provitamin A activity of β-carotene. This might contribute to an explanation fo
the vitamin A deficiency that is found in some developing countries in spite of
high intake of β-carotene by leafy vegetables.

In conclusion, the dioxygenase assay method described in this report with S-9 a
enzyme source seems an appropriate method to measure cleavage activity unde
different feeding conditions. However, for other purposes adaptation of th
method is required. Simple addition of intake of all provitamin A carotenoid
even taking into account their provitamin A activity, may overestimate the rea
provitamin A activity of the diet.

Acknowledgements

The authors want to thank André Wolterbeek for kindly providing us wit
hamster intestines. We thank Dr. Guido Haenen and Dr. Wim de Kort fo
critical reading of the manuscript and Dirk van der Heij for editorial advice.

References

1. van Poppel G. Carotenoids and cancer - an update with emphasis on human intervention studies. Eur J Cancer 1993;29A:1335-1344.
2. Olson JA and Hayaishi O. The enzymatic cleavage of β-carotene into vitamin A by soluble enzymes of rat liver and intestine. Proc Natl Acad Sci USA 1965;54:1364-1370.
3. Goodman DS and Huang HS. Biosynthesis of vitamin A with rat intestinal enzymes. Science 1965;149:879-880.
4. Lakshman MR, Mychkovsky I and Attlesey M. Enzymatic conversion of all-*trans*-β-carotene by a cytosolic enzyme from rabbit and rat intestinal mucosa. Proc Natl Acad Sci USA 1989;86:9124-9128.
5. Gronowska-Senger A and Wolf G. Effect of dietary protein on the enzyme from rat and human intestine which converts β-carotene to retinal. J Nutr 1970;100:300-308.
6. Villard L and Bates CJ. Carotene dioxygenase (EC 1.13.11.21) activity in rat intestine: effects of vitamin A deficiency and of pregnancy. Br J Nutr 1986;56:115-122.
7. Singh H and Cama HR. Enzymatic cleavage of carotenoids. Biochim Biophys Acta 1974; 370:49-61.
8. Lakshmanan MR, Chansang H and Olson JA. Purification and properties of carotene 15,15′-dioxygenase of rabbit intestine. J Lipid Res 1972;13:477-482.
9. Sklan D. Carotene-cleavage activity in chick intestinal mucosa cytosol: association with a high-molecular-weight lipid-protein aggregate fraction and partial characterization of the activity. Br J Nutr 1983;50:417-425.
10. Fidge NH, Smith FR and Goodman DS. Vitamin A and carotenoids. The enzymatic conversion of β-carotene into retinal in hog intestinal mucosa. Biochem J 1969;114: 689-694.
11. Olson JA. The alpha and the omega of vitamin A metabolism. Am J Clin Nutr 1969;22: 953-962.
12. Wang X-D, Tang G-W, Fox JG, Krinsky NI and Russell RM. Enzymatic conversion of β-carotene into β-apo-carotenals and retinoids by human, monkey, ferret, and rat tissues. Arch Biochem Biophys 1991;285:8-16.
13. Wang X-D, Krinsky NI, Tang G and Russell RM. Retinoic acid can be produced from excentric cleavage of β-carotene in human intestinal mucosa. Arch Biochem Biophys 1992; 293:298-304.
14. Crain FD, Lotspeich FJ and Krause RF. Biosynthesis of retinoic acid by intestinal enzymes of the rat. J Lipid Res 1967;8:249-254.
15. Napoli JL and Race KR. Biogenesis of retinoic acid from β-carotene: Differences between the metabolism of β-carotene and retinal. J Biol Chem 1988;263:17372-17377.
16. van Vliet T, van Schaik F, van Schoonhoven J and Schrijver J. Determination of several retinoids, carotenoids and E vitamers by high-performance liquid chromatography. Application to plasma and tissues of rats fed a diet rich in either β-carotene or canthaxanthin. J Chromatogr 1991;553:179-186.
17. Goodman DS, Huang HS, Kanai M and Shiratori T. The enzymatic conversion of all-*trans* β-carotene into retinal. J Biol Chem 1967;242:3543-3554.
18. Kakkad BP and Ong DE. Reduction of retinaldehyde bound to cellular retinol-binding protein (type II) by microsomes from rat small intestine. J Biol Chem 1988;263: 12916-12919.
19. Fidge NH and Goodman DS. The enzymatic reduction of retinal to retinol in rat intestine. J Biol Chem 1968;243:4372-4379.
20. Wang X-D, Russell RM, Marini RP, Tang G, Dolnikowski GG, Fox JG and Krinsky NI. Intestinal perfusion of beta-carotene in the ferret raises retinoic acid level in portal blood. Biochim Biophys Acta 1993;1167:159-164.

21. Moffa DJ, Lotspeich FL and Krause RF. Preparation and properties of retinal-oxidizing enzyme from rat intestinal mucosa. J Biol Chem 1970;245:439-447.
22. Tang G, Wang X-D, Russell RM and Krinsky NI. Characterization of β-apo-13-carotenone and β-apo-14′-carotenal as enzymatic products of the excentric cleavage of β-carotene. Biochemistry 1991;30:9829-9834.
23. Goodman DS and Olson JA. The conversion of all-Trans β-carotene into retinal. Methods Enzymol 1969;15:462-475.
24. Hansen S and Maret W. Retinal is not formed in vitro by enzymatic central cleavage of β-carotene. Biochemistry 1988;27:200-206.
25. Sharma RV, Mathur SN and Ganguly J. Studies on the relative biopotencies and intestinal absorption of different apo-β-carotenoids in rats and chickens. Biochem J 1976; 158:377-383.
26. Wenzl E, Sjaastad MD, Weintraub WH and Machen TE. Intracellular pH regulation in IEC-6 cells, a cryptlike intestinal cell line. Am J Physiol 1989;257:G732-G740.
27. Bauernfeind JC. Carotenoid vitamin A precursors and analogs in foods and feeds. J Agr Food Chem 1972;20:456-473.
28. High E and Day HG. Effects of different amounts of lutein, squalene, phytol and related substances on the utilization of carotene and vitamin A for storage and growth in the rat. J Nutr 1951;43:245-260.
29. High E and Day HG. Fate of lycopene in the rat and its effects on the utilization of carotene and vitamin A. J Nutr 1952;48:369-376.
30. Premachandra BR, Vasantharajan VN and Cama HR. Availability of β-carotene from high-β tomato hybrids to vitamin A-deficient rats & utilization of β-carotene in the presence of other carotenoids. Indian J Exp Biol 1978;16:473-477.
31. Ershov YV, Dmitrovskii AA and Bykhovskii VY. Characterization of the interaction of beta-carotene-15,15′-dioxygenase from rabbit small intestine with lycopene, 15,15′-dehydro-beta-carotene, lutein, and astaxanthine. Biochemistry - Russia 1993;58:483-487.
32. Mangels AR, Holden JM, Beecher GR, Forman MR and Lanza E. Carotenoid content of fruits and vegetables: an evaluation of analytic data. J Am Diet Assoc 1993;93:284-296.
33. Chug-Ahuja JK, Holden JM, Forman MR, Mangels AR, Beecher GR and Lanza E. The development and application of a carotenoid database for fruits, vegetables, and selected multicomponent foods. J Am Diet Assoc 1993;93:318-323.

Chapter 5

Uptake but no cleavage of β-carotene by cultures of rat and human intestinal cell lines

Trinette van Vliet and Roeselien Jaspars

Abstract

The aim of the study described was to investigate potential cleavage of β-carotene by intestinal epithelial cells *in vitro*. The rat intestinal epithelial cell lines IEC-6 and IEC-18 and the human colon carcinoma cell line Caco-2, undergoing enterocyte-like differentiation, were used. No cleavage products of β-carotene (retinal, retinol, retinoic acid, retinyl esters or apocarotenals) could be demonstrated after incubation with β-carotene added as beadlet solution in water in serum-free medium for 4 to 24 h in any of the cell lines. In the dioxygenase assay (an *in vitro* β-carotene cleavage assay), no cleavage activity was seen in 9,000 *g* supernatant preparations of IEC-18 or Caco-2 cells, suggesting the absence or a very low expression of the cleavage enzyme in these cell cultures.

Introduction

In earlier *in vitro* experiments using a rat intestinal homogenate, retinal wa found as the sole cleavage product[1]. However, *in vivo* retinal will be furthe metabolized. From other *in vitro* studies reported it seems that the reactio products, and possibly also the type of cleavage (central or eccentric), depend t a large extent on the assay conditions used. Therefore studying the cleavag products and the cleavage mechanism in intact intestinal cells might be mor appropriate than using cell homogenates. The aim of this study was therefore t investigate potential cleavage of β-carotene by intestinal epithelial cells *in vitro*

The rat intestinal epithelial cell lines IEC-6 and IEC-18, both establishe and characterized by Quaroni et al.[2,3], as well as the human Caco-2 cell lin were used. The Caco-2 cell line is a colon carcinoma cell line that undergoe spontaneous enterocyte-like differentiation in culture, forming a polarize monolayer with well developed apical brush borders. Caco-2 cells exhibit durin growth an increased production of brush-border enzymes typical of the adul human small intestine, including sucrase isomaltase, alkaline phosphatase an aminopeptidase[4]. As recently reviewed by Artursson[5], good correlations betwee Caco-2 cell culture and *in vivo* studies have been established for drug an peptide absorption. Quick and Ong[6] already reported conversion of β-carotene i Caco-2 cells, although only below passage 35 and with a very low efficiency.

As described in this report, apparent uptake of β-carotene by both rat an human intestinal cells, but no cleavage, could be demonstrated.

Materials and methods

Materials

IEC-6 and IEC-18 cells were obtained from the American Type Cultur Collection (ATCC, Rockville, MD) and used at passage 16 - 20 (IEC-6) or 25 - 3 (IEC-18). Caco-2 cells (ATCC HTB 37) of a high passage (around 200) wer kindly donated by Dr. Koninkx (Utrecht University, The Netherlands) and cell of passage 14 and 71 by Professor van Os and Dr. Fransen (Catholic Universit of Nijmegen, The Netherlands), respectively.

Plastic culture flasks of 25 cm^2 and 75 cm^2, plastic culture dishes of 55 cm^2 6-well plates and collagen-treated Transwell cell culture chambers 24.5 mm i diameter (4.71 cm^2 surface area) and 0.4 µm pore size were purchased fron Costar (Badhoevedorp, The Netherlands). Dulbecco's modified Eagle's medium

(DMEM, with 20 mmol/L Hepes, without glutamine and without sodium bicarbonate), foetal bovine serum, sodium bicarbonate 7.5% (w/v), L-glutamine non-essential amino acids, gentamycine, penicillin, streptomycin and trypsin were from Flow Laboratories (Irvine, Scotland). Phosphate-buffered saline (PBS) consisted of 8 g/L NaCl and 0.2 g/L KCl in a 0.01 mol/L phosphate buffer EDTA was obtained from Merck (Darmstadt, Germany). Insulin, crystalline β-carotene, retinol, retinal, retinoic acid and α-tocopheryl acetate were obtained from Sigma (St Louis, MO). β-Carotene as 10% water soluble beadlets was a gift from Roche (Mijdrecht, The Netherlands). All organic solvents used were pro-analysis grade, accept for acetonitrile which was HPLC grade.

Preparation of β -carotene solution

The β-carotene beadlet solution was prepared in sterilized water and not filtered, since filter sterilization of the solution (Millex FG 0.2 µm filters, Millipore, Molsheim, France) resulted in variable losses of β-carotene of 65 to 85%. Since experiments normally lasted 8 to 16 h and never exceeded 24 h no infections were to be expected.

Cell culture conditions

IEC cells were grown in DMEM supplemented with 0.83 g/L $NaHCO_3$, 4 mmol/L glutamine, 5% heat-inactivated foetal calf serum (30 min 56°C), 5 mg/L insulin, 50,000 IU/L penicillin and 50 mg/L streptomycin. For Caco-2 cells the medium was supplemented with 0.83 g/L $NaHCO_3$, 4 mmol/L glutamine, 20% heat-inactivated foetal calf serum, 1% non-essential amino acids and 50 mg/L gentamycine. Both IEC and Caco-2 cells were grown as monolayers in 75 cm^2 culture flasks at 37°C in a humidified atmosphere of 5% CO_2/95% air. Medium was changed three times a week. Cells were passaged before reaching confluency by detachment from the culture flasks with 0.25% trypsin in PBS-EDTA (EDTA 0.54 mmol/L for Caco-2 cells and 20 mmol/L for IEC cells), resuspension, and seeding at ca. 2×10^4 and 4×10^4 cells/cm^2 for IEC cells and Caco-2 cells, respectively, in new flasks.

For experiments cells were seeded into culture dishes of 55 cm^2 with 10 - 12 mL medium, 6-well plates with 2 mL medium per well or Transwell inserts with 3 mL medium in both the upper and the lower compartment. Experiments were carried out 1 day (IEC cells) or 4 - 17 days (Caco-2 cells) after reaching confluency. Medium was suctioned off and cells were washed three

times with PBS whereupon serum-free medium containing β-carotene (0.1 t 4.7 μmol/L) was added. After incubation for 1 to 24 h medium was collecte cells were washed three times with PBS (PBS was added to the collecte medium) and the cells were carefully scraped off and taken up in 2 mL PB The cell suspension was sonicated two times for 10 s, with an ultrasonic sonifi (Soniprep 150, MSE Scientific Instruments, Crawley, UK) with a microprob Medium and cell homogenates were analysed for β-carotene and cleava products. β-Carotene uptake was corrected for adhesion to the cells, estimate by measuring the amount of β-carotene associated with the cell fraction aft 5 min incubation (in general 0 - 2% of β-carotene added). In cleava experiments incubations without β-carotene were used as controls.

All handlings with β-carotene-containing materials were carried out und subdued or yellow light.

Cell fractionation

IEC-18 cells of 6 culture dishes, cultured in β-carotene-supplemented mediu (0.1 μmol/L) for 16 h were harvested and homogenized as described under ce culture conditions. All subsequent procedures were performed at 4°C. Th homogenate was centrifuged at 700 *g* for 10 min, yielding a pellet of cru nuclei and a postnuclear supernatant. The postnuclear supernatant wa centrifuged for 10 min at 5,000 *g*, yielding a pellet of crude mitochondria. Th supernatant was centrifuged at 105,000 *g* for 80 min, yielding a pellet of cru microsomes and a supernatant of cytosol. To obtain washed mitochondria, th crude mitochondria were resuspended in PBS and centrifuged at 24,000 *g* f 10 min using a Beckmann ultracentrifuge with 50.2 TL rotor.

Analyses

For analysis of β-carotene and cleavage products in culture medium, ce homogenates or cell fractions, samples were extracted with ethanol and hexa as described[1]. The organic layer was removed, taken to dryness under nitroge and redissolved in mobile phase for HPLC analysis of β-carotene, and i cleavage experiments for analysis of retinal, retinol, retinoic acid an apocarotenals as described[7]. In cleavage experiments aliquots of the mediu and cell fraction were also extracted with diisopropyl ether after alkali saponification[8], to analyse total vitamin A (retinol together with retinyl esters).

In order to study the functional polarization of the Caco-2 cells, alkaline phosphatase, a brush border enzyme, was measured, using a commercially available enzymatic colorimetric assay (Baker, Deventer, The Netherlands) with *p*-nitrophenyl phosphate as substrate. In the cultures on Transwell filters the integrity of the Caco-2 cell monolayers was assessed by measuring the transepithelial electrical resistance, using a millicell-ERS epithelial Voltohmeter (Millipore, Bedford, USA). Protein was determined using the BioRad protein assay (München, Germany) with BSA type V (Sigma, St Louis, MO) as standard.

Results

β-Carotene uptake in IEC-18 was very low (≤ 2% after 16 h) when the described culture conditions were used. Absorption was increased to about 16% when serum-free medium was used, as was done in all subsequent experiments. β-Carotene stability under serum-free conditions was somewhat lower than in the presence of serum. Total β-Carotene recovery after incubation for 16 h in the presence of cells was 70 - 90%, but was almost complete after 8 h in culture.

IEC-cells

IEC-18 cells usually formed confluent monolayers within 4 days and IEC-6 cells within 7 days as assessed by phase-contrast microscopy. **Figure 1** shows the time dependence of β-carotene uptake in IEC-18 cells during the first 8 h of incubation. Subcellular fractionation of cells incubated with β-carotene for 16 h showed that most β-carotene was associated with the crude mitochondria and microsome fractions (32% and 43% of β-carotene recovered in each cell fraction, respectively), while only 13% was present in the cytosol and 12% in the crude nuclei fraction. Absorption of β-carotene in IEC-6 cells was 11% during a 16 h incubation with 0.07 µmol/L β-carotene.

No β-carotene cleavage products (retinal, retinol, retinyl esters or retinoic acid) could be demonstrated in IEC-6 and IEC-18 cells or the culture medium after incubation with β-carotene for 16 h. Detection limits were such that less than 0.5% of the β-carotene absorbed in the IEC-18 cells and less than 5% in the IEC-6 cells was converted to one of these products.

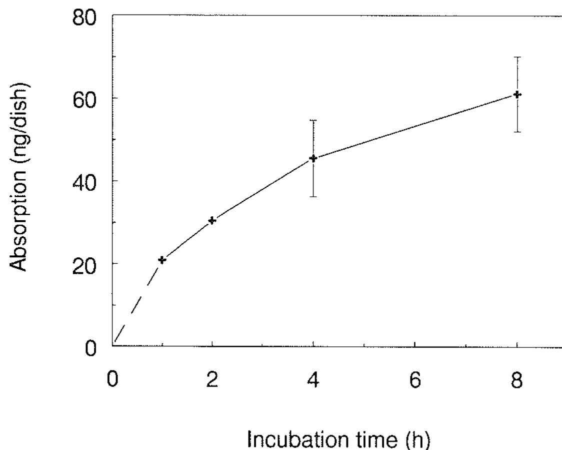


Figure 1. Uptake of β-carotene in IEC-18 cells incubated with 650 ng β-carotene per culture dish (0.1 µmol/L) for 1 to 8 h (one dish corresponds to about 8×10^{6} cells). Results are means with SD of two incubations.

Caco-2 cells

Since Quick and Ong[6] reported β-carotene cleavage in Caco-2 cells only belo passage 35, we used cells from different passages (and different laboratories All cells usually formed confluent monolayers within 4 days as assessed b phase-contrast microscopy. Alkaline phosphatase activity increased with time i culture until about 11 days after reaching confluency to a value of 125 - 165 U/ protein, after which only small increases were seen. The integrity of cells grow on Transwell filters was followed by measuring the transepithelial electrica resistance, which increased to 1000 - 1100 Ω·cm^2 23 days after reachin confluency (corrected for the control value, ca. 130 Ω·cm^2).

Dose dependence of β-carotene uptake during 4, 8 or 24 h incubation of cells o passage 19 is shown in **Figure 2**. The insert shows the time dependence fo incubation with 3.3 µmol/L. A linear increase with dose and time was seen up t 8 h. The decrease seen at 24 h may be caused by instability of β-caroten Absorption in cells of passage 76 after incubation for 16 h in 1.5 µmol/ β-carotene was 11% and compared rather well with absorption in cells of th other passages. After incubation of cells of passage 21 grown on Transwells wit 3.7 µmol/L β-carotene for 10 h, 4.3% of β-carotene was present in the cells an 17% in the basolateral compartment.

No cleavage products of β-carotene could be demonstrated in cells or mediu in any of the experiments. Detection limits for the percentage of conversion o β-carotene taken up in the cells was about 1% (into one product).

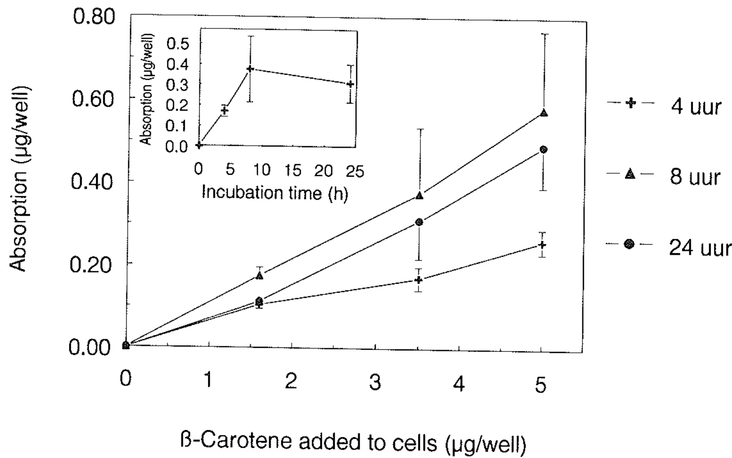


Figure 2. Uptake of β-carotene in Caco-2 cells incubated with various doses of β-carotene for 4, 8, or 24 h. Insert: time dependent uptake of the 3.5 µg dose of β-carotene. One well corresponds to about 2.4×10^6 cells. Results are means with SD of duplo incubations.

Discussion

In this study potential cleavage of β-carotene by IEC-18, IEC-6 and Caco-2 cells was investigated. Although β-carotene apparently was taken up in the cells, no cleavage products could be demonstrated in the cells or the culture medium.

The two main causes for not measuring β-carotene cleavage products are the absence or very low expression of the cleavage enzyme, and inappropriate experimental conditions for the cleavage reaction. To distinguish between these two explanations we tested 9,000 *g* supernatant fractions of cells for cleavage activity in the dioxygenase assay described before[1]. Neither in a preparation of a monolayer of IEC-18 cells, nor of Caco-2 cells of passage 19 (17 days after confluency), cleavage activity could be demonstrated. Thus, the β-carotene cleavage enzyme was not present or only present in very small amounts in the culture systems we used.

The absence of the dioxygenase enzyme in the IEC-18 cells might be explained by their ileal origin[3] because we demonstrated with the *in vitro* cleavage assay that the distal part of the intestine does not show cleavage activity. Since IEC-6 cells, established from the duodenum, also showed no cleavage activity, another explanation might be that both cell lines originate from the crypts[2,3]. Whether crypt cells already show β-carotene cleavage activity is unknown.

The Caco-2 cell line is known to consist of a heterogeneous population cells. The ratios among the various subpopulations may change, for instance, b a difference in growth rate of the subpopulations[5]. This effect might explain th disappearance of cleavage activity above passage 35 reported by Quick an Ong[6]. Different batches of Caco-2 cells may also vary considerably in the morphologic characteristics as reported by Herold et al.[9], and it is unclea whether these cells also differ with respect to functional and biochemica parameters. To overcome these problems we used 3 different batches of cell but still could not demonstrate cleavage activity. Alternatively, cultu conditions might have been inappropriate for expression of the cleava enzyme. However, standard culture conditions were used in our study.

Insufficient differentiation of Caco-2 cells may also result in the absence the enzyme. Comparison of alkaline phosphatase activity in this study wit values reported is complicated since in most studies reported the enzym activity was measured in the brush border fraction of cells. Pinto et al.[4] reporte a maximum activity in Caco-2 cells 19 days in culture of 764 U/g protein of brush border membrane fraction compared to 1793 U/g in a similar fractio from the small intestinal mucosa. Osypiw et al.[10] reported a plateau value aft 21 days in culture of 980 U/g protein, being a 6-fold enrichment over th non-brush border fraction. Thus alkaline phosphatase activity in ou experiments seemed normal for Caco-2 cells, but lower then in normal sma intestine. However, activity of a brush border enzyme may not be representati for the cytosolic dioxygenase enzyme. Caco-2 cells grown on filters we reported to differentiate better than those grown on plastic, probably due to th access to nutrients also from the basolateral side[5]. Even in cells grown on filte no cleavage activity could be demonstrated, although these cells were not teste in the dioxygenase assay using cell homogenates.

Omission of serum during the experiments is not expected to affe β-carotene cleavage. Incubation for 16 h in serum-free medium did not affe alkaline phosphatase activity in our studies. Moreover, the cells used for th dioxygenase assay were not incubated with serum-free medium. Another reaso to use serum-free medium is that serum would introduce retinol in the cultu medium.

Cleavage activity may have been too low to detect possible cleava products. Quick and Ong[6] reported the formation of 12 - 20 pmol retinyl este per hour when incubating the cells with 3 μmol/L β-carotene. Our detecti

limit for total vitamin A formation (retinol + retinyl esters) and for retinal was about 20 pmol, either in one dish or in a pooled sample of 5 dishes. In the latter situation our detection limit seems appropriate.

Scita et al.[11] reported somewhat higher β-carotene cleavage in hBRIE 380 cells (hybrid intestinal epithelial cell line of the rat). Of β-carotene taken up in the cells 17% was converted into retinol and 5% into retinoic acid. They reported a β-carotene absorption of about 450 pmol/10^6 cells after incubation for 16 h with 5 μmol/L β-carotene, which compares rather well with our value of uptake in Caco-2 cells of 440 pmol/10^6 cells after incubation for 8 h with 4.7 μmol/L. β-Carotene may in part be adsorbed to the cells and not taken up. However, in the Caco-2 cell cultures grown on filters 17% of added β-carotene was present in the basolateral compartment, suggesting that β-carotene actually is taken up by the cells. Subcellular fractionation of IEC-18 cells incubated with β-carotene showed β-carotene mainly in the microsomes and mitochondria fractions. Since no further characterization of these fractions occurred, the presence of cell membrane fractions cannot be excluded. Subcellular distribution of β-carotene in intestinal cells has, to our knowledge, not been reported, and comparison with other tissues seems not useful since distribution seems to depend on the tissue studied[12].

In conclusion, β-carotene cleavage activity could not be detected in any of the cell lines tested. Neither could cleavage products be detected in the cells of monolayer cultures nor could we demonstrate cleavage activity in S-9 preparations of the cells in the dioxygenase assay. Further attempts to develop a cell culture model for β-carotene cleavage may focus on culture conditions for Caco-2 cells grown on filters and on other cell lines, for instance the hBRIE 380 cells. The use of the dioxygenase assay during the development of a system seems very useful.

Acknowledgements

The authors thank John Groten and Fons Rutten for critical reading of the manuscript.

References

1. van Vliet T, van Schaik F, Schreurs WHP and van den berg H. *In vitro* measurement of β-carotene cleavage activity: methodological considerations and the effect of other carotenoids on β-carotene cleavage. Chapter 4 of this thesis.
2. Quaroni A, Wands J, Trelstad RL and Isselbacher KJ. Epitheliod cell cultures from rat intestine: Characterization by morphologic and immunologic criteria. J Cell Biol 1979;80: 248-265.
3. Quaroni A and Isselbacher KJ. Cytotoxic effects and metabolism of benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene in duodenal and ileal epithelial cell cultures. JNCI 1981 67:1353-1362.
4. Pinto M, Robine-Leon S, Appay M, Kedinger M, Triadou N, Dussaulx E, Lacroix B, Simon Assman P, Haffen K, Fogh J and Zweibaum A. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol Cell 1983;47:323-330
5. Artursson P. Cell cultures as models for drug absorption across the intestinal mucosa. Cr Rev Ther Drug 1991;8:305-330.
6. Quick TC and Ong DE. Vitamin A metabolism in the human intestinal Caco-2 cell line. Biochemistry 1990;29:11116-11123.
7. van Vliet T, van Schaik F, van Schoonhoven J and Schrijver J. Determination of several retinoids, carotenoids and E vitamers by high-performance liquid chromatography. Application to plasma and tissues of rats fed a diet rich in either β-carotene or canthaxanthin. J Chromatogr 1991;553: 179-186.
8. van Vliet T, Fentener van Vlissingen M, van Schaik F and van den Berg H. β-Carotene absorption and cleavage in the rat is affected by the vitamin A content of the diet. Chapter 7 of this thesis.
9. Herold G, Rogler D, Rogler G and Stange EF. Morphology of CaCo-2 cells varies in different cell batches. In Vitro Cell Dev Biol 1994;30A:289-291 (Letter).
10. Osypiw JC, Gleeson D, Lobley RW, Pemberton PW and Mcmahon RFT. Acid-base transport systems in a polarized human intestinal cell monolayer: Caco-2. Exp Physiol 1994;79:723-739.
11. Scita G, Aponte GW and Wolf G. Uptake and cleavage of β-carotene by cultures of rat small intestinal cells and human lung fibroblasts. J Nutr Biochem 1992;3:118-123.
12. Wamer WG, Wei RR, Matusik JE, Kornhauser A and Dunkel VC. β-Carotene uptake, metabolism, and distribution in BALB/c 3T3 cells. Nutr Cancer 1993;19:31-41.

Chapter 6

Effect of a low vitamin A or high β-carotene diet on intestinal and liver β-carotene cleavage activity. A pilot study with hamsters.

Trinette van Vliet, Frank van Schaik and Henk van den Berg

Abstract

A pilot study with hamsters was carried out to study the effect of consuming a diet with a low vitamin A content or a high β-carotene content on the *in vitro* β-carotene cleavage activity measured with the dioxygenase assay. Three groups of 10 hamsters were fed a diet containing 400 IU vitamin A per kg (Low A), 4000 IU vitamin A per kg (Norm) or 4000 IU vitamin A + 10 g β-carotene per kg (High βC) for three months. The low vitamin A diet increased the intestinal dioxygenase activity 2.3-fold compared to the Norm diet. The High βC diet decreased the activity in one subgroup of the animals, but had no effect in the other subgroup. Cleavage activity in the liver was not affected by the Low A diet, but was increased by the High βC diet. In conclusion the vitamin A and β-carotene content of the diet do modulate the *in vitro* dioxygenase activity in hamsters.

Introduction

In an earlier study[1] it was concluded that the dioxygenase assay method see an appropriate method to measure β-carotene cleavage activity under differe feeding conditions. Two potential conditions affecting β-carotene cleava activity are a low vitamin A intake or a high β-carotene intake. When vitamin intake is very low an increased conversion of β-carotene may be useful provide sufficient vitamin A. Indeed a vitamin A deficiency has been reported enhance *in vitro* β-carotene cleavage activity in rats[2,3]. When β-carotene inta is very high down regulation of β-carotene cleavage activity may prevent vitamin A intoxication. This may explain the absence of any sign of vitamin intoxication in patients with photosensitivity diseases consuming large doses β-carotene for long periods of time[4]. Thus we hypothesized that the vitamin and β-carotene content of the diet modulate the intestinal dioxygenase activity

Within an ongoing project on vitamin A and respiratory tract cancer in t TNO Institute, a study was carried out in which hamsters were fed a l vitamin A diet, a normal diet or a high β-carotene diet and were intratrachea intubated with benzo[a]pyrene. Small intestine and liver of hamsters at the e of the adaptation period, i.e. before treatment with benzo[a]pyrene w available for a pilot study. We studied the effects of a low vitamin A diet o high β-carotene diet on the intestinal β-carotene cleavage activity measu with the dioxygenase assay. In addition liver cleavage activity was determin to evaluate the possible role of the liver in β-carotene cleavage.

Materials and methods

Animals and diets

Male weanling Syrian golden hamsters (Central Institute for the Breeding Laboratory Animals, Zeist, The Netherlands) were housed per two in stainl steel cages. The animals had free access to food and tap water. Individual bo weights were assessed every two weeks and feed intake per cage every f weeks. Animals were checked daily for clinical signs of health disturbances.

The hamsters were fed a pelleted purified diet containing different amou of vitamin A and β-carotene for each of the experimental groups. Vitamin A w added as retinyl palmitate in oil, β-carotene was added as 10% water solu beadlets (both gifts from Roche, Mijdrecht, The Netherlands).

Study protocol

For this pilot study the adaptation period of a study on vitamin A and respiratory tract cancer reported by Wolterbeek et al.[5] was used. Three groups of 10 hamsters each were fed a diet containing 400 IU vitamin A per kg (Low A), 4000 IU vitamin A per kg (Norm) or 4000 IU vitamin A and 10 g (1.7×10^7 IU) β-carotene per kg (High βC) for three months. At the end of three months hamsters were killed by exsanguination under diethyl ether anaesthesia. The small intestine, from stomach to ileum, was collected, flushed with ice-cold 0.9% NaCl, packed in aluminium foil, frozen in liquid nitrogen and stored at –80°C. Liver was collected, frozen on dry-ice and stored at –80°C. Tissues were stored until further processing for maximal 9 months. For practical reasons the experiment was split-started in two groups of 15 animals three months apart, further mentioned as group 1 and group 2.

Tissue processing and analyses

The intestines were thawed on ice and further processed to a 9,000 *g* supernatant (S-9) of the mucosa as described before[1] (all on the same day). Nine livers of group 1 (three per diet) were thawed on ice, cut in pieces and homogenized with a Teflon pestle in 6 mL 0.15 mol/L tris buffer pH 8.2 containing 0.01 mol/L cysteine, and 0.01 mol/L nicotinamide. Subsamples of the homogenates were frozen in liquid nitrogen and stored at –80°C for analysis of β-carotene and possible cleavage products (within 1 month). The remaining homogenates were used to prepare S-9 by centrifugation at 9,000 *g* for 20 min. S-9 was used for the dioxygenase assay the same day and an aliquot of the S-9 was used for protein analysis.

The intestinal and liver dioxygenase activities were measured as described elsewhere[1] (as final method). Except that 3.25 mg protein S-9 was used per incubation and β-carotene was dissolved in ethanol. Incubations were carried out in triplicate and an aliquot of the S-9 was directly extracted for analyses of β-carotene and retinal.

Analyses of incubation mixtures, aliquots of S-9 and liver homogenates were carried out after extraction using ethanol and hexane, by high-performance liquid chromatography (HPLC) as described previously[6]. Six livers of group 1 and of group 2, not used for other analyses, were extracted with diisopropylether after alkaline saponification as described previously[7]. β-Carotene was measured as describe before[6]. Vitamin A was measured using a

Polygosil 60-5 column (Machery-Nagel, Düren, Germany) and a mobile phase
hexane - methylene chloride - iso-propyl alcohol (900:90:12, v/v/v). A flow
2 mL per min was used. Fluorometric detection was carried out at an excitati
wavelength of 333 nm and an emission wavelength of 470 nm.

Protein was analysed using BioRad protein assay (München, Germany) w
BSA type V (Sigma, St Louis, MO) as standard.

Statistics

Dioxygenase activity, liver vitamin A contents and liver β-carotene conte
were log transformed before statistical evaluation to overcome the problem
unequal variances between groups or variances correlated with the me
Overall effects of the diet were tested with a one way analysis of variance. Wh
ANOVA indicated significant differences, individual group means w
compared using t-tests. P values < 0.05 were considered statistically significa
All analyses were performed with the BMDP Statistical Software packa
version 1990 (VAX/VMS).

Results

Although the hamsters of group 1 and 2 were treated according to a simi
protocol, unexplained differences were observed between results for b
groups. Therefore the results for group 1 and 2 were treated as separ
experiments.

Within each group no effect of the diet on the growth was found. Howev
the starting body weights of the hamsters were significantly lower in grou
than in group 2 (64 and 75 g, respectively), whereas the growth during
study period was significantly greater in group 1 than in group 2 (74 and 47 g
13 weeks, respectively).

The intestinal cleavage activities are presented in **Figure 1**. Although
results for group 1 and 2 were somewhat different, for both groups a significa
overall effect of the diet was found (P = 0.004 and P = 0.006, respectively) a
cleavage activity was significantly higher in the animals on the Low A d
compared to the High βC diet. As can be seen in the figure, the tendency of
increased cleavage activity in hamsters of group 2 on the High βC diet is due
one extreme high value in this group, for which we have no explanation.

Cleavage activity in the liver was significantly higher in the hamsters
the High βC diet than in the other hamsters, as is shown in **Table 1**. This ta

also shows the vitamin A and β-carotene contents of the livers, which reflected the dietary intake.

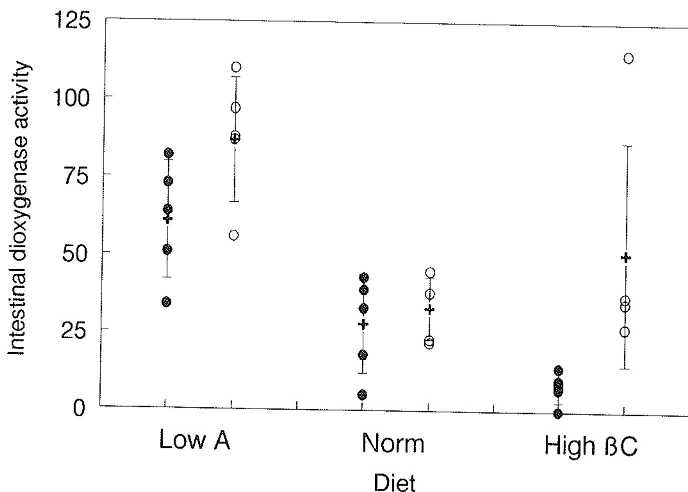


Figure 1. Intestinal β-carotene cleavage activity (ng retinal formed per h per 3.25 mg protein S-9) in hamsters fed either a low vitamin A diet, a normal diet or a high BC diet for 3 months. Two identical experiments, started 3 months apart, individual values (circles) with the mean (cross) and SD, n = 5 per diet per group, closed circles for group 1 and open circles for group 2.

Table 1. Cleavage activity and vitamin A and β-carotene content of the liver after 3 months on the different diets[1].

	Dioxygenase activity[2]	Total vitamin A[3]		β-Carotene	
		nmol/g	nmol/liver	nmol/g	nmol/liver
Group 1					
Low A	13 ± 5[a]	87 ± 31[a]	531 ± 157[a]	0.2 ± 0.1[a]	1.5 ± 0.5[a]
Norm	9 ± 4[a]	241 ± 98[b]	1358 ± 740[b]	0.8 ± 1.1[a]	5.4 ± 8.6[a]
High BC	29 ± 8[b]	778 ± 66[c]	4363 ± 412[c]	37 ± 62[b]	201 ± 333[b]
Group 2					
Low A	nm[4]	279, 206	1033, 953	0.1, 0.3	0.4, 1.4
Norm	nm	366, 353	1742, 1337	0.3, 0.4	1.4, 1.5
High BC	nm	740, 1145	4855, 4136	11, 20	69, 70

[1] Group 1 mean ± SD, n = 3 for dioxygenase activity, n = 5 for other parameters. Values with different superscripts within a column are significant different. Group 2: individual values, no statistical analysis were carried out.

[2] ng retinal formed per h per 3.25 mg protein S-9.

[3] Measured as retinol after saponification.

[4] nm, not measured.

Discussion

This pilot study was carried out to study whether the reported induction dioxygenase activity with a low vitamin A diet could be confirmed and, on t other side of the spectrum, whether a high β-carotene intake could reduced t dioxygenase activity. Indeed a low vitamin A diet fed for 3 months enhanced t intestinal dioxygenase activity. The 2.3-fold increase in activity found in tl study in hamsters is much more than the ca. 1.2-fold increase in rats report by Villard and Bates[2], but compares rather well with the about 2-fold increa found by Gronowska-Senger and Wolf[8] in rats fed a 10% protein diet.

The high β-carotene diet significantly decreased intestinal dioxygena activity in group 1, but not in group 2. We have no explanation for tl difference in response. The β-carotene content of the diet was very high, so do not expect that the difference in food intake, and thus β-carotene intal between the groups (65 vs. 74 mg β-carotene per hamster per day) played a ro There was a difference in β-carotene present in S-9; 1704 ± 388 ng/3.25 protein for group 1 and 497 ± 186 for group 2. Thus more than 3 μg substra was present in the assay. However, since we showed previously[1] an increase the amount of substrate from 3 to 5 μg decreased retinal formation with or 9%, this seems no explanation for the difference seen between the groups. Sir the preparation of S-9 was carried out at the same time, the only difference sample treatment between the groups seems the storage times of the intestir before preparation of S-9 being 8 and 5 months. Although storage of S-9 10 months did not affect cleavage activity, we cannot exclude that a decrease cleavage activity occurs during storage of the complete intestine. In conclusi further studies have to determine whether a high β-carotene intake decrea intestinal dioxygenase activity.

Remarkably, cleavage activity in the liver was higher in the animals on t High βC diet than in the other animals of group 1. As the regular (laborato diet of the hamster does not contain appreciable amounts of β-carotene, the li is generally not exposed to β-carotene. Besides, intestinal β-carotene cleavage almost complete. The appearance of β-carotene in the liver due to feeding a d with a very high β-carotene content apparently activates the cleavage enzyme

In this study we demonstrated that a low vitamin A intake during 3 mon increased *in vitro* intestinal β-carotene cleavage activity, while a hi β-carotene intake had either no effect or decreased this cleavage activity. Sir we do not know whether the dioxygenase activity is the rate limiting step

in vivo β-carotene metabolism, the consequences of changes in this activity for *in vivo* β-carotene metabolism are not clear.

Acknowledgement

The authors want to thank André Wolterbeek for kindly providing us with hamster intestines and livers and the growth data.

References

1. van Vliet T, van Schaik F, Schreurs WHP and van den Berg H. In vitro measurement of β-carotene cleavage activity: methodological considerations and the effect of other carotenoids on β-carotene cleavage. Chapter 4 of this thesis.
2. Villard L and Bates CJ. Carotene dioxygenase (EC 1.13.11.21) activity in rat intestine: effects of vitamin A deficiency and of pregnancy. Br J Nutr 1986;56:115-122.
3. Gronowska-Senger A and Wolf G. Effect of dietary protein on the enzyme from rat and human intestine which converts β-carotene to retinal. J Nutr 1970;100:300-308.
4. Mathews-Roth MM. Beta-carotene: Clinical aspects. Curr Top Nutr Dis 1989;22:17-38.
5. Wolterbeek APM, Rutten AAJJL, Feron VJ. High survival rate of hamsters given intratracheal instillations of benzo[a]pyrene and ferric oxide and kept on a high beta-carotene diet. Carcinogenesis 1994;15:133-136.
6. van Vliet T, van Schaik F, van Schoonhoven J and Schrijver J. Determination of several retinoids, carotenoids and E vitamers by high-performance liquid chromatography. Application to plasma and tissues of rats fed a diet rich in either β-carotene or canthaxanthin. J Chromatogr 1991;553: 179-186.
7. Speek AJ, Temalilwa CR, Schrijver J. Determination of β-carotene content and vitamin A activity of vegetables by high performance liquid chromatography and spectrophotometry. Food Chem 1986;19:65-74.
8. Beems RB, van Beek L, Rutten AAJJL and Speek AJ. Subchronic(106-day) toxicology and nutrition studies with vitamin A and β-carotene in Syrian hamsters. Nutr Rep Int 1987; 35:765-770.

Chapter 7

β-Carotene absorption and cleavage in the rat is affected by the vitamin A content of the diet

Trinette van Vliet[1,2] Martje Fentener van Vlissingen[1], Frank van Schaik[1] and Henk van den Berg[1]

[1] TNO Nutrition and Food Research Institute, Zeist, The Netherlands; [2] Department of Experimental Zoology, University of Amsterdam, Amsterdam, The Netherlands

Submitted for publication

Abstract

The purpose of this study was to examine whether intestinal β-carotene cleavage activity, measured with the dioxygenase assay, is affected by vitamin A intake and whether this *in vitro* activity is a determinant of β-carotene cleavage in vivo, measured in lymph-cannulated rats. Six groups of 10 - 20 rats were fed a diet containing 400, 4,000 or 40,000 IU vitamin A per kg for 14 to 18 wk, either supplemented or not with 50 mg β-carotene per kg in the last 6 wk. Intestinal dioxygenase activity was 1.9-fold higher ($P < 0.05$) in the animals fed the un-supplemented low-A diet than in the animals fed the un-supplemented high-A diet, while β-carotene supplementation significantly decreased intestinal dioxygenase activity. The ratio between retinyl ester and β-carotene content in lymph collected over 8 h after a single intestinal dose of β-carotene (250 μg) was correlated with intestinal dioxygenase activity ($r = 0.66$, $P = 0.003$). Dioxygenase activity in the liver was not affected by the vitamin A content of the diet, and was 1.7-fold higher in the β-carotene supplemented rats. Based on the difference in liver vitamin A contents between β-carotene supplemented and unsupplemented rats β-carotene conversion factors of 9:1 and 4:1 were calculated for the rats fed the high-A diet or the norm-A/low-A diets, respectively. In conclusion, intestinal β-carotene cleavage activity is higher in vitamin A-deficient rats than in rats with a high intake of either vitamin A or β-carotene. The intestinal dioxygenase activity as measured *in vitro* is an adequate indicator of *in vivo* β-carotene cleavage activity.

Introduction

Only part of β-carotene consumed is absorbed in the intestine. Most of tl
β-carotene absorbed (in man 60 - 70%[1]) is converted in the enterocytes, main
to retinal by the enzyme β-carotene 15,15′-dioxygenase and further to retin
and retinyl esters. The remaining β-carotene is absorbed intact. Wheth
regulation of cleavage activity may occur is still largely unknown as are facto
affecting cleavage activity. Upregulation of β-carotene cleavage could be
biological relevance under conditions of a deficient or marginal vitamin
status, while on the other side increased absorption of intact β-carotene mig
be beneficial in the light of the potential protective effect of β-carotene again
cardiovascular disease and certain types of cancer[2,3]. Although vitamin A
known to be toxic in high amounts, no toxicity has been found for β-caroter
Patients with the light-sensitive disease erythropoietic protoporphyria ha
been treated with large doses of β-carotene for long periods of time without a
sign of vitamin A toxicity[4], suggesting down-regulation of cleavage activi
and/or controlled absorption.

Regulation may occur at the level of the intestinal β-carotene cleava
enzyme. Indeed, a low vitamin A intake in rats was found to be associated wi
increased *in vitro* intestinal cleavage activity[5,6]. In a pilot study, we found
2.3-fold higher cleavage activity in hamsters fed a diet containing 400 I
vitamin A (as retinyl palmitate) per kg for 3 months than in the control gro
receiving 4000 IU vitamin A per kg[7]. Although a 'low' vitamin A status
generally assumed to increase β-carotene cleavage, this has only be
demonstrated *in vitro*, and the relationship between *in vitro* and *in vi*
cleavage activity has, to our knowledge, not yet been demonstrated.

The purpose of the present study was to examine whether feeding rats a l
or a high vitamin A diet (as retinyl palmitate) affects intestinal β-carote
cleavage activity as assessed *in vitro* and whether this *in vitro* cleavage activi
is indeed a predictor of *in vivo* β-carotene absorption and cleavage rate.

Materials and methods

Animals and diets

Male weanling Wistar-WU rats (n = 105) were obtained from Harlan, Sprag
Dawley (Zeist, The Netherlands). The rats were housed in groups of five each,
stainless steel cages in a well ventilated room at 21 ± 2°C, with a relati

humidity of 60 ± 10%, and a 12 h - 12 h light-dark cycle. Animals had free access to food and tap water. Individual body weights and feed intake per cage were assessed weekly. Animals were checked daily for clinical signs of health disturbances.

Experimental diets consisted of the basal semi-synthetic powdered diet (**Table 1**) with different amounts of vitamin A and β-carotene for each of the experimental groups. Vitamin A was added as retinyl palmitate in oil, and β-carotene was added as 10% water-soluble beadlets (both gifts from Roche, Mijdrecht, The Netherlands). Diets were prepared every 6 wk and stored at –20°C.

Table 1. Composition of the experimental diet[1]

Component	Amount g/kg
Casein[2]	200
DL-methionine	3
Wheat starch	530
Maize oil	50
Cellulose[3]	50
Mineral mixture, AIN-76AM	35
Vitamin mixture, AIN-76AM, vitamin A deficient	10
$CaHPO_4$	15
Choline bitartrate	2
Vitamin A mixture[4]	105
β-carotene 10% water-soluble beadlets[5]	0.5

1 Adapted from Rutten and Groot[8];
2 Acid-precipitated, containing 89.1% protein (N × 6.38), moisture 8.9%, ash 4.67%;
3 Dicacel, highly purified and bleached fibrous filter powder, consisting of 87 - 90% pure α-cellulose; average length of fibres ca. 44 μm; water 4%; ash 0.12 - 0.15%; lignin 0.04%;
4 Vitamin A premix contained retinyl palmitate (10^6 IU/g) 0.08% and pre-gelatinized wheat starch 99.92%. Vitamin A mixture for Low A (400 IU/kg) consisted of 0.5 g vitamin A premix and 104.5 g wheat starch; for Norm A (4,000 IU/kg), 5 g vitamin A premix and 100 g wheat starch; and for High A (40,000 IU/kg), 50 g vitamin A premix and 55 g wheat starch;
5 Only in diets supplemented with β-carotene.

Study protocol

The study protocol is schematically shown in **Figure 1**. Rats were randomly assigned to six groups after an acclimatization period of 2 wk. A few rats were reallocated in order to equalize initial mean body weight in the various groups. All rats first received a diet containing 1200 IU vitamin A per kg. After 4 wk 3 levels of vitamin A were fed, i.e. 400, 4,000 or 40,000 IU vitamin A per kg (as

retinyl palmitate), each level to 2 groups. After 8 wk on these diets 5 animals o
each diet were killed by aortic exsanguination under diethyl ether anaesthesi
Blood and liver were collected for vitamin A analyses to control for th
effectiveness of the diets in creating different vitamin A liver storage states. Fo
clarity, from this point on the study will be described as two sub-experiments.
Experiment 1: dioxygenase activity and tissue vitamin A and β-carotene
contents. Three groups (of 10 rats each) were kept on their diet for anoth
6 wk, while the other three groups (of 10 rats each) received their di
supplemented with 50 mg β-carotene per kg (83,333 IU, assuming 1 IU equa
0.6 μg β-carotene) for 6 wk. During the last experimental week (wk 17) ra
were housed individually. After an adaptation period of 3 d, faeces we
collected and food intake was measured for a 4-d period. At the end of th
experimental period (in wk 18), after a 15 to 18 h food-deprivation, all anima
(n = 60) were killed by aortic exsanguination under diethyl ether anaesthesi
Intestine, liver and lungs were collected and weighed and treated as describe
below (Tissue processing).
Experiment 2: lymph cannulation and dioxygenase activity. Three groups (of
rats each) were kept on their diet containing 400, 4,000 or 40,000 IU vitamin
per kg for another 6 to 10 wk. These rats were used for the collection
intestinal lymph, after which their intestine was collected for dioxygena
assay. The experimental protocol was approved by the TNO-Institute's Anim
Care and Use Committee.

Plasma and tissue processing

Blood was collected from the abdominal aorta in heparinized tubes and store
on ice in the dark. Plasma was separated by centrifugation within 2 h an
stored at –80°C. The proximal 60 cm of the small intestine was flushed wi
ice-cold potassium phosphate buffer, 100 mmol/L, pH 7.7, containing 4 mmol
$MgCl_2$, 30 mmol/L NAD and 1 mmol/L dithiotreitol (DTT). The intestine was c
lengthwise and the mucosa was scraped off with a glass slide. The intestin
scrapings were homogenized with a Teflon pestle in 2.5 mL of the potassiu
phosphate buffer. Livers of the interim killing were packed in aluminium fo
snap-frozen in liquid nitrogen and stored at –80°C for determination
vitamin A. Liver and lungs of the final killing were homogenized in one volum
of buffer with an Ultra-Turrax homogenizer (Wilten Woltil, De Bilt, Th
Netherlands) and further with a Teflon pestle. The same buffer was used for th

lungs and the intestinal mucosa, while for the liver a 100 mmol/L potassium phosphate buffer pH 7.4 with 50 mmol/L KCl was used. Aliquots of the liver and lung homogenates were snap-frozen in liquid nitrogen and stored at –80°C for β-carotene and vitamin A analysis. The intestinal mucosal tissue and subsamples from the liver and lungs were used to prepare a 9,000 g supernatant (S-9) by centrifugation for 20 min using a Beckmann ultracentrifuge with 50.2 TL rotor. All handlings were carried out on ice and using a refrigerated centrifuge (4°C). A small subsample of the S-9 was stored at –20°C for protein analyses. The main sample of S-9 was directly frozen in liquid nitrogen and stored at –80°C for measurement of dioxygenase activity.

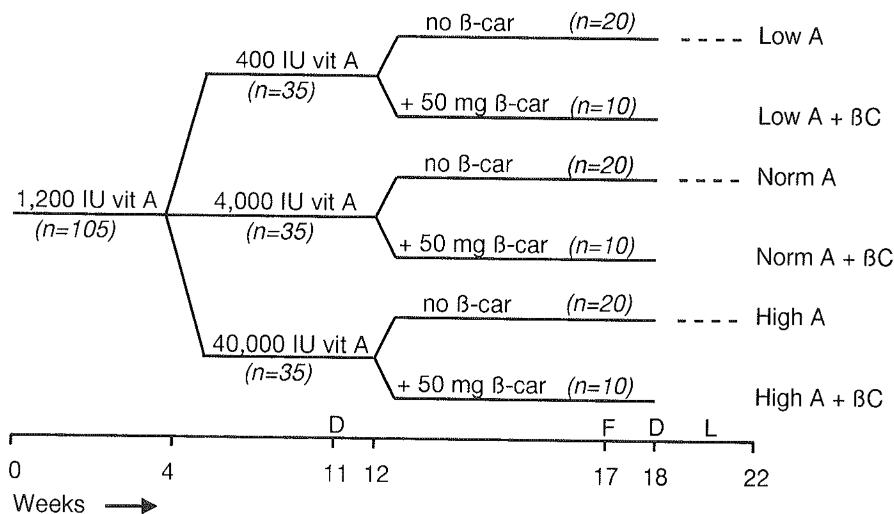


Figure 1. Study design. Overview of the diets consumed by the different groups of rats in the different study periods. Composition of the diets expressed per kg of diet. D, dissection; F, faeces collection; L, lymph cannulation.

Storage

Samples were stored for 10 months at most before analysis. In previous experiments in our laboratory retinal-forming activity in the dioxygenase assay was unaffected by storage of the enzyme preparation at –80°C for up to 10 months (unpublished results). The samples for measurement of intestinal dioxygenase activity were stored 2 months at most. Retinol and β-carotene are known to remain stable in plasma during storage at –70°C for at least

28 months[9]. Comparable stability was assumed for retinol, retinyl esters ar β-carotene in tissue homogenates stored at –80°C. Since faeces were stored –20°C, we can not exclude some degradation of β-carotene in these samples.

Dioxygenase assay

The assay was carried out with 2.8 μmol/L β-carotene added in acetone substrate and 2.0 mg of intestinal S-9 protein or 3.0 mg liver or lung S-9 prote as enzyme source. Incubation was carried out in reaction vials in 2 m potassium phosphate buffer (100 mmol/L), pH 7.7, containing nicotinamic (15 mmol/L), $MgCl_2$ (2 mmol/L), glutathione (5 mmol/L), SDS (1.7 mmol/I taurocholic acid 6 mmol/L), L-a-phosphatidylcholine (0.2 g/L) and α-tocopher (0.125 g/L), in a shaking water bath at 37°C in the dark for 1 h. To stop tl reaction 2 mL of ethanol was added to the incubation mixture. Control via were run in all experiments, containing all reactants except for β-caroten Under the conditions used retinal was the only product we could demonstra and retinal formation was proportional to the amount of enzyme present.

Lymph cannulation

Rats were fed a vitamin A-free diet for 24 h and subsequently deprived of foc for 12 to 16 h before surgery. About 1 h before surgery rats were given 1.5 mL a fatty mixture (90 g/L sunflower oil, 12 mmol/L sodium taurocholate ar 4 mmol/L KCl in 6 g/L NaCl, homogenized by sonication) by gastric intubatic enabling a better visualization of the intestinal lymphatics by enhancing tl chylomicron content of the lymph. Animals were anaesthetized with a mixtu of halothane, N_2O and O_2. The superior mesenteric lymph duct was cannulate according to the procedure of Bollman et al.[10] with the following modification The cannula was fixed in position with a minimal amount of tissue glu (Delta-Acryl, Hendriks, Weesp, The Netherlands) and exteriorized through stab wound in the right flank. The cannula was filled with heparin (10^5 U/L i 9 g/L NaCl) to prevent clotting. A second cannula was placed in the duodenu about 2 cm distal from the fundus, and secured by a purse-string suture. Th tube was also exteriorized through the right flank. The abdomen was close routinely and the rats were allowed to recover from anaesthesia. The rats we kept in restraining cages and infused duodenally (0.5 mL every half hour) wit a glucose-salt solution (280 mmol/L glucose, 145 mmol/L NaCl, 4 mmol/L KCl One hour after surgery a single dose of 250 μg crystalline β-carotene (Merc

Darmstadt, Germany) mixed with 0.25 mL sunflower oil was given through the duodenal cannula, followed by 1.25 mL of a taurocholate solution (18 mmol/L in the glucose-salt solution). The β-carotene dose was about one third of the daily intake of rats on diets supplemented with β-carotene. Lymph was collected in 1-h portions on ice in tubes containing 75 μL 130 mmol/L EDTA to prevent clotting (final concentration of EDTA in lymph was about 10 mmol/L). Lymph collection was carried out under subdued light and tubes were placed on ice and covered with aluminium foil. Immediately after 1 h collection, lymph was frozen in liquid nitrogen and stored at –80°C before analysis within 3 months.

After lymph collection for 8 to 10 h, rats were killed using Nembutal. The gastro-intestinal tract was inspected for passage of its contents and the lymph cannula was inspected for proper positioning. Only results from rats with apparent gastro-intestinal motility and proper positioning of the lymph cannula were included in the analyses. The proximal 60 cm of the intestine was collected, flushed with ice-cold 9 g/L NaCl, packed in aluminium foil, snap-frozen in liquid nitrogen and stored at –80°C for preparation of S-9 after 9 months, as described under Tissue processing.

Analyses

Protein was determined using the BioRad protein assay (München, Germany) with BSA type V (Sigma, St Louis, MO) as standard. The dioxygenase incubation mixture, plasma and aliquots of each lymph sample (for retinyl ester analysis) were deproteinated with one volume ethanol containing internal standard (retinyl acetate, α-tocopheryl acetate, and α-carotene, respectively), and extracted using two volumes hexane. Faeces were homogenized using a coffee grinder. Faeces, feed and the livers obtained from the interim killing were extracted with diisopropylether after alkaline saponification as described previously[11]. Liver and lung homogenates and an aliquot of each lymph sample (for β-carotene analysis) were saponified and extracted with the same method, adapted for analysis of small samples. All extracts were evaporated under nitrogen at room temperature. Residues were dissolved in water-free eluent and transferred into brown HPLC injection vials.

HPLC. HPLC analyses were performed using methods described in detail before[12] with slight modifications. In short, retinal and other retinoids were separated on a 125 × 4.6 mm column packed with Nucleosil 120-3 C_{18} (Machery-Nagel, Düren, Germany). The mobile phase consisted of

acetonitrile - 100 mmol/L ammonium acetate (75:25, v/v). The ammoniu
acetate was acidified with acetic acid to pH 4.65 to obtain a sharp peak fo
retinal. Retinol and β-carotene in plasma and in tissue samples were separate
on a 125 × 4.6 mm column packed with Hypersil ODS 3 μm (Shandon Souther
Products, Astmoor, UK) with a mobile phase consisting of acetonitrile
methanol - methylene chloride - water (70:15:10:5, v/v/v/v). For analyses o
β-carotene in faeces and lymph, and retinyl esters in lymph, a 125 × 4.6 mm
column packed with Superspher 100 RP-18 (Merck, Darmstadt, Germany) wa
used. The mobile phase consisted of acetonitrile - methanol - methylene chlorid
(70:10:15, v/v/v). Detection was carried out using programmable absorbanc
detectors, spectroflow 783. Retinal was monitored at 380 nm, the other retinoid
at 325 nm, α-tocopheryl acetate at 292 nm and β-carotene at 445 nm.

Standards of α-carotene, retinol, retinal, retinyl acetate and α-tocophery
acetate were obtained from Sigma (St Louis, MO), β-carotene from Merc
(Darmstadt, Germany) and retinyl palmitate from Fluka (Buchs, Switzerland
Retinyl stearate containing small amounts of palmitate, for which we correcte
was a gift from Hoffmann-La Roche (Basel, Switzerland).

The β-carotene and vitamin A contents of the diets were checked by HPL
analysis. The diets contained no (< 0.1 mg) or 43 ± 3 mg β-carotene per kg an
405, 3750 and 40,100 IU vitamin A per kg.

Statistics

To overcome the problem of unequal variances between groups or variance
correlated with the mean, all variables except for the growth, liver weight an
lung weight were log-transformed before statistical evaluation. Overall effects o
vitamin A and β-carotene in the diet were tested by two-way analysis o
variance. When analysis of variance (ANOVA) indicated significant differences
individual group means were compared by t-tests. Growth curves for th
different diet groups were compared by testing growth from wk 4 (start of th
different diets) to the end of the study by a two-way ANOVA. The Pearso
correlation coefficient was used to assess the relationship between the results o
the intestinal dioxygenase assay and the of lymph cannulation. P values < 0.0
were considered statistically significant. All analyses were performed using th
BMDP Statistical Software package (version 1990 (VAX/VMS), Los Angeles
CA).

Results

The vitamin A content of the diet affected weight gain of the rats ($P = 0.016$). The mean body weight at the start of the study was 150 g, and the weight gain between wk 4 and 18 was significantly less in the rats fed the low-A diet than in the rats fed the high-A diet (136 g vs. 148 g). Total food intake in this same period was slightly less in the Low A group than in the High A group (228 g vs. 234 g, based on measurements per cage).

Experiment 1

Mean intestinal and liver dioxygenase activities of the rats killed after 18 wk are shown in **Figure 2**. Intestinal cleavage activity was affected by both the vitamin A and the β-carotene content of the diet (overall effects $P = 0.009$ and $P = 0.019$, respectively). In contrast to intestinal cleavage activity, liver cleavage activity was only affected by the β-carotene content of the diet ($P < 0.0001$). Cleavage activity in the liver was significantly higher in rats fed the β-carotene supplemented diets at all three vitamin A levels. No cleavage activity could be demonstrated in the lungs (< 2.5 ng retinal per h per mg protein S-9, n = 11).

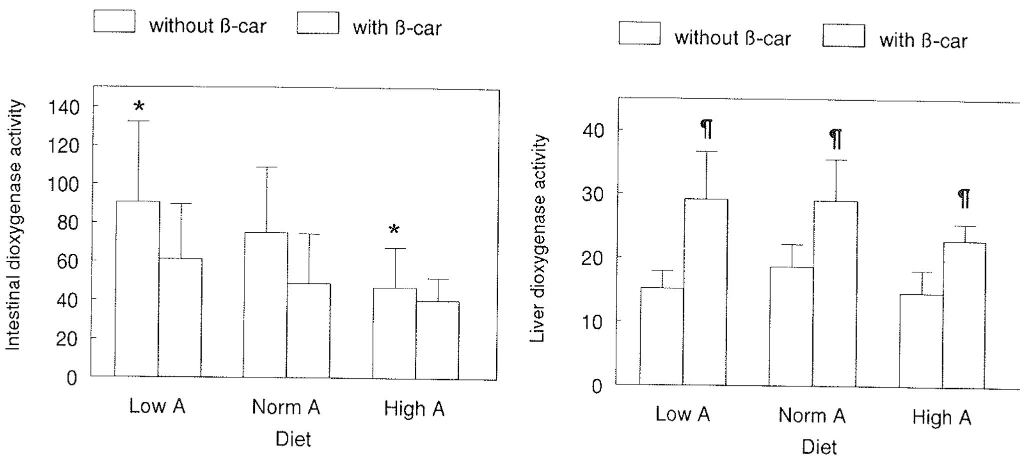


*Figure 2. β-Carotene cleavage activity (expressed as ng retinal formed per h per mg protein S-9) in rats fed different amounts of vitamin A with or without β-carotene. Means with SD. Left: intestinal dioxygenase activity, n = 10 per group, overall significant effect of the vitamin A content of the diet ($P = 0.009$) and of the β-carotene content of the diet ($P = 0.019$), * significantly different from each other. Right: liver dioxygenase activity, n = 5 per group, overall significant effect of β-carotene supplementation ($p < 0.0001$), ¶ significantly different from the same diet without β-carotene.*

Amounts of β-carotene and retinoids accumulated in liver, lungs and plasma ar summarized in **Table 2**. The results of the interim killing illustrate that th different diets had indeed resulted in different vitamin A storage states. At th end of the study β-carotene was found in the liver only in very small amount apparently independent of the retinyl palmitate content of the diet, while could not be demonstrated in plasma and lungs. As expected, the amounts vitamin A in lungs and liver reflected both the retinyl palmitate and th β-carotene content of the diets. The difference in vitamin A storage betwee groups fed the same level of vitamin A either with or without β-caroten supplementation was similar for the Low A and Norm A groups (i.e. 10,161 an 9,302 nmol respectively), but was much less for the High A groups (1,155 nmol This indicates a lower β-carotene cleavage and/or absorption at a higher retin palmitate intake. The plasma retinol concentration was lower in the Low group than in the other groups.

Based on the amount of β-carotene recovered in the faeces unexpectedly hig apparent absorptions (74%, 68% and 75%) were calculated for the Low A + βC the Norm A + βC and the High A + βC group, respectively.

Experiment 2

The lymph cannulation experiment was conducted to study the effect of th vitamin A content of the diet on the *in vivo* absorption and cleavage of a singl dose of β-carotene; therefore, only animals from the β-carotene-free diet group were used. Retinyl palmitate and stearate were the only cleavage products tha could be demonstrated in the lymph together with small amounts of β-carotene Despite the vitamin A-free diet for 14 h and food-deprivation overnight befor the operation, the first 1-h samples (i.e. before dosing) of the High A and th Norm A group, but not of the Low A group, contained small amounts of retiny esters (48 ± 27 pmol and 34 ± 28 pmol, respectively). These amounts correspon with less than 2% of the total amount of retinyl esters measured in the lymph i the 8 h after dosing. A typical example of the lymph curves obtained fo β-carotene and retinyl esters (sum of retinyl palmitate and retinyl stearate) i shown in **Figure 3**.

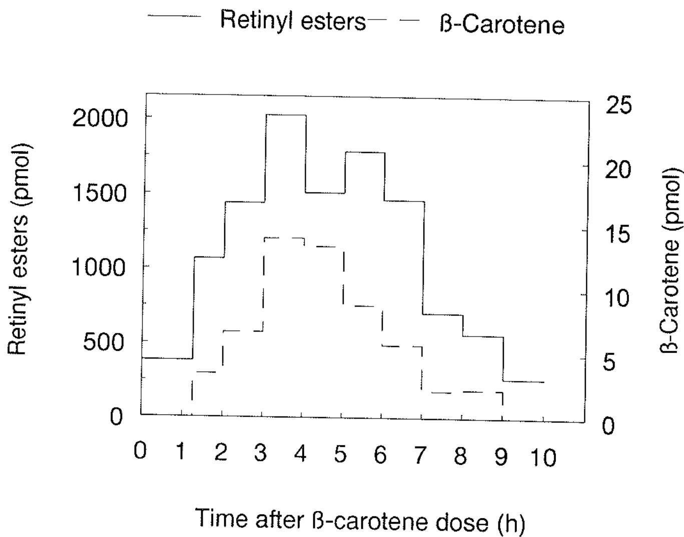


Figure 3. Typical example of the amount of retinyl esters and β-carotene in the lymph collected 1 to 10 h after a single intestinal dose of 250 μg β-carotene.

For quantitative comparisons, the total amount of retinyl esters and β-carotene excreted in the lymph during the first 8 h after the β-carotene dose were calculated (see **Table 3** for results). Retinyl ester excretion in lymph tended to be higher in the Low A group than in the High A group, whereas the inverse was seen for β-carotene, resulting in a significantly higher ratio of retinyl esters to β-carotene in the Low A than in the High A group. The ratio was calculated as an indicator for β-carotene cleavage to enable comparison with the dioxygenase activity. The intestinal dioxygenase activities in the rats used in the cannulation experiment (see Table 3) were similar to the activities measured in the non-cannulated rats of experiment 1 (see Figure 2); cleavage activity in rats fed the low-A diet was significantly higher than in rats fed the high-A diet. The relationship between the dioxygenase activity and the ratio of retinyl esters to β-carotene in the lymph is shown in **Figure 4**. Although variations in both measures were considerable, they were significantly correlated ($r = 0.66$, $P = 0.003$).

Table 2. Vitamin A content of plasma, lung and liver, and β-carotene content of liver of rats fed different levels of vitamin A for 7 or 14 weeks[1].

Diet	After 7 weeks on diets[2]			After 14
	Plasma	Liver		Plasma
	retinol (µmol/l)	weight (g)	total vit A[4] (µmol)	retinol (µmol/L)
Low A	0.55 ± 0.15[a]	8.4 ± 0.7	0.028 ± 0.042[a]	0.40 ± 0.03[a]
Low A + βC				1.28 ± 0.14[bc]
Norm A	1.09 ± 0.15[b]	8.7 ± 1.0	1.18 ± 0.18[b]	1.32 ± 0.12[bc]
Norm A + βC				1.34 ± 0.15[b]
High A	1.11 ± 0.36[b]	8.7 ± 1.4	25.8 ± 3.9[c]	1.24 ± 0.07[bc]
High A + βC				1.21 ± 0.13[c]
ANOVA, P-value				
A effect	0.005	ns	< 0.001	< 0.001
βC effect				< 0.001
Interaction				< 0.001

[1] Values are means ± SD. β-Carotene could not be detected in plasma (< 0.01 µmol/l) and lung (< 0.1 nmol), values with different superscripts within a column are significantly different. ns; not significant.
[2] n = 5 per diet group.
[3] n = 10 per diet group.
[4] Measured as retinol after saponification.
[5] Mean lung weight 1.26 g.

Table 3. Lymph retinyl ester and β-carotene content and intestinal dioxygenase activity in rats fed diets with different levels of vitamin A for 15 to 18 weeks[1].

Diet (n)	Lymph		
	retinyl esters (nmol/8 h)	β-carotene (nmol/8 h)	retinyl esters/β-carotene
Low A (4)	13.0 ± 10.7	0.016 ± 0.014	858 ± 384[a]
Norm A (8)	9.9 ± 8.5	0.017 ± 0.015	817 ± 526[a]
High A (6)	6.6 ± 4.6	0.061 ± 0.057	178 ± 130[b]
ANOVA, P-value	ns	ns	0.001

[1] Values are mean ± SD. Values with different superscripts within a column are significant different. ns; not significant.
[2] Calculated assuming cleavage of 1 mol β-carotene (βC) into 2 mol retinyl esters (RE), 8 h absorption: (nmol RE/2 + nmol βC)/βC dose, 8 h cleavage: (nmol RE/2)/(nmol RE/2 + nmol βC)

weeks on diets (β-carotene supplementation for 6 weeks)[3]			
Liver			Lung[5]
weight (g)	β-carotene (μmol)	total vitamin A[4] (μmol)	total vitamin A[4] (μmol)
8.6 ± 1.2[a]	-	0.021 ± 0.056[a]	$6x10^{-4} \pm 7x10^{-4a}$
10.0 ± 0.7[b]	0.013 ± 0.005	10.2 ± 1.8[b]	0.021 ± 0.012[b]
9.6 ± 1.0[b]	-	3.03 ± 0.66[c]	0.012 ± 0.004[c]
10.0 ± 0.4[b]	0.011 ± 0.005	12.3 ± 1.1[d]	0.030 ± 0.013[d]
9.6 ± 0.8[b]	-	37.6 ± 2.9[e]	0.043 ± 0.019[d]
9.8 ± 1.6[b]	0.017 ± 0.011	38.8 ± 5.8[e]	0.062 ± 0.023[e]
ns	ns	< 0.001	< 0.001
0.014	< 0.001	< 0.001	< 0.001
ns	ns	< 0.001	< 0.001

Lymph		Intestinal dioxygenase activity
absorption[2] %	cleavage[2] %	ng retinal/(h.mg protein)
1.4 ± 1.1	99.7 ± 0.1	81.2 ± 9.2[a]
1.1 ± 0.9	99.7 ± 0.2	63.9 ± 21.4[a]
0.7 ± 0.5	97.8 ± 2.4	28.6 ± 14.1[b]
ns	ns	0.001

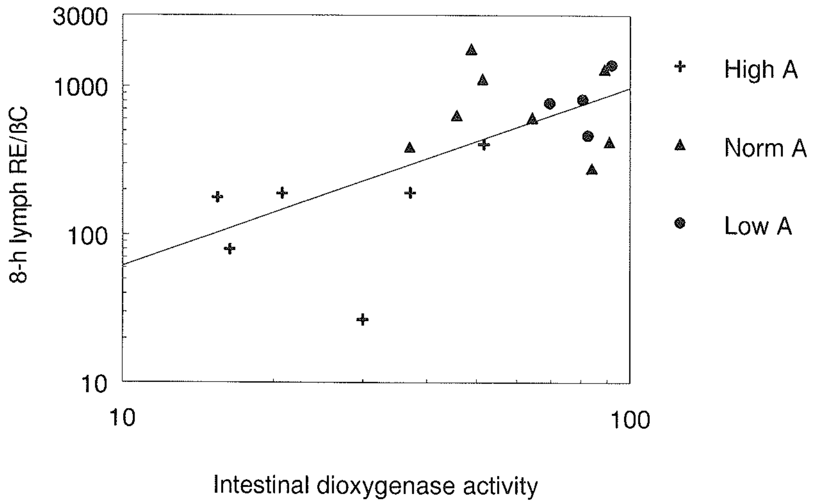


Figure 4. Correlation between intestinal dioxygenase activity (expressed as ng retinal formed per h per mg protein S-9) and the 8-h lymph molar ratio between retinyl esters (RE) and β-carotene (βC), r = 0.66, P = 0.003.

Discussion

Although the β-carotene cleavage enzyme was already described in 1965[13,14], th enzyme has not yet been purified and several details with respect to the actu reaction mechanism (central or eccentric cleavage) and factors controlling th cleavage activity still remain to be elucidated. Previous studies alread indicated that a low vitamin A intake increases *in vitro* β-carotene cleavag activity, but it is as yet not known whether a high vitamin A intake vice vers results in down-regulation of β-carotene cleavage activity. Gronowska-Seng and Wolf[5], using rats on a 10% protein diet, reported a two-fold higher cleavag activity in rats fed a vitamin A deficient diet for 4 wk than in rats fed this di for 2 wk after which they were refed a sufficient dose of vitamin A. Villard an Bates[6] measured cleavage activity in rats fed a diet containing either 270 IU 5170 IU vitamin A per kg for 10 wk. Activity in the rats on the low-A diet wa 1.2-fold higher than in rats on the normal diet (the effect seemed to be strong in pregnant rats).

In this study we confirmed that a low vitamin A intake results in a higher *i vitro* intestinal cleavage activity. Furthermore we found a lower cleavag activity in rats fed the high-A diet. Subsequent supplementation of the die with β-carotene decreased cleavage activity. This decrease was most marked i the Low A group, most probably because the vitamin A deficiency in this grou

was restored. Since the decrease is stronger than can be explained by restoring vitamin A status and is also observed in the Norm A group, β-carotene supplementation seems to decrease cleavage activity independently, protecting the organism against an unwanted high retinoid accumulation. In this respect the study reported by Lakshman et al.[15] is of interest as these authors observed that pre-feeding rats with β-carotene for 2 weeks caused a 1.9-fold stimulation of β-carotene absorption as well as its conversion to retinyl esters, as measured in lymph. Their hypothesis that this increase is caused by induction of the intestinal cleavage enzyme is not confirmed by our results.

In contrast to intestinal cleavage activity, liver cleavage activity increased after β-carotene supplementation and was not affected by the retinyl palmitate content of the diet. The higher activity in the β-carotene fed rats cannot be explained by the presence of β-carotene in the enzyme preparations, since in control incubations without added substrate no activity was demonstrated. Shapiro et al.[16] already suggested that liver β-carotene cleavage activity might be induced at increasing β-carotene levels in the liver. They found a decrease in liver β-carotene storage after an initial increase in rats during supplementation with β-carotene for 21 wk. Liver cleavage activity might seem relatively unimportant as the 'normal' diet of laboratory rats does not contain provitamin A carotenoids, and intestinal cleavage of β-carotene is almost complete. However, total cleavage activity in the liver is higher than in the intestine (ca. 16 vs. 3 μg retinal per total tissue in the Norm A group).

Extra-intestinal β-carotene cleavage may especially be of relevance to compensate for 'local' vitamin A deficiencies induced for instance by carcinogens or by inadequate levels of retinol-binding protein (RBP) and transthyretin (TTR) due to insufficient dietary protein[17]. Edes et al.[18] observed that administration of the carcinogen benzo[a]pyrene induced lower levels of retinol in liver and intestine of rats, which could be prevented by the administration of β-carotene, but not retinol. Since the lungs are a potential target tissue for cancer, we were interested to know whether cleavage activity could be demonstrated and, if so, could be affected by retinyl palmitate or β-carotene intake. We could not demonstrate cleavage activity (i.e. retinal formation) in lungs, also not after supplementation with β-carotene. However, cleavage activity in lung tissue has been reported by others using different enzyme preparations for the dioxygenase assay and measuring other products, i.e. apocarotenals with a 800*g* supernatant[19] or retinol with cytosol[20]. Thus, lung

tissue apparently contains cleavage activity, but most likely not with retinal a
the reaction product. The differences in vitamin A storage in the lungs betwee
diet groups (see Table 2) probably can be explained by differences in dispositio
of vitamin A originating from the liver.

Our data of the lymph cannulation experiment again confirm that, at th
administered doses, the cleavage of β-carotene in the rat is almost complete a
was reported before by Huang and Goodman[21] and Goodman et al.[22]. The forme
group collected lymph after a dose of ^{14}C-β-carotene and found 0 - 2% of lymp
radioactivity to be present as β-carotene, while the latter reported 87 and 91
of the activity to be present as retinyl esters. Lakshman and coworkers[1
collecting lymph for 24 h after a dose of ^{14}C-β-carotene, found less (only 80% o
the label) in the retinyl ester fraction.

β-Carotene absorption in lymph in our study varied from 0.1 to 6.6% (mea
1.0%) assuming central cleavage of β-carotene, or about 2% assuming eccentri
cleavage. Although retinyl esters were the only products demonstrated
eccentric cleavage cannot be excluded as retinyl esters could also be formed vi
apo-carotenals. This absorption (1% or 2%) is somewhat lower than reported b
Huang and Goodman[21]; 3 - 6% of a 100 µg dose, Goodman et al.[22]; 3 - 4% of
94 µg dose, and Lakshman et al.[15]; 3.6% of a 200 µg dose infused over a 1-
period. The relatively low absorption in our study might be explained by th
relatively high single dose of β-carotene used. We cannot exclude that th
surgical operation decreased intestinal activity or lymph flow.

Absorption calculated from faecal analysis (balance method) was muc
higher (about 70%) than observed in the lymph cannulation studies. This ma
be explained by destruction of β-carotene by enzymes, bacteria or oxidation.

Feeding the rats a vitamin A-free diet for one day before the cannulatio
experiment apparently did not affect cleavage activity since dioxygenas
activity in the cannulated rats was comparable with that measured in th
non-cannulated rats.

The lymph is expected to be the main route for β-carotene uptake in rat
and only small amounts of polar metabolites such as retinoic acid (if formed
will be transported through the portal vein. In lymph-cannulated rats 15% o
β-carotene absorbed from a ^{14}C-β-carotene dose (3-6%) was recovered in th
lymph and only 0.2% bypassed the lymph route and was found in the liver[2
However, in ferrets most of a dose of ^{14}C-β-carotene absorbed was absorbe

through the portal vein, not only polar metabolites, but also retinyl esters[23]. We followed β-carotene and retinyl ester concentrations in plasma obtained through a vena jugularis cannula from one rat with and one rat without a lymph cannula. Only in the rat without the lymph cannula was an increase in retinyl esters seen (3-fold), while in neither of the rats could β-carotene be demonstrated in plasma, suggesting that lymph transport is indeed the main route and intermediate between absorption and plasma appearance.

The ratio of retinyl esters to β-carotene in the lymph was calculated as an indicator for *in vivo* β-carotene cleavage and to enable a comparison with the *in vitro* dioxygenase activity. Although the variations in retinyl ester and β-carotene concentrations in the collected lymph were considerable, the ratio of retinyl esters to β-carotene indicated similar differences between the three groups as found for intestinal dioxygenase activity. Furthermore, the significant correlation between both measures (Figure 4) suggests that *in vitro* intestinal dioxygenase activity is indeed an indicator for *in vivo* β-carotene cleavage activity.

Since absorbed β-carotene is almost completely converted in rat and the liver is the main storage site of vitamin A, the liver vitamin A content seems a good measure of 'overall' β-carotene availability, i.e. absorption, cleavage and excretion. From the relation between vitamin A intake and liver vitamin A storage in the rats not fed β-carotene, the vitamin A intake required to give the same liver vitamin A storage as for the β-carotene fed rats was calculated by extrapolation. The conversion factor was calculated taking into account the amount of preformed vitamin A consumed, resulting in a factor of 4 for the low-A and norm-A diets, and of 9 for the high-A diet (μg β-carotene resulting in the same liver vitamin A storage as 1 μg vitamin A). Whether this difference is due to differences in absorption or cleavage or a combination of both cannot be concluded from this experiment. Comparison of the calculated conversion factors with those reported before is complicated since the vitamin A potency of β-carotene depends on a variety of factors, such as the dose and source of β-carotene, the carrier used, the quantity and nature of the fat given, as well as the assay used[24]. For the higher levels of supplementation, as we used, Bauernfeind et al.[24] reported a factor of 6 to 10, while Brubacher and Weiser[25] reported a factor of 3.3 for oily solutions and 6 for vegetables. Although the dependency of the conversion factor on the β-carotene intake is well described,

the dependency we demonstrated on the vitamin A intake has, to ou knowledge, not been reported before.

The results of this study do not allow clear distinction between effects o absorption and effects on cleavage of β-carotene, but the data suggest that bot might be affected by the retinyl palmitate content of the diet. Since β-carotene thought to be absorbed by passive diffusion, at high doses the absorption rat may depend on the rate of disappearance from the enterocyte. Since in rat almost all β-carotene absorbed is converted, it may well be that the decrease cleavage of β-carotene to retinol decreased the β-carotene concentratio difference across the luminal wall and thus decreased absorption. The suggeste relation between cleavage activity and absorption cannot be demonstrated b the results of the lymph cannulation experiment.

In this study we demonstrated that both the vitamin A and β-caroten intake affect β-carotene cleavage. We can only speculate about the signal an the mechanism involved. Liver vitamin A content may play an important rol This is supported by the observation that in the Low A group the 7 rats wit completely vitamin A depleted livers had a mean intestinal dioxygenase activit of 112 ± 28 ng retinal formed per h per mg protein S-9, while the 3 rats sti having a small amount of vitamin A in their liver showed a much lower cleavag activity of 40 ± 10 (t-test, P = 0.003). However, in the Norm A group no relatio between the liver vitamin A content and the dioxygenase activity could b demonstrated, although a wide range of cleavage activities was found (19 136 ng retinal per h per mg protein S-9). Furthermore, in a previous study wit hamsters fed a low vitamin A diet for 3 months, a 2.3-fold increase in intestina cleavage activity was found, while the livers were not depleted of vitamin A The plasma retinol concentration seems not involved. Plasma retinol wa substantially decreased in all rats fed the low-A diet, but not related to th dioxygenase activity. Furthermore, as expected, plasma retinol was not affecte by a high vitamin A intake, whereas the dioxygenase activity was. Othe regulatory factors might be the vitamin A content of the enterocytes, th concentration of retinoid glucuronides in the intestine, or retinoic acid forme from β-carotene (in small amounts that escaped detection in this study). Wang found strong indications for a regulatory effect of retinoic acid on retin metabolism in ferrets.

Another mechanism playing a role in control of the β-carotene cleavag might proceed through the activity of retinal reductase, or the availability c

cellular retinol-binding protein type II (CRBP II), the protein that binds retinal in the intestinal mucosa to be reduced to retinol. Vitamin A deficiency may increase the CRBP II content as Rajan et al.[26] reported a higher level of CRBP II mRNA in the small intestine of vitamin A deficient rats than in those of control rats.

In conclusion, in rats dioxygenase activity measured *in vitro* seems an adequate indicator of *in vivo* β-carotene cleavage. Both *in vitro* and *in vivo* measured β-carotene cleavage activity in the intestine is higher in rats with a low vitamin A intake than in rats with a high intake of either vitamin A or β-carotene. Further studies are needed to elucidate the regulator(s) of cleavage activity and the mechanism of activity changes. Furthermore, extrapolation to other species, especially humans, has to be studied.

References

1. Goodman DS, Blomstrand R, Werner B, Huang HS and Shiratori T. The intestinal absorption and metabolism of vitamin A and β-carotene in man. J Clin Invest 1966;45: 1615-1623.
2. Gey KF, Moser UK, Jordan P, Stahelin HB, Eichholzer M and Ludin E. Increased risk of cardiovascular disease at suboptimal plasma concentrations of essential antioxidants - An epidemiological update with special attention to carotene and vitamin C. Am J Clin Nutr 1993;57:S787-S797.
3. van Poppel G. Carotenoids and cancer: an update with emphasis on human intervention studies. Eur J Cancer 1993;29A:1335-1344.
4. Mathews-Roth MM. Beta-carotene: Clinical aspects. Curr Top Nutr Dis 1989;22:17-38.
5. Gronowska-Senger A and Wolf G. Effect of dietary protein on the enzyme from rat and human intestine which converts β-carotene to retinal. J Nutr 1970;100:300-308.
6. Villard L and Bates CJ. Carotene dioxygenase (EC 1.13.11.21) activity in rat intestine: effects of vitamin A deficiency and of pregnancy. Br J Nutr 1986;56:115-122.
7. van Vliet T, van Schaik F and van den Berg H. Beta-carotene metabolism: the enzymatic cleavage to retinal. Voeding 1992;53:186-190.
8. Rutten AAJJL and de Groot AP. Comparison of cereal-based diet with purified diet by short-term feeding studies in rats, mice and hamsters, with emphasis on toxicity characteristics. Food Chem Toxicol 1992;30:601-610.
9. Craft NE, Brown ED and Smith JC. Effects of storage and handling conditions on concentrations of individual carotenoids, retinol, and tocopherol in plasma. Clin Chem 1988;34:44-48.
10. Bollman JL, Cain JC and Grindlay JH. Techniques for the collection of lymph from the liver, small intestine, or thoracic duct of the rat. J Lab Clin Med 1948;33:1349-1352.
11. Speek AJ, Temalilwa CR and Schrijver J. Determination of β-carotene content and vitamin A activity of vegetables by high performance liquid chromatography and spectrophotometry. Food Chem 1986;19:65-74.
12. van Vliet T, van Schaik F, van Schoonhoven J and Schrijver J. Determination of several retinoids, carotenoids and E vitamers by high-performance liquid chromatography. Application to plasma and tissues of rats fed a diet rich in either β-carotene or canthaxanthin. J Chromatogr 1991;553:179-186.

13. Olson JA and Hayaishi O. The enzymatic cleavage of β-carotene into vitamin A by solubl enzymes of rat liver and intestine. Proc Natl Acad Sci USA 1965;54:1364-1370.
14. Goodman DS & Huang HS. Biosynthesis of vitamin A with rat intestinal enzymes. Scien 1965;149:879-880.
15. Lakshman MR, Asher KA, Attlesey MG, Satchithanandam S, Mychkovsky I and Coutlak PJ. Absorption, storage, and distribution of beta-carotene in normal and beta-carotene-fe rats: roles of parenchymal and stellate cells. J Lipid Res 1989;30:1545-1550.
16. Shapiro SS, Mott DJ and Machlin LJ. Kinetic characteristics of β-carotene uptake and depletion in rat tissue. J Nutr 1984;114:1924-1933.
17. Carlier C, Etchepare M, Ceccon J-F, Mourey M-S and Amédée-Manesme O. Efficacy of massive oral doses of retinyl palmitate and mango (*mangifera indica* L.) 8consumption to correct an existing vitamin A deficiency in Senegalese children. Br J Nutr 1992;68: 529-540.
18. Edes TE, Gysbers DG, Buckley CS and Thornton WH. Exposure to the carcinogen benzopyrene depletes tissue vitamin A: β-carotene prevents depletion. Nutr Cancer 1991 15:159-166.
19. Wang X-D, Tang G-W, Fox JG, Krinsky NI and Russell RM. Enzymatic conversion of β-carotene into β-apo-carotenals and retinoids by human, monkey, ferret, and rat tissues Arch Biochem Biophys 1991;285:8-16.
20. Napoli JL and Race KR. Biogenesis of retinoic acid from β-carotene: Differences between the metabolism of β-carotene and retinal. J Biol Chem 1988;263:17372-17377.
21. Huang HS and Goodman DS. Vitamin A and carotenoids. I. Intestinal absorption and metabolism of ^{14}C-labeled vitamin A alcohol and β-carotene in the rat. J Biol Chem 1965; 240:2839-2844.
22. Goodman DS, Huang HS and Shiratori T. Mechanism of the biosynthesis of vitamin A from β-carotene. J Biol Chem 1966;241:1929-1932.
23. Wang XD. Absorption and metabolism of beta-carotene. J Am Coll Nutr 1994;13:314-325
24. Bauernfeind JC, Adams CR and Marusich WL. Carotenes and other vitamin A precursor in animal feed. In: Bauernfeind JC, ed. Carotenoids as colorants and vitamin A precursors. New York: Academic Press, 1981.
25. Brubacher GB and Weiser H. The vitamin A activity of β-carotene. Int J Vitam Nutr Res 1985;55:5-15.
26. Rajan N, Blaner WS, Soprano DR, Suhara A and Goodman DS. Cellular retinol-binding protein messenger RNA levels in normal and retinoid-deficient rats. J Lipid Res 1990;31: 821-829.

Chapter 8

Intestinal β-carotene absorption and cleavage in men: response of β-carotene and retinyl esters in the triglyceride-rich lipoprotein fraction after a single oral dose of β-carotene

Trinette van Vliet[1,2], Wil H.P. Schreurs[1,2] and Henk van den Berg[1]

[1] TNO Nutrition and Food Research Institute, Zeist, The Netherlands; [2] Department of Experimental Zoology, University of Amsterdam, Amsterdam, The Netherlands

American Journal of Clinical Nutrition 1995;62:110-116

Abstract

Postprandial response curves of β-carotene and retinyl esters in a triglyceride-rich lipoprotein (TRL) fraction were evaluated as a potential measure of β-carotene uptake and cleavage. β-Carotene, retinyl ester and triglyceride concentrations in the TRL fraction (density < 1.006 kg/L) and plasma were measured in 10 men for 8 or 16 h after an oral dose of 15 mg β-carotene. The β-carotene response unlike the triglyceride and retinyl ester response can be evaluated in the TRL fraction but not in plasma. Intraindividual variations in the triglyceride-adjusted response of β-carotene and retinyl palmitate in TRL fractions were 23% and 20% and interindividual variations were 42% and 36% respectively. A low β-carotene response was associated with a high ratio between retinyl palmitate and β-carotene responses ($r = -0.56$, $P = 0.013$). In conclusion, the measurement of β-carotene and retinyl esters in the TRL fraction after a dose of β-carotene with a vitamin A-free meal may be an appropriate method to study β-carotene uptake and cleavage.

Introduction

Epidemiological studies indicate that the consumption of foods rich i
carotenoids is associated with a reduced risk for certain types of cancer an
cardiovascular disease[1,2]. β-Carotene may be preventive as an antioxidant[3] or a
a precursor for vitamin A. The factors determining the balance betwee
absorption of intact β-carotene and β-carotene cleavage in humans are sti
largely unknown. The identification of these factors is not only important i
controlling β-carotene antioxidant status, but also in controlling vitamin
status, which is especially relevant in many parts of the developing world whe
β-carotene is more readily available in the diet than vitamin A. To identify thes
factors, a reproducible method to assess intestinal β-carotene absorption an
cleavage in humans is needed.

Most of the studies focused on β-carotene absorption and availability, use
the β-carotene plasma response curve. A wide variability for this response ha
been reported[4-11]. Sometimes the retinyl ester response is also measured[6,8,10,
However, because in most studies the test meal contained vitamin A, it
unclear whether retinyl esters originate from cleaved β-carotene or fro
vitamin A in the test meal.

Interpretation of plasma β-carotene response curves is hampered by the fa
that most of the circulating β-carotene is of endogenous origin and has a lo
half-life of about 5 days[4,12]. Chylomicrons, however, contain only absorbe
β-carotene and its cleavage products. Therefore chylomicrons may be a bett
model for studying intestinal absorption and cleavage of β-caroten
Chylomicron fractions were analysed after oral dosing in two studies onl
These studies consisted of only a limited number of samples and cleava
products could not be demonstrated[11] or were not measured[13].

A problem with the use of plasma response curves may be nonrespons
recently reported by Johnson and Russell[11] in a relatively high percentage
their subjects. Nonresponders showed no plasma β-carotene response and only
small response in chylomicrons after a large oral dose of 120 mg β-carotene.
was also suggested that the plasma β-carotene response was affected by
delayed release of β-carotene from the enterocyte into the circulation, possib
as a consequence of a second meal as reported by Henderson et al.[6].

To evaluate postprandial chylomicron curves as a reliable method to stud
factors affecting β-carotene intestinal absorption and cleavage in humans, w
studied the intra- and interindividual variabilities of this method. Therefore w

determined the response curves of β-carotene, retinyl esters and triglycerides in a triglyceride-rich lipoprotein (TRL) fraction and plasma of volunteers after a single oral dose of β-carotene on two occasions, 16 days apart. In addition, the possibility of a delayed response was tested by assessing the response after a second meal without β-carotene, 8 h after the β-carotene dose.

Subjects and methods

Subjects

Twelve healthy, non-smoking, young men living in the Utrecht, The Netherlands, area participated. All volunteers underwent a screening procedure that included a health and lifestyle questionnaire, a physical examination and a routine blood clinical chemistry profile testing. Volunteers had no history or biochemical evidence of liver or pancreatic disease, active bowel disease, resection, or abnormal fat metabolism and did not use medication suspected of interfering with fat-soluble-vitamin absorption. Excessive alcohol consumers (> 40 g/d) were excluded and the volunteers did not use vitamin or carotenoid supplements in the 2 months before the study.

The study protocol was approved by the Medical Ethical Committee of the TNO Institute and the participants provided written informed consent.

Study design

All participants were instructed to avoid food products with a high vitamin A or provitamin-A-carotenoid content in the week before the study; they were given a list of products to exclude from the diet. During the last 2 days before the experiment, all participants were supplied with a standardized diet, low in vitamin A (70 μg/d) and β-carotene (11 μg/d), to minimize the presence of β-carotene and dietary retinoids in the intestine which could interfere with the results.

The study consisted of 2 experimental days, 16 days apart. Day 1 (the first test day) included two consecutive experimental periods of 8 h, the first starting with a meal with β-carotene and the second with the same meal without β-carotene. Ten subjects participated but the results for β-carotene in the TRL fraction are based on eight subjects; curves could not be made for two subjects because of missing data. Day 16 (the second test day) consisted of one 8-h period, starting with a meal with β-carotene for 10 subjects and a meal without β-carotene for two subjects (control subjects).

All participants arrived at the institute on the evening before th experimental day and received the standardized evening meal. The nex morning, after a 12 h fast, an indwelling catheter (obturator locked) wa inserted in the antecubital vein. After a fasting blood sample had been collecte (t = 0), all volunteers consumed a meal consisting of 400 g skimmed yoghur 50 g arachidic oil, 20 g sugar and either 15 mg β-carotene or no β-caroten (control subjects). The meal was freshly prepared and thoroughly mixed f about 3 min with a mixer. β-Carotene was added as 10% water-soluble beadle (Roche, Mijdrecht, The Netherlands). The energy content of the meal wa 2750 kJ, the meal contained 8.7 mg α-tocopherol, and no vitamin A could b detected (< 3 μg). The near absence of β-carotene in the test meal given to th control subjects (as was expected) was confirmed by HPLC (< 1 μg). Bloo samples were collected into tubes containing EDTA 2, 3, 4, 5, 6, 7 and 8 h afte every meal. During this period and in the evening before the experimental da no food was allowed and participants were only permitted to drink tap wate and unsweetened coffee or tea with no added cream.

Isolation of TRLs

Blood samples were immediately placed in the dark and further samp handling was performed in subdued light to protect β-carotene and retin esters. Plasma was collected after centrifugation at 2000 *g* for 10 min at 4°C within 0.5 h after the blood samples were collected. About 0.5 mL was stored a 4°C for triglyceride analysis (within 1 d), whereas the remaining plasma wa frozen on dry ice and stored at –80°C until the TRLs were isolated, within 10 For isolation of the TRLs, plasma samples were thawed while being gent shaken in water at 10°C. A 2.5-mL aliquot of plasma was transferred into 4.4-mL polyallomer tube and overlayered with 1 mL NaCl solution (density 1.006 kg/L). The samples were subjected to ultracentrifugation for 30 min a 100,000 *g* in a swing-out rotor type TFT 41.14 (Kontron Instruments, Mila Italy). Tubes were sliced at a fixed position, and 0.5 mL of the TRL-containin supernatant was removed and brought to a final volume of 1.3 mL with salin yielding the TRL fraction.

The procedure of isolating the TRL fraction from frozen plasma samples wa validated by measuring the retinyl palmitate, β-carotene and triglyceric contents of the TRL fraction obtained from fresh plasma as well as from th same plasma sample after the plasma had been frozen for ≥ 1 d. Plasm

samples were collected from nine volunteers after an overnight fast and 5 h after a meal, as in the main study. Only small amounts of β-carotene and retinyl palmitate (both < 8 nmol/L plasma) and triglycerides (< 0.1 mmol/L plasma) could be demonstrated in both the freshly isolated TRL fraction and the TRL fraction from frozen plasma collected after subjects had fasted. For non-fasting plasma the amounts ($\bar{x}$ ± SEM) of β-carotene, retinyl palmitate and triglycerides in the TRL fraction isolated from frozen plasma, expressed as a percentage of the amount in the freshly isolated TRL fraction, were 118 ± 6%, 101 ± 4%, and 95 ± 4%, respectively. Amounts in fresh and frozen samples did not differ significantly for any of the compounds analysed (paired t-test).

Analytical methods

Cholesterol and triglyceride concentrations were measured by using commercially available enzymatic colorimetric assays (CHOD-PAP and GPO-PAP respectively; Boehringer, Mannheim, Germany).

For retinyl ester, β-carotene and vitamin E analyses 1 mL of the TRL fraction or 1 mL plasma was mixed with 1 mL ethanol (containing 16 - 32 μmol α-tocopheryl acetate/L as internal standard). After 10 min, 2 mL hexane was added and the sealed tubes were vortexed for 4 min. After centrifugation for 10 min at 3000 *g* at 4°C, the hexane layer was separated and evaporated under nitrogen at room temperature. The residue was dissolved in 0.4 mL HPLC solvent and transferred into brown HPLC injection vials.

Retinyl palmitate, retinyl stearate, β-carotene and vitamin E were quantified by HPLC, using a modified version of a method previously described[14]. Briefly, a Superspher 100 RP-18 column (Merck, Darmstadt, Germany) was used. The mobile phase consisted of acetonitrile - methylene chloride - methanol (70:15:10, v/v/v) and the flow rate was 1 mL/min. Two absorbance detectors in series were used, one switching from 292 to 445 nm for detection of tocopherols and β-carotene respectively, and the other switching from 292 to 325 nm for detection of tocopherols and retinyl esters, respectively. Limits of detection for the TRL fraction were 1.6 and 3.7 nmol/L plasma for β-carotene and retinyl palmitate respectively, and for plasma these limits were 3.0 and 5.5 nmol/L plasma.

Standards of α-tocopherol and α-tocopheryl acetate were obtained from Sigma (St. Louis, MO), β-carotene from Merck, and retinyl palmitate from Fluka (Buchs, Switzerland). Retinyl stearate containing small amounts of palmitate was a gift from Hoffmann-La Roche (Basel, Switzerland). The

stearate content was determined by correcting spectrophotometric readings of
stearate solution for the HPLC-determined palmitate content, using an $E_{1\,cm}^{1\,\%}$
(the absorbance of a 1% solution) of 975 for palmitate and 940 for stearate, bot
in ethanol at 325 nm[15]. Retinyl palmitate is the most abundant retinyl ester ar
the amount of stearate was a fixed percentage of palmitate, independent
subject or time point (on average 34%). Because retinyl stearate was difficult
quantitate at early and late time points, only the results of retinyl palmitate a
given. The sum of retinyl palmitate and retinyl stearate, mentioned as retin
esters, is used for estimation of β-carotene conversion and absorption.

Statistics

Results were expressed as mean ± SEM. Results for days 1 and 16 we
compared by using the paired Student's t test. Pearson's correlation coefficien
were used to assess the relation between results for day 1 and those for day 1
and between different responses. Two-sided P values < 0.05 were considere
statistically significant. Estimates of the intra- and interindividual SDs we
calculated from the pooled data by one way analysis of variance. Changes in th
ratio of retinyl esters to β-carotene in TRLs with time were evaluated by fittin
orthogonal polynomials for all subjects. A Student's t-test was used to te
whether the mean quadratic coefficient of the polynomials was significant
greater than zero, indicating the presence of a U-shaped component.

Results

Characteristics of the subjects are presented in **Table 1**. Normal ranges fro
the laboratory for fasting plasma concentrations established with apparent
healthy blood donors aged 16 - 64 y were 15 - 43 μmol/L for vitamin E ar
100 - 800 nmol/L for β-carotene. Generally reported cut-off points of < 2.3 ar
6.5 mmol/L were adopted for triglycerides and cholesterol, respectively. For a
volunteers the vitamin E and cholesterol concentrations were within the norm
range. One volunteer was accepted with a screening triglyceride concentratio
of 2.6 mmol/L (< 2.3 mmol/L on both test days). The β-carotene concentrations
4 subjects were below the lower limit of the normal range, probably becau
subjects consumed a diet low in β-carotene for 1 wk before the test days. N
differences were observed between the 10 experimental subjects and the
control subjects.

Table 1. Subject characteristics[1].

	Value	
Age (y)	22.2 ± 0.3	(20 - 24)
Body mass index (kg/m^2)	21.7 ± 0.6	(19.4 - 24.5)
Fasting plasma concentrations		
Total cholesterol (mmol/L)	4.8 ± 0.2	(3.6 - 5.7)
Triglyceride (mmol/L)	1.3 ± 0.2	(0.7 - 2.6)
β-Carotene (nmol/L)	159 ± 27	(47 - 447)
Retinyl palmitate (nmol/L)	14.7 ± 3.1	(4.6 - 59)
Vitamin E (μmol/L)	23.4 ± 1.5	(15 - 35)

[1] $\bar{x}$ ± SEM; range in parentheses. n = 12.

Response in TRLs

For all subjects given β-carotene, there was a positive response of triglycerides, β-carotene and retinyl palmitate in the TRL fraction, although the variation in response was large. Mean curves for triglycerides, β-carotene and retinyl palmitate in the TRL fraction on days 1 and 16 are given in **Figure 1.** The mean triglyceride curves peaked early at around 2 h whereas a second peak was seen 5 - 6 h after the first meal. Curves for β-carotene and retinyl palmitate both showed one peak at 5 - 6 h after the first meal; the β-carotene peak was symmetrical whereas the retinyl palmitate peak seemed skewed to the right.

For quantitative comparisons between and within subjects, and to estimate β-carotene absorption, the areas under the concentration time curves (AUCs) were calculated by using trapezoidal approximation after subtracting baseline concentrations. The results are presented in **Table 2**. Although the triglyceride response tended to be higher on day 16 than on day 1, no significant differences were found between the experimental days (paired t-test). The control subjects, who only participated on day 16, showed a triglyceride response comparable with that of the subjects (1.75 and 3.15 mmol·h/L), whereas one control subject showed no retinyl palmitate and β-carotene responses and the other showed very low responses, never exceeding 18% of the mean value for subjects on day 16.

The second meal on day 1 resulted in a triglyceride response not different from the response to the first meal; the AUC at 0 - 8 h was 1.93 ± 0.47 mmol·h/L and the AUC at 8 - 16 h was 1.73 ± 0.27 mmol·h/L. For both β-carotene and retinyl palmitate the mean curves showed no increase in response to the second meal. Seven subjects displayed no response to the second meal, whereas three

subjects showed a response with an AUC 73%, 29%, and 13% (mean of th β-carotene and the retinyl palmitate response) of the AUC after the first meal.

Plasma response

Mean plasma curves for triglycerides, β-carotene and retinyl palmitate o days 1 and 16 are shown in **Figure 2**. The curves for triglycerides and retin palmitate were quite similar to the curves of the TRL fraction because baseli plasma concentrations were low. Fasting plasma β-carotene concentrations we much higher; therefore, the β-carotene response in plasma was less clear. A increase in the β-carotene concentration until 16 h was seen in plasma but n in the TRL fraction. The increase in plasma may have been due to resecretion β-carotene taken up by the liver. For retinyl palmitate the AUC of plasma w calculated (Table 2).

Table 2. Areas under response curves on days 1 and 16[1].

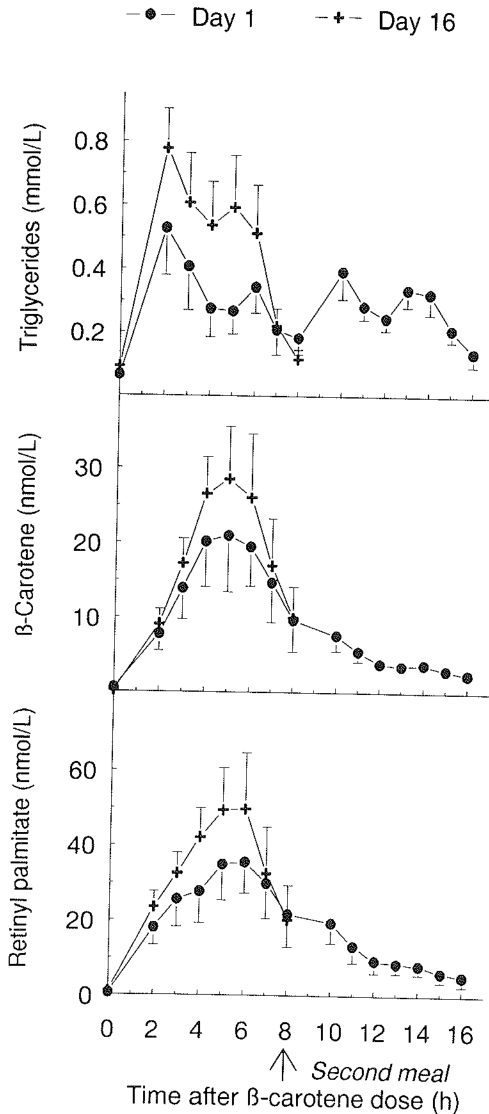
	Day 1[2]		Day 16	
Triglyceride-rich lipoproteins				
Triglyceride (mmol·h/L)	1.93 ± 0.47	(0.82 - 5.86)	3.05 ± 0.64	(1.16 - 7.60
β-Carotene (nmol·h/L)	100 ± 25[3]	(31 - 248)	149 ± 34	(47 - 328)
β-Carotene/triglyceride	52 ± 10[3]	(34 - 114)	52 ± 8	(17 - 85)
Retinyl palmitate (nmol·h/L)	188 ± 42	(40 - 480)	245 ± 54	(82 - 577)
Retinyl palmitate/triglyceride	99 ± 11	(46 - 172)	83 ± 12	(41 - 177)
Plasma retinyl palmitate (nmol·h/L)	267 ± 52	(81 - 554)	325 ± 62	(117 - 654)

[1] $\bar{x}$ ± SEM; range in parentheses. n = 10. There were no significant differences between day and 16 (paired t-test).

[2] First meal only; [3] n = 8.

Intra- and interindividual variation

Intra- and interindividual variabilities for the AUCs of triglycerides, β-carote and retinyl palmitate in TRLs and for fasting plasma concentrations a presented in **Table 3**, together with the ratios of intra- to interindividu variation. Both the β-carotene and retinyl palmitate responses in TRLs we strongly correlated with the triglyceride response (both days together, r = 0. and 0.92 respectively; P < 0.0001). Because the triglyceride response vari widely between the 2 experimental days, the responses of β-carotene and retin palmitate were adjusted for the triglyceride response. As shown, adjustme reduced intraindividual and to a lesser extent interindividual variations a improved the ratio of intra- to interindividual variability.



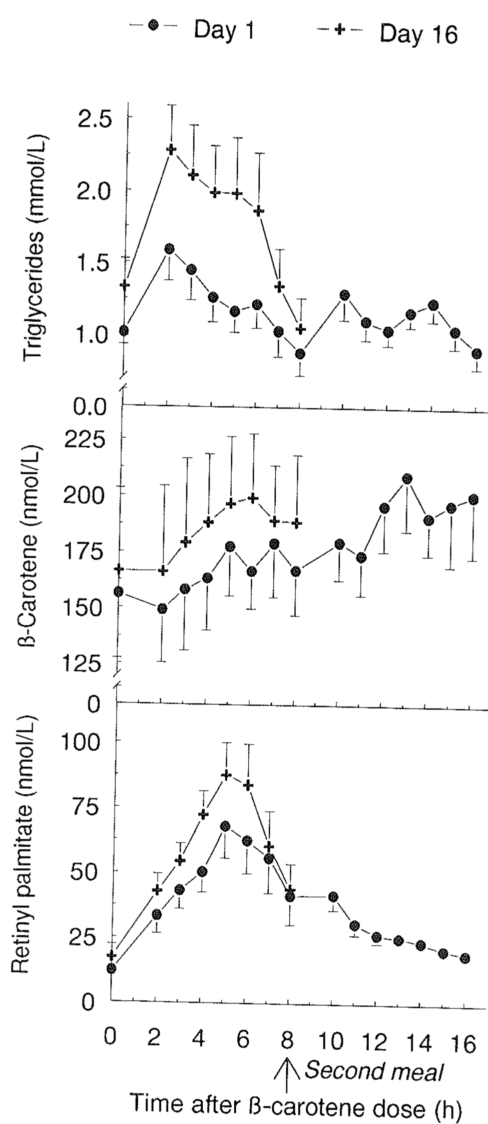


Figure 1. Response of triglyceride, β-carotene and retinyl palmitate in the triglyceride-rich lipoprotein (TRL) fraction after a single oral dose of 15 mg β-carotene with a test meal and a second meal (β-carotene-free) 8 h later, $\bar{x}$ ± SEM; n = 10 (n = 8 for β-carotene on day 1).

Figure 2. Response of triglyceride, β-carotene and retinyl palmitate in plasma after a single oral dose of 15 mg β-carotene with a test meal and a second meal (β-carotene-free) 8 h later, $\bar{x}$ ± SEM; n = 10.

Table 3. Intra- and interindividual variabilities[1].

		SD		
	$\bar{x}$	Intra [CV, %]	Inter [CV, %]	Intra/Ir
AUC for TRL				
Triglyceride (mmol•h/L)	2.49	1.55 [62]	0.98 [39]	1.58
β-Carotene (nmol•h/L)[2]	124	65.0 [52]	59.0 [47]	1.10
Retinyl palmitate (nmol•h/L)	216	85.7 [40]	128 [59]	0.67
Retinyl ester/β-carotene[2]	2.68	0.52 [19]	0.89 [33]	0.58
β-Carotene/triglyceride[2]	52.3	12.1 [23]	22.2 [42]	0.55
Retinyl palmitate/triglyceride	91.3	18.7 [20]	32.7 [36]	0.57
AUC for plasma retinyl palmitate (nmol•h/L)	296	72.9 [25]	168 [57]	0.43
Fasting plasma				
Triglyceride (mmol/L)	1.17	0.42 [36]	0.28 [24]	1.49
β-Carotene (nmol/L)	160	35.0 [22]	98.4 [62]	0.36
Retinyl palmitate (nmol/L)	14.7	7.01 [48]	10.8 [74]	0.65

[1] n = 10. AUC, area under the curve; TRL, triglyceride-rich lipoprotein.
[2] n = 8.

Ratio of retinyl esters to β -carotene

To get an impression of the intestinal β-carotene cleavage activity the ratio AUCs for retinyl esters to AUCs for β-carotene in the TRL fraction w calculated. This ratio varied widely between subjects from 1.07 to 4.85 ($\bar{x}$ SEM: 2.92 ± 0.41 for day 1 and 2.43 ± 0.30 for day 16), but was reproduci between both days (r = 0.87, P = 0.0021).

Efficient conversion, defined as a high ratio between retinyl esters a β-carotene, was related to a low AUC for β-carotene, as is shown in **Figur** (r = –0.56, P = 0.013). No relation between conversion and the sum of the reti ester and β-carotene response adjusted for triglyceride response could demonstrated.

As mentioned before, the shapes of the β-carotene and retinyl palmit curves were not identical. To further study this difference the mean ratio retinyl esters to β-carotene at all time points after the meal with β-carotene shown in **Figure 4**. After a decline during the first hours after the mea gradual increase was seen. The mean quadratic component of the fit individual polynomials was significantly greater than zero (P < 0.0(indicating that the curves do contain a U-shaped component.

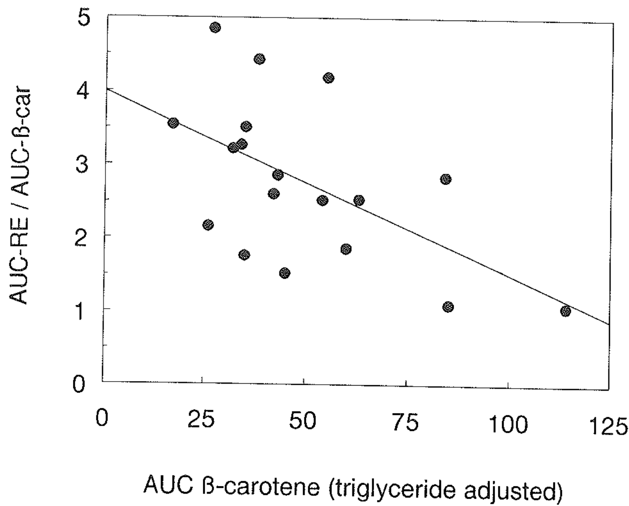


Figure 3. Correlation between the triglyceride-adjusted β-carotene response in triglyceride-rich lipoproteins (TRLs) and the ratio of the response of retinyl ester to β-carotene in TRLs for both test days (day 1, n = 8; day 16, n = 10). r = –0.56, P=0.013. AUC, area under the curve.

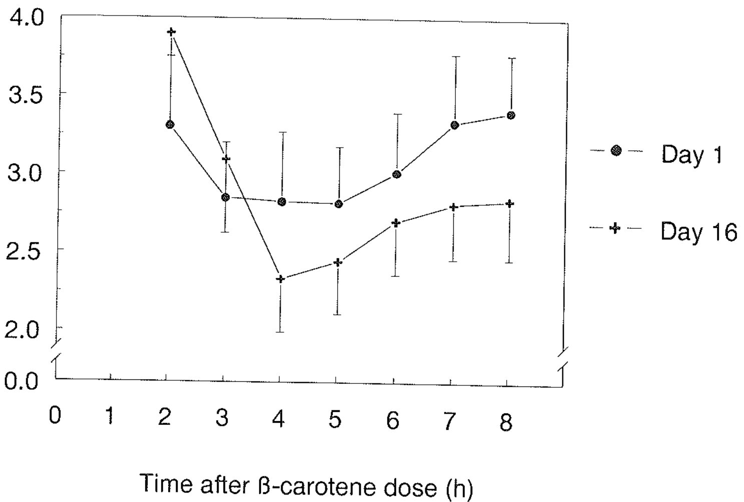


Figure 4. Ratio of retinyl esters to β-carotene in triglyceride-rich lipoproteins (TRLs) after a single oral dose of 15 mg β-carotene with a test meal. $\bar{x}$ *± SEM, n = 8 (day 1), n = 10 (day 16).*

Discussion

In this study we measured the individual responses of β-carotene and retin esters in TRL fractions and plasma after a single oral dose of β-carotene. Th data show that the β-carotene response unlike the response of triglyceride an retinyl ester, after a single oral dose of β-carotene can be evaluated in TRLs bu not in plasma.

Reported β-carotene plasma responses from other studies are highl variable[4-11], because of differences in the dose and administration β-carotene[5,6,13], test meal composition[7,9,16], meal pattern during the test[6] an subject characteristics[8,16,17]. Because of this variability quantitative compariso with our results is not useful.

The use of TRL response curves as a measure for β-carotene uptake an cleavage is based on the assumption that the TRL fraction contains mainl intestinally derived lipoproteins (chylomicrons and their remnants) and som liver derived lipoproteins (very-low-density lipoproteins. However, Cohn al.[18,19] and Schneeman et al. [20] reported rather high contributions of liver derive lipoproteins (apo B-100 containing) to the TRL fraction. The followin observations in our study support the presence of mainly intestinally derive lipoproteins in our TRL fraction. The TRL fraction from fasting plasm contained no β-carotene, control subjects showed no response or only a very lo response, and the increase of plasma β-carotene seen up to 16 h after the fir meal (probably because of liver derived lipoproteins) was not seen in the TR fraction.

Recovery of intestinally derived lipoproteins in TRL is reported to b incomplete. In the first 8 h after a meal 80 - 90% of plasma retinyl esters wa recovered in the TRL fraction[21-23]. In our study the recovery of retinyl palmitat in the TRL fraction as a percentage of the increase in plasma 2, 3, 4, 5 and 8 after the β-carotene dose was 97%, 90%, 69%, 63% and 68%, respectively. On explanation, supported by the decrease of the percentage with time, is that pa of the remnants were not isolated in the TRL fraction. Another explanatio could be an exchange from chylomicrons to other lipoproteins. Exchange β-carotene does not appear to occur rapidly[24]. For retinyl esters an exchange 13% after 6 h has been reported *in vitro* in rabbits[25]. Incomplete recovery intestinally derived lipoproteins makes the method somewhat less sensitiv however, since the recovery for retinyl esters and β-carotene is supposed to b the same, this does not affect our conclusions.

Another way to evaluate recovery of intestinally derived lipoproteins in the TRL fraction is to estimate β-carotene absorption using the TRL response curves. We estimated the mean absorption by dividing the sum of the mean AUC for β-carotene and retinyl esters in TRL by the estimated AUC β-carotene after an intravenous dose. This AUC after an intravenous dose was calculated using a plasma volume of 3300 mL (927 + 31.47 × mean body weight[26] and the mean of reported half-lives of remnants, i.e. 11.5 min[22,27,28]. As this study does not allow any conclusion on the type of cleavage, i.e., central versus eccentric cleavage, absorption was calculated for both variants. Mean absorption (β-carotene plus retinyl esters) was 11% assuming central cleavage, i.e. 2 molecules of retinyl esters formed per molecule of β-carotene, or 17% assuming eccentric cleavage, i.e., 1 molecule of retinyl ester formed per molecule of β-carotene. We realize that this is a rather rough estimate, that can only be made on group level, but the results are in reasonable agreement with the absorption of 9 - 17% (with one extreme of 52%) found in lymph cannulation studies in humans[29,30].

For comparative studies on factors affecting β-carotene absorption and cleavage, a reproducible measure, i.e. a relatively low intraindividual as compared to interindividual variability, is required. As shown in Table 3, best results for β-carotene and retinyl palmitate in TRL were obtained after adjustment for the triglyceride response. The large intraindividual variability in the triglyceride response in our study (62%) as compared to reported values of 19%[31] and 6.4%[23] could be explained by differences in fasting plasma triglyceride concentrations. The fasting plasma triglyceride concentration is reported to be an important determinant of the triglyceride response[32]. Adjustment of our triglyceride response by dividing the AUC for triglyceride by the fasting plasma triglyceride concentration resulted in AUCs of 1.83 ± 0.34 and 2.28 ± 0.29 mmol·h/L for days 1 and 16, respectively; a reduction of the intraindividual variability from 62% to 28%; and a reduction of the ratio from 1.58 to 0.68.

SDs in fasting plasma concentrations of β-carotene, retinyl palmitate and triglyceride found in our study (Table 3) were in good agreement with reported values[33,34].

Limitations of the method such as nonresponse or delayed response, seemed unimportant in our study. All subjects that received β-carotene showed a response and only one subject showed an appreciable response of β-carotene and retinyl ester to the second meal as compared to the first meal. The relatively low

dose of β-carotene given in combination with a fair amount of fat resulted maximal absorption with the first meal.

To further evaluate our method, β-carotene conversion was estimated using the ratios between the AUCs for retinyl esters and β-carotene in t TRLs. Assuming a similar clearance for retinyl esters and β-carotene, the rati found correspond with 35 - 71% of β-carotene conversion when only centr cleavage occurred and 52 - 83% when only eccentric cleavage occurred, i. there was only one molecule of retinyl ester formed per molecule β-caroter These percentages are similar to those (67 - 94%) calculated from the results the lymph cannulation studies mentioned before[29,30].

The assumed similarity of retinyl palmitate and β-carotene clearance w supported by the fact that maximum concentrations of β-carotene and retin palmitate were reached at the same time. Furthermore, calculated half-lives f both compounds in the TRLs over the period from the maximum concentrati to 8 h after the dose were not different (paired t test) and correlated well (bo days together, $r = 0.68$, $P = 0.0015$).

The balance between cleavage and absorption of intact β-carotene differ between subjects. A low β-carotene response seemed to be related to a hi retinyl ester response (Figure 3), but not to a low total response (sum β-carotene and retinyl ester responses). The absence of a β-carotene respon may be explained by very efficient conversion, although this was not seen in o study.

Intestinal metabolism of β-carotene may not be a continuous proce (Figure 4). The decline of the ratio in the first hours after the β-carotene do can possibly be explained by product inhibition or by saturation of the enzym The establishment of a new equilibrium may explain the increase of the rat after 4 h. Some form of regulation of the enzyme activity is expected because high β-carotene intake does not lead to vitamin A intoxication[35]. Anoth possible explanation for the decline is the faster incorporation of retinyl este into chylomicrons, compared with β-carotene. As described by Ong[36], retin formed from intestinal cleavage of β-carotene is probably bound to cellul retinol-binding protein II and efficiently converted to retinol and subsequent to retinyl esters and incorporated in chylomicrons. The mechanism of β-carote transport through the cell is not known, but may be less efficient. This theo however can not explain the increase of the ratio from 4 h on.

In summary, our study shows that the use of response curves of β-carotene and retinyl palmitate in TRLs after a single oral dose of β-carotene is an appropriate method to evaluate intestinal absorption and cleavage of β-carotene in humans. More reproducible data were obtained after adjustment of the β-carotene and retinyl palmitate responses (expressed as the AUC) for the triglyceride response. This method enables exploratory studies on factors affecting β-carotene absorption and cleavage. The ratio of the response of retinyl esters to β-carotene may be a good indicator for intestinal β-carotene conversion.

Acknowledgements

We thank Henk Hendriks for critical review of the manuscript, Dirk van der Heij for editorial advice and Hans Mocking and Renske Brune for skilful assistance in lipoprotein isolation.

References

1. van Poppel G. Carotenoids and cancer: an update with emphasis on human intervention studies. Eur J Cancer 1993;29A:1335-1344.
2. Gey KF, Moser UK, Jordan P, Stahelin HB, Eichholzer M and Ludin E. Increased risk of cardiovascular disease at suboptimal plasma concentrations of essential antioxidants - an epidemiological update with special attention to carotene and vitamin-C. Am J Clin Nutr 1993;57(suppl):787S-797S.
3. Krinsky NI. Antioxidant functions of carotenoids. Free Radic Biol Med 1989;7:617-635.
4. Dimitrov NV, Boone CW, Hay MB, Whetter P, Pins M, Kelloff GJ and Malone W. Plasma beta-carotene levels-kinetic patterns during administration of various doses of beta-carotene. J Nutr Growth Cancer 1986;3:227-237.
5. Brown ED, Micozzi MS, Craft NE, Bieri JG, Beecher G, Edwards BK, Rose A, Taylor PR and Smith JC. Plasma carotenoids in normal men after a single ingestion of vegetables or purified β-carotene. Am J Clin Nutr 1989;49:1258-65.
6. Henderson CT, Mobarhan S, Bowen P, Stacewicz-Sapuntzakis M, Langenberg P, Kiani R, Lucchesi D and Sugerman S. Normal serum response to oral beta-carotene in humans. J Am Coll Nutr 1989;8:625-635.
7. Rock CL and Swendseid ME. Plasma β-carotene response in humans after meals supplemented with dietary pectin. Am J Clin Nutr 1992;55:96-99.
8. Sugerman SB, Mobarhan S, Bowen PE, Stacewicz-Sapuntzakis M, Langenberg P, Henderson C, Kiani R, Friedman H and Lucchesi D. Serum time curve characteristics of a fixed dose of β-carotene in young and old men. J Am Coll Nutr 1991;10:297-307.
9. Prince MR and Frisoli JK. Beta-carotene accumulation in serum and skin. Am J Clin Nutr 1993;57:175-181.
10. Mobarhan S, Bowen P, Andersen B, Evans M, Stacewicz-Sapuntzakis M, Sugerman S, Simms P, Lucchesi D and Friedman H. Effects of β-carotene repletion on β-carotene absorption, lipid peroxidation, and neutrophil superoxide formation in young men. Nutr Cancer 1990;14:195-206.
11. Johnson EJ and Russell RM. Distribution of orally administered β-carotene among lipoproteins in healthy men. Am J Clin Nutr 1992;56:128-135.

12. Kübler W and von Reinersdorff D. The dose dependency of plasma carotenoids. Biokineti studies in men. In: Schlemmer U, ed. Proceedings of the international conference bioavailability '93 - Nutritional, chemical and food processing implications of nutrient availability. Ber Bundesforschungsanst Ernähr 1993;part 2:331-335.
13. Cornwell DG, Kruger FA and Robinson HB. Studies on the absorption of beta-carotene and the distribution of total carotenoid in human serum lipoproteins after oral administration. J Lipid Res 1962;3:65-70.
14. van Vliet T, van Schaik F, van Schoonhoven J and Schrijver J. Determination of several retinoids, carotenoids and E vitamers by high-performance liquid chromatography. Application to plasma and tissues of rats fed a diet rich in either β-carotene or canthaxanthin. J Chromatogr 1991;553:179-186.
15. Fragner J. Vitamine, Chemie und Biochemie, 1964, Gustav Fischer Verlag, Jena.
16. Maiani G, Mobarhan S, Ceccanti M, Ranaldi L, Gettner S, Bowen P, Friedman H, De Lorenzo A and Ferro-Luzzi A. Beta-carotene serum response in young and elderly female Eur J Clin Nutr 1989;43:749-761.
17. Albanes D, Virtamo J, Rautalahti M, Haukka J, Palmgren J, Gref CG and Heinonen OP Serum beta-carotene before and after beta-carotene supplementation. Eur J Clin Nutr 1992;46:15-24.
18. Cohn JS, McNamara JR, Krasinski SD, Russell RM and Schaefer EJ. Role of triglyceride rich lipoproteins from the liver and intestine in the etiology of postprandial peaks in plasma triglyceride concentration. Metabolism 1989;38:484-490.
19. Cohn JS, Johnson EJ, Millar JS, Cohn SD, Milne RW, Marcel YL, Russell RM and Schaefer EJ. Contribution of apoB-48 and apoB-100 triglyceride-rich lipoproteins (TRL) t postprandial increases in the plasma concentration of TRL triglycerides and retinyl este J Lipid Res 1993;34:2033-2040.
20. Schneeman BO, Kotite L, Todd KM and Havel RJ. Relationships between the responses triglyceride-rich lipoproteins in blood plasma containing apolipoproteins B-48 and B-100 a fat-containing meal in normolipidemic humans. Proc Natl Acad Sci USA 1993;90: 2069-2073.
21. Krasinski SD, Cohn JS, Russell RM and Schaefer EJ. Postprandial plasma vitamin A metabolism in humans: a reassessment of the use of plasma retinyl esters as markers fo intestinally derived chylomicrons and their remnants. Metabolism 1990;39:357-365.
22. Berr F, Eckel R and Kern F. Plasma decay of chylomicron remnants is not affected by heparin-stimulated plasma lipolytic activity in normal fasting man. J lipid Res 1985;26: 852-859.
23. Berr F, Eckel RH and Kern F. Contraceptive steroids increase hepatic uptake of chylomicron remnants in healthy young women. J Lipid Res 1986;27:645-651.
24. Clevidence BA and Bieri JG. Association of carotenoids with human plasma lipoproteins. Methods Enzymol 1993;214:33-46.
25. Zilversmit DB, Morton RE, Hughes LB and Thompson KH. Exchange of retinyl and cholesteryl esters between lipoproteins of rabbit plasma. Biochim Biophys Acta 1982;712 88-93.
26. Grundy SM and Mok HYI. Chylomicron clearance in normal and hyperlipidemic man. Metabolism 1976;25:1225-1239.
27. Redgrave TG, Ly HL, Quintao ECR, Ramberg CF and Boston RC. Clearance from plasm of triacylglycerol and cholesteryl ester after intravenous injection of chylomicron-like lipi emulsions in rats and man. Biochem J 1993;290:843-847.
28. Cortner JA, Coates PM, Le N-A, Cryer DR, Ragni MC, Faulkner A and Langer T. Kineti of chylomicron remnant clearance in normal and in hyperlipoproteinemic subjects. J Lipi Res 1987;28:195-206.

29. Goodman DS, Blomstrand R, Werner B, Huang HS and Shiratori T. The intestinal absorption and metabolism of vitamin A and β-carotene in man. J Clin Invest 1966;45: 1615-1623.
30. Blomstrand R and Werner B. Studies on the intestinal absorption of radioactive β-carotene and vitamin A in man. Conversion of β-carotene into vitamin A. Scand J Clin Lab Invest 1967;19:339-345.
31. Cohen JC, Noakes TD and Benade AJS. Serum responses to fatty meals: effects of meal fat content. Am J Clin Nutr 1988;47:825-827.
32. Patsch JR. Postprandial lipaemia. Baillières Clin Endocrinol Metab 1987;1:5515-5580.
33. Gallagher SK, Johnson LK and Milne DB. Short-term and long-term variability of selected indices related to nutritional status. 2. Vitamins, lipids, and protein indices. Clin Chem 1992;38:1449-1453.
34. Tangney CC, Shekelle RB, Raynor W, Gale M and Betz EP. Intra- and interindividual variation in measurements of β-carotene, retinol, and tocopherols in diet and plasma. Am J Clin Nutr 1987;45:764-769.
35. Mathews-Roth MM. Beta-carotene: clinical aspects. Curr Top Nutr Dis 1989;22:17-38.
36. Ong DE. Cellular transport and metabolism of vitamin A: roles of the cellular retinoid-binding proteins. Nutr Rev 1994;52:S24-31.

Chapter 9

General discussion

β-Carotene is important as provitamin A in many parts of the developing world where β-carotene is more readily available in the diet than pre-formed vitamin A, whereas in industrialized countries the main focus is on the role of β-carotene in the prevention of certain chronic diseases. For a better understanding of the provitamin A activity of β-carotene and of the possible mechanisms of action of β-carotene in the prevention of diseases, more information on β-carotene metabolism is needed.

The research described in this thesis focused on the intestinal absorption and cleavage of β-carotene. In this chapter the results of the work described in this thesis will be discussed in relation to the following questions: 1) How to quantitate β-carotene uptake and cleavage in rats; 2) Which cleavage products are formed from β-carotene in the intestine, and is cleavage central or eccentric; 3) Does dietary vitamin A or β-carotene intake moderate intestinal absorption and cleavage of β-carotene; 4) What is the relevance of peripheral compared to intestinal cleavage; 5) How to assess intestinal β-carotene uptake and cleavage in humans; 6) What is the relevance of the different findings to humans.

In addition, the conclusions of the work described in this thesis and recommendations for future research are given.

How to quantitate β-carotene uptake and cleavage in rats

As discussed in Chapter 2, possible approaches for studying β-carotene metabolism in humans are limited. Thus for studies on mechanisms an animal model has to be used. However, the choice of an animal model is complicated since all models have their limitations and no model is fully representative of the human situation in one or more aspects. When the work described in this thesis was started, models such as the ferret, the preruminant calf and the gerbil had not yet been described. For this work, focused on intestinal β-carotene metabolism, the rat seemed a useful model. However, the rat seems less appropriate for studying extra-intestinal β-carotene metabolism because it

is an efficient β-carotene converter. A main advantage of using the rat is t
large amount of information already available on β-carotene metabolism and
vitamin A metabolism in general.

To study the intestinal β-carotene cleavage reaction in rat, the dioxygena
assay was implemented. Although first described in 1965, the enzyme has st
not been purified and fully characterized, indicating the methodologi
problems with the assay, as is discussed in Chapter 4. Retinal was found as t
only cleavage product in this work. As is concluded in Chapter 4, the produ
formed in the assay are largely dependent on the enzyme preparation a
incubation conditions used and do not necessarily reflect the *in vivo* conditio
where retinal will be further metabolized. An important question was wheth
the *in vitro* cleavage rate, which is the maximum activity under optimu
conditions, reflects *in vivo* cleavage probably under less optimal conditio
Therefore, the effects of the vitamin A and β-carotene intake on both *in vi*
and *in vivo* β-carotene cleavage were measured in a study with rats. As sho
in Chapter 7, activity measured with the dioxygenase assay seems indeed
adequate indicator of *in vivo* β-carotene cleavage, at least when high doses
β-carotene are given. This indicates that the conversion of β-carotene i
retinal is the rate-limiting step of *in vivo* β-carotene cleavage. Thus, t
dioxygenase assay is a useful method to study factors affecting *in vivo* cleava
in rat.

The lymph cannulation method can also be applied for studying *in v*
cleavage products of β-carotene as discussed below. Since β-carotene conversi
in the rat is almost complete and the liver is the main storage site of vitamin
the liver vitamin A content seems a good measure of 'overall' β-carote
availability and can therefore be used to estimate β-carotene conversion facto
Although conversion factors differ quantitatively between rats and huma
qualitative changes of the factors induced by, for instance, dietary factors m
be of relevance to humans.

Intestinal β-carotene cleavage; central or eccentric?

As discussed in Chapter 2, the cleavage mechanism (central or eccentric) a
the actual products formed during *in vivo* intestinal cleavage of β-carotene a
still a subject of debate. Using the *in vitro* enzyme assay we found retinal as t
sole cleavage product. However, as discussed in Chapter 2, retinal can
formed from β-carotene through β-apocarotenals. To evaluate this possibili

the stoichiometry of the reaction has to be determined. The low activity of th enzyme in the *in vitro* assay combined with a slight instability of β-caroten makes it very difficult to arrive at a correct estimate of the reactio stoichiometry. From the two experiments carried out with ^{3}H-labelle β-carotene, described in Chapter 4, we estimated the molar ratio of retina formed to β-carotene consumed to be 1.2. This value was calculated from th differences in radioactivity recovered in the β-carotene and the retinal fractio between the incubations with and without enzyme. This is a rough estimate since the total recovery of radioactivity was only 70%, but is similar to the valu reported by Olson[1]. A value above 1 indicates that at least part of β-carotene i cleaved centrally. The remainder can either be destroyed by oxidation or cleave eccentrically.

In the *in vivo* study with lymph-cannulated rats described in Chapter 7 after a single dose of β-carotene, no β-apocarotenals, retinal, retinol or retinoi acid could be demonstrated, although retinoic acid formation cannot be exclude since this compound is unlikely to be transported through the lymph. Afte long-term supplementation of rats and hamsters with β-carotene in the studies described in Chapters 6 and 7, no β-apocarotenals could be demonstrated in the livers.

In conclusion, in rats and hamsters retinal is the main cleavage product in the in vitro dioxygenase assay, whereas retinyl esters are the main cleavage products of β-carotene *in vivo*. However, we cannot exclude that β-apocarotenals were formed as intermediates in the cleavage reaction or that small amounts of retinoic acid were formed.

Does dietary vitamin A or β-carotene intake affect the β-carotene cleavage and absorption?

As discussed in Chapter 2, dietary factors can affect intestinal β-carotene metabolism at various levels. In principle, interaction can occur in the intestinal lumen at the level of fat emulsification and incorporation of β-carotene in the mixed micelles, thus actually at the absorption level. Once absorbed in the enterocyte, dietary factors could affect the dioxygenase enzyme activity, and play a role in the incorporation of β-carotene and/or cleavage products in the chylomicrons.

Cleavage

As described in Chapter 7, vitamin A intake affected both *in vitro* and *in vi* β-carotene conversion. Since both were affected and the effect seen in th lymph-cannulated rats appeared after dosing with only β-carotene, this effect most likely mediated via β-carotene cleavage enzyme activity. The decrease liver vitamin A stores found in the rats fed the diet rich in vitamin A may part be caused by the decreased enzyme activity in these rats, but also by inhibitio of intestinal β-carotene conversion due to the simultaneous presence vitamin A in the enterocyte. Alternatively, the content of cellul retinol-binding protein type II (CRBP II, not studied in this thesis) may involved. High levels of retinol may saturate CRBP II. As discussed Chapter 2, CRBP II is probably important for the subsequent conversion retinal formed from β-carotene. It may be suggested that free retinal inhibi the β-carotene cleavage reaction, whereas CRBP II-bound retinal does no Alternatively, retinol may give a feedback inhibition of β-carotene cleavag either directly or via retinal reductase.

We can only speculate about the underlying mechanism of the changes enzyme activity found. One effect of vitamin A deficiency might be an increa in intestinal CRBP II since Rajan et al.[2] reported an increase in CRBP II mRN in vitamin A-deficient rats.

The regulatory factor for the β-carotene cleavage enzyme activity or th CRBP II content could be of intestinal or extra-intestinal origin. Intestin factors might include the actual β-carotene or vitamin A content in th enterocyte, but also the concentration of retinoid glucuronides in the intestin mucosa or the retinoic acid formed in the enterocyte. This is not unlikely retinoic acid is known to regulate vitamin A metabolism [3,4] and plays a role gene expression[5]. An extra-intestinal regulatory factor might be liver vitamin storage. As discussed in Chapter 7, an apparent relationship was seen betwee liver vitamin A stores and cleavage activity in the low-vitamin A diet group, b not in the other groups, whereas such a relationship was not observed hamsters on a low-vitamin A diet. To elucidate the mechanism of increased decreased cleavage, further research is needed.

Absorption

Absorption of β-carotene occurs by passive diffusion and thus will depend on i concentration in both the intestinal lumen and the enterocyte. In the situatio

of similar concentrations, the intracellular processing rate, which is mainly determined by the cleavage rate, will determine absorption. β-Carotene absorption in the lymph-cannulated rats (β-carotene and retinyl esters in the lymph) indeed seemed to be related to cleavage activity: 0.7, 1.1 and 1.4% for the groups on a diet rich, intermediate and poor in vitamin A, respectively. However, this relation was no longer apparent when considering the individual data of all rats. Another finding in favour of such a relationship is the higher conversion factor observed in rats on the diet rich in vitamin A, while no extra β-carotene was stored in their livers.

Relevance of intestinal versus peripheral cleavage

As mentioned in Chapter 2, β-carotene cleavage activity has been demonstrated in a number of tissues other than the intestine. In both hamster and rat liver homogenates specific cleavage activities were lower, but total tissue cleavage capacity was higher than in intestinal mucosal homogenates. *In vivo*, the intestine (as first passage way) is quantitatively most important for β-carotene cleavage, but extra-intestinal cleavage may be a useful complementary source of retinoids, although its relevance *in vivo* still has to be established.

As discussed in Chapter 7, extra-intestinal cleavage may especially be important in case of inadequate levels of retinol-binding protein (RBP) and transthyretin due to insufficient dietary protein[6]. Extra-intestinal cleavage may also be important to compensate for local vitamin A deficiencies induced by carcinogens because Edes et al.[7] reported the prevention of a benzo[a]pyrene-induced reduction of liver retinol levels by the administration of β-carotene, but not retinol. In addition, retinoic acid might be formed from β-carotene, which may be important because it bypasses retinol as precursor and the formation might be regulated independent of the retinol pathway[5].

An important question is whether retinol formed extra intestinal from β-carotene can be transported to other tissues. This seems likely for the liver, since β-carotene is mainly stored in the parenchymal cells[8], from which retinol is normally excreted.

How to assess intestinal β-carotene uptake and cleavage in humans

The *in vitro* enzyme assay is not an appropriate model for *in vivo* cleavage where further conversion of retinal is to be expected. The use of intestinal cell

lines may therefore be a useful alternative, offering the opportunity to stud cleavage in human cells. However, we could not demonstrate cleavage activit in the human intestinal cell line Caco-2 (Chapter 4).

There is actually no suitable animal model for β-carotene metabolism i humans and a wide range of factors involved in metabolism complicate extrapolation to humans. In humans, chylomicron responses after a single or dose of β-carotene, as described in Chapter 8, can be used to assess β-caroten absorption and cleavage. Since only relative absorption and cleavage can b studied, the method seems especially appropriate for studying factors affectin intestinal β-carotene absorption and cleavage within persons. Factors related t the matrix, processing, meal composition and some nutrient status effects ca be studied, with the exception of factors that may affect chylomicron clearanc Conversion factors for β-carotene may also be estimated with this method b comparing retinyl ester responses between dosages of vitamin A and β-caroten A slight underestimation of the factors is to be expected, since extra-intestin cleavage is not taken into account.

Alternatively, for studying β-carotene absorption plasma levels can b measured before and after supplementation with a certain product c combination under controlled dietary conditions. However, this would requir stable isotopes to measure cleavage products.

A principal drawback in human studies remains restrictions as to samplin of blood and faeces, and therefore mechanistic human studies are in general nc possible. For such questions one still has to rely on animal studies.

Relevance of the different findings to the human situation

Whether vitamin A and β-carotene intake affects β-carotene cleavage i humans, as was found for rats, needs confirmation. One aspect that need further study is the dose-response relation of the effects.

Possible advantage of an increased intestinal dioxygenase activity

Increased cleavage activity is especially of relevance for people mainl dependent on carotenoids for their vitamin A provision. Regulation through th vitamin status seems therefore effective, whereas regulation at the level c enterocyte β-carotene or retinoid content seems less effective, since intake ma vary from day to day. The relevance of extra-intestinal cleavage a 'complementary' source of retinoids mainly depends on its *in vivo* efficiency. A

increase of cleavage activity in the liver upon β-carotene consumption, as wa seen in hamsters and rats, is unlikely to occur in humans since β-carotene is normal component of the human diet.

It is important to realize that increased intestinal β-carotene cleavag activity is only helpful for β-carotene already absorbed, indicating that th source of β-carotene eaten may be much more important for vitamin A provisio than intestinal cleavage activity. In addition, vegetable-rich diets contain othe carotenoids that may negatively affect β-carotene cleavage, as is suggested fo lutein in Chapter 4.

Possible advantage of a decreased intestinal dioxygenase activity

Assuming that for the possible protective function of β-carotene maximization o intact β-carotene absorption is important, a low cleavage activity seems mos appropriate. However, the large interindividual variation in the ratio of retiny esters to β-carotene as discussed in Chapter 8 (1.1 to 4.9) for subjects not using supplements, suggests that vitamin A and β-carotene intake are not very strong determinants of cleavage activity.

An important implication of decreased cleavage activity upon high β-carotene intakes may be that β-carotene is a safe source of vitamin A. Indeed, no sign of vitamin A toxicity is reported in subjects with a high β-carotene intake. However, it cannot be excluded that 'normal' cleavage activity already prevents vitamin A intoxication upon high β-carotene consumption. An alternative explanation may be a diminished absorption under conditions of high intake. Since absorption is expected to occur through passive diffusion no regulation is to be expected, but, as was discussed for the rats, absorption may be affected by cleavage activity. However, in the human study described in Chapter 8, no relation between the ratio of retinyl esters to β-carotene (assumed to be a measure of cleavage activity) and the sum of the retinyl ester and β-carotene responses (assumed to be a relative measure of absorption) was found. Since β-carotene absorption has been reported to increase linearly with the dose up to about 30 mg[10,11], β-carotene cleavage activity may affect β-carotene absorption only at high dose levels.

Although the precise mechanism still needs to be elucidated, β-carotene is a safe form of vitamin A. This seems especially of relevance for pregnant women, elderly people and neonates, in whom the range between an adequate and a toxic intake of vitamin A is rather small. Neonates have been reported to show

β-carotene cleavage activity, measured with the dioxygenase assay[12], bu
cleavage activity needs to be further studied to evaluate the ability of neonate
to use β-carotene as vitamin A source.

Conclusions

- *In vitro* dioxygenase activity in intestinal homogenates is an adequat indicator of *in vivo* β-carotene cleavage rate in rats.
- Retinal is the main cleavage product of β-carotene in the *in vitr* dioxygenase assay, whereas *in vivo* retinal is further metabolized in th intestine into retinyl esters as the main cleavage product. Whether th cleavage mechanism is central or eccentric cannot be concluded.
- Intestinal β-carotene cleavage activity is higher in vitamin A-deficient rat than in rats with a high intake of either vitamin A or β-carotene.
- Chylomicron response curves of β-carotene and retinyl esters seem a usefu method to study intestinal β-carotene metabolism in comparative huma studies.

Recommendations for future research

From the above discussion it is clear that several questions remain to b answered. A systematic approach to characterize the intestinal processe involved in β-carotene absorption seems useful. For this purpose an *in vitr* model to study intestinal digestion, the incorporation into micelles an subsequent absorption might be helpful. With such a model, effects of the foo matrix, meal composition, interactions between carotenoids and betwee β-carotene and other vitamins at the absorption level can be studied in a simpl and cost-effective way. Also regulatory control of intestinal cleavage activit needs to be studied into more detail.

Although several important questions on β-carotene metabolism remain t be answered, highest priority may be given to the study of conversion factor and carotene isomers. The commonly used conversion factors need further stud and possibly adaptation. If an appropriate classification can be made, it may b useful to establish differentiated conversion factors for various groups o products. Investigation of the effect of carotene isomerization on the absorptio and cleavage process seems of great importance as during processing, an maybe even in the gastro-intestinal tract, isomerization may occur.

References

1. Olson JA. The alpha and the omega of vitamin A metabolism. Am J Clin Nutr 1969;22: 953-962.
2. Rajan N, Blaner WS, Soprano DR, Suhara A and Goodman DS. Cellular retinol-binding protein messenger RNA levels in normal and retinoid-deficient rats. J Lipid Res 1990;31: 821-829.
3. Ong DE, Newcomer ME and Chytil F. Cellular retinoid-binding proteins. In: Sporn MB, Roberts AB and Goodman DS, eds. The retinoids. Biology, chemistry, and medicine. 2nd ed. New York: Raven Press, 1994:283-317.
4. Wang XD, Krinsky NI and Russell RM. Retinoic acid regulates retinol metabolism via feedback inhibition of retinol oxidation and stimulation of retinol esterification in ferret liver. J Nutr 1993;123:1277-1285.
5. Ross AC and Ternus ME. Vitamin A as hormone: recent advances in understanding the actions of retinol, retinoic acid, and beta-carotene. J Am Diet Assoc 1993;93:1285-1290.
6. Carlier C, Etchepare M, Ceccon J-F, Mourey M-S and Amédée-Manesme O. Efficacy of massive oral doses of retinyl palmitate and mango (*mangifera indica* L.) consumption to correct an existing vitamin A deficiency in Senegalese children. Br J Nutr 1992;68: 529-540.
7. Edes TE, Gysbers DG, Buckley CS and Thornton WH. Exposure to the carcinogen benzopyrene depletes tissue vitamin A: β-carotene prevents depletion. Nutr Cancer 1991; 15:159-166.
8. Lakshman MR, Asher KA, Attlesey MG, Satchithanandam S, Mychkovsky I and Coutlakis PJ. Absorption, storage, and distribution of beta-carotene in normal and beta-carotene-fed rats: roles of parenchymal and stellate cells. J Lipid Res 1989;30:1545-1550.
9. Mathews-Roth MM. Beta-carotene: Clinical aspects. Curr Top Nutr Dis 1989;22:17-38.
10. Dimitrov NV, Boone CW, Hay MB, Whetter P, Pins M, Kelloff GJ and Malone W. Plasma beta-carotene levels-kinetic patterns during administration of various doses of β-carotene. J Nutr Growth Cancer 1986;3:227-237.
11. Henderson CT, Mobarhan S, Bowen P, Stacewicz-Sapuntzakis M, Langenberg P, Kiani R, Lucchesi D and Sugerman S. Normal serum response to oral beta-carotene in humans. J Am Coll Nutr 1989;8:625-635.
12. Lakshman MR, Johnson LH, Okoh C, Attlesey M, Mychkovsky I and Bhagavan HN. Conversion of all trans beta-carotene to retinal by an enzyme from the intestinal mucosa of human neonates. J Nutr Biochem 1993;4:659-663.

Summary

β-Carotene is a yellow pigment that occurs in yellow and orange fruits an vegetables and in green vegetables. The first known function of β-carotene i humans was as provitamin A, which function is still very important for peopl consuming a diet low in vitamin A. Renewed interest for β-carotene appeare since epidemiological studies indicated that consumption of foods rich i carotenoids is associated with a reduced risk for certain types of cancer an cardiovascular disease. β-Carotene may be protective as antioxidant or a provitamin A. For a better understanding of the mechanism of action o β-carotene, more information on β-carotene metabolism is needed. The worl described in this thesis focused on how to assess intestinal β-carotene uptak and cleavage in rats and in humans, which cleavage products are formed fron β-carotene in the intestine, and whether dietary vitamin A or β-carotene intak can affect intestinal absorption and cleavage of β-carotene.

Chapter 2 provides an overview of current knowledge on carotenoid absorptior and cleavage in animal models and humans, with the emphasis on β-carotene Several aspects of β-carotene metabolism still need to be elucidated, such as the mechanism of the cleavage reaction (eccentric or central) and dietary factors affecting cleavage. A number of animal models are being used, but all remair limited and a poor model to study β-carotene metabolism in humans.

First of all, high-performance liquid chromatography (HPLC) methods were adapted to enable the analysis of possible cleavage products of β-carotene. Chapter 3 describes two HPLC systems, one to measure different β-apocarotenals, α-tocopherol, α- and β-carotene, and one to measure different retinoids. As a first application, the tissues and plasma of rats fed a diet supplemented with either β-carotene or canthaxanthin (2 g/kg) for 2 weeks were analysed. β-Carotene or canthaxanthin, but no β-apocarotenals (products of eccentric cleavage of β-carotene), could be demonstrated in the tissues.

To study intestinal β-carotene cleavage, an *in vitro* enzyme assay (the so-called dioxygenase assay) was implemented and optimized as described in Chapter 4. Retinal was the only cleavage product demonstrated; no

β-apocarotenals, retinoic acid or retinol were found. It appeared that the ce
fraction with the highest cleavage activity was the 9,000 *g* supernatant (S-9
Maximal retinal formation was obtained with a combination of detergents in th
incubation mixture and β-carotene dissolved in acetone. Retinal formatio
increased proportionally with the amount of protein S-9 used and with time u
to 40 - 60 min. Incubation with α-carotene or β-cryptoxanthin resulted in retina
formation of 29 and 55% of the amount formed from β-carotene. Addition
lutein to the incubation with β-carotene reduced retinal formation, whil
lycopene had no effect, indicating that the simultaneous presence
non-provitamin A carotenoids may affect β-carotene cleavage.

The β-carotene cleavage assay with S-9 as enzyme source was found to be
useful tool to study (dietary) determinants of β-carotene cleavage activity an
was subsequently applied in a pilot study with hamsters (Chapter 6) and
study with rats (Chapter 7). Products formed in the assay largely depend on th
enzyme preparation and incubation conditions used. Since *in vivo* retinal will b
further metabolized, the assay seems less appropriate to study *in vivo* cleavag
products and mechanism. Therefore, both rat (IEC-6 and IEC-18) and huma
(Caco-2) intestinal cell lines were tested for their ability to convert β-carotene
As described in Chapter 5, no cleavage activity could be demonstrated wit
these cultures. The absence of cleavage activity in 9,000 *g* supernatan
preparations of IEC-18 and Caco-2 cells in the dioxygenase assay suggests th
absence, or very low expression, of the cleavage enzyme in these cell cultures.

To investigate whether the level of vitamin A and/or β-carotene in the die
affects β-carotene cleavage, a pilot study with hamsters was carried ou
(Chapter 6). Three groups of hamsters were fed a diet containing 400 I
vitamin A per kg (Low A), 4000 IU vitamin A per kg (Norm) or 4000 I
vitamin A + 10 g β-carotene per kg (High BC) for three months. The Low A die
increased intestinal dioxygenase activity 2.3-fold compared to the Norm die
The High BC diet decreased the activity in one subgroup of the animals, but ha
no effect in the other subgroup. In this study the vitamin A and β-caroten
content of the diet were shown to modulate the *in vitro* dioxygenase activity.

To study the significance of changes in the *in vitro* cleavage activity for *i
vivo* β-carotene metabolism, *in vitro* measured activity was compared wit
activity *in vivo* (Chapter 7). For this purpose six groups of rats were fed a die
containing 400, 4000 or 40,000 IU vitamin A per kg for 14 to 18 weeks, eithe
supplemented or not with 50 mg β-carotene per kg in the last 6 weeks

Intestinal dioxygenase activity was 1.9-fold higher ($P < 0.05$) in the animals fed the unsupplemented Low A diet than in the animals fed the unsupplemented High A diet, while β-carotene supplementation significantly decreased intestinal dioxygenase activity. To measure *in vivo* β-carotene cleavage lymph-cannulated rats received a single intestinal dose of β-carotene. In the lymph collected over 8 h after the dose β-carotene and retinyl esters were measured. The ratio of retinyl esters to β-carotene in the lymph, as a measure for cleavage activity, was shown to correlate with intestinal dioxygenase activity ($r = 0.66$, $P = 0.003$). Thus, *in vitro* measured intestinal dioxygenase activity seems an adequate indicator of *in vivo* β-carotene cleavage activity. Dioxygenase activity in the liver was not affected by the vitamin A content of the diet, and was 1.7-fold higher in the β-carotene-supplemented rats. β-Carotene conversion factors of 9:1 and 4:1 were calculated for the rats fed the High A diet or the Norm A/Low A diets, respectively, based on the total vitamin A deposition in the liver.

Since no animal model truly reflects β-carotene metabolism in humans, it was considered important to develop methods to assess intestinal β-carotene metabolism in humans. The evaluation of such a method is described in Chapter 8. β-Carotene, retinyl ester and triglyceride responses in the triglyceride-rich lipoprotein (TRL) fraction and plasma were measured in 10 men after an oral dose of 15 mg β-carotene. The β-carotene response, unlike the triglyceride and retinyl ester response, can be evaluated in the TRL fraction but not in plasma. Intra-individual variations in the triglyceride-adjusted response of β-carotene and retinyl palmitate in TRL fractions were estimated to be 23% and 20%, with inter-individual variations of 42% and 36%, respectively (as coefficients of variation). Measurement of β-carotene and retinyl esters in the TRL fraction after a dose of β-carotene with a vitamin A-free meal may be an appropriate method to study β-carotene uptake and cleavage. A low β-carotene response was associated with a high ratio between retinyl palmitate and β-carotene responses ($r = -0.56$, $P = 0.013$).

The results of these studies are discussed in Chapter 9 in relation to the original research questions and related questions such as the importance of extra-intestinal cleavage activity and the relevance of described findings for human nutrition. The following overall conclusions were drawn:

- *In vitro* dioxygenase activity in intestinal homogenates is an adequate indicator of *in vivo* β-carotene cleavage rate in rats.

- Retinal is the main cleavage product of β-carotene in the *in vitr* dioxygenase assay, whereas *in vivo* retinal is further metabolized in th intestine into retinyl esters as the main cleavage product. Whether th cleavage mechanism is central or eccentric cannot be concluded.
- Intestinal β-carotene cleavage activity is higher in vitamin A-deficient rat than in rats with a high intake of either vitamin A or β-carotene.
- Chylomicron response curves of β-carotene and retinyl esters seem a usefu method to study intestinal β-carotene metabolism in comparative huma studies.

For further studies on β-carotene absorption and metabolism the developmen of an *in vitro* system might be helpful. Priority in further studies may be give to the study of conversion factors, metabolism of carotene isomers and possibl interactions between carotenoids and between β-carotene and other fat-solubl vitamins.

Samenvatting

β-Caroteen is een gele kleurstof die voornamelijk voorkomt in worteltjes, groen
bladgroenten en een aantal vruchten. De bekendste functie van β-caroteen voo
de mens is als provitamine A, welke functie vooral van belang is voor mensen
met een lage inname van vitamine A. Er is lang gedacht dat dit de enige functie
is van β-caroteen voor de mens, maar er zijn nu aanwijzingen dat β-caroteen
bijdraagt aan een verminderd risico op bepaalde vormen van kanker en hart- en
vaatziekten. Het is nog onduidelijk of β-caroteen zelf bescherming biedt (b.v. als
antioxidant), of na omzetting in vitamine A. Om deze en andere vragen over de
werking van β-caroteen te kunnen beantwoorden is meer kennis over het
metabolisme van β-caroteen nodig. Het doel van het onderzoek beschreven in dit
proefschrift is om de volgende vragen over het metabolisme van β-caroteen te
beantwoorden:

- Hoe kan de opname en splitsing van β-caroteen in de darm worden bestudeerd bij de rat en bij de mens.
- Welke produkten worden in de darm uit β-caroteen gevormd.
- Kan de absorptie en splitsing van β-caroteen worden beïnvloed door de inname van vitamine A of β-caroteen.

In hoofdstuk 2 wordt een overzicht gegeven van de huidige kennis over functies en metabolisme van β-caroteen. Dit overzicht laat o.a. zien dat het mechanisme van de β-caroteen splitsingsreactie (centraal of excentrisch) nog niet is opgehelderd en dat nog onduidelijk is of voedingsfactoren het metabolisme van β-caroteen kunnen beïnvloeden. Ook wordt duidelijk dat er geen diermodel beschikbaar is met een β-caroteen metabolisme vergelijkbaar met dat van de mens.

Om de mogelijke splitsingsprodukten van β-caroteen te kunnen analyseren is een HPLC methode opgezet zoals beschreven in hoofdstuk 3; een systeem voor de analyse van carotenoïden, apocarotenalen en vitamine E en een systeem voor de analyse van retinoïden. De methode is allereerst gebruikt om bloed en weefsels te analyseren van ratten die 2 weken voer kregen met β-caroteen of canthaxanthine (beide 2 g per kg voer).

Voor het bestuderen van de β-caroteen splitsingsreactie is een enzym test, d zogenaamde dioxygenase assay, geïmplementeerd en geoptimaliseerd zoal beschreven in hoofdstuk 4. In deze test wordt β-caroteen geïncubeerd met ee darmmucosa preparaat waarna de gevormde produkten worden gemeten Retinal bleek het enige produkt; andere mogelijke splitsingsprodukten zoal apocarotenalen, retinol of retinoïnezuur werden niet gevonden. De hoogst splitsingsactiviteit was aanwezig in de 9000 *g* supernatant fracti (bovenstaande vloeistof na centrifugeren bij 9000 *g*) van het darmmucos homogenaat. Voor maximale activiteit was verder een combinatie va detergentia nodig en werd β-caroteen toegevoegd in aceton. Wanneer incubatie werden uitgevoerd met de provitamine A carotenoïden α-caroteen (β-cryptoxanthine werd ook retinal gevormd (29% en 55% respectievelijk van d hoeveelheid uit β-caroteen). De carotenoïden luteïne en lycopeen hebben gee provitamine A activiteit, maar luteïne leek wel de omzetting van β-caroteen t verminderen terwijl lycopeen geen effect had.

Uit de experimenten met de dioxygenase assay kan worden geconcludeer dat de gevonden splitsingsprodukten en splitsingsactiviteit met name worde bepaald door het gekozen enzym preparaat en de incubatie condities. De assa lijkt dus wel bruikbaar voor de vergelijking van splitsingsactiviteit onde verschillende voeder condities, maar niet voor het bepalen van de *in viv* splitsingsprodukten van β-caroteen.

Als model voor de *in vivo* splitsing lijkt een systeem met intacte celler bijvoorbeeld een cellijn, meer geschikt. In dunne-darmcellen van de rat (IEC- en IEC-18) en in humane colon-carcinoma cellen (Caco-2) kon echter gee β-caroteen splitsingsactiviteit worden aangetoond (hoofdstuk 5).

Met behulp van de dioxygenase assay is in een pilot-studie met hamster nagegaan of een lage vitamine A inname (400 IU per kg voer) of een hog β-caroteen inname (10 g per kg voer) de splitsingsactiviteit kunnen beïnvloeden De splitsingsactiviteit in de hamsters met de lage vitamine A inname wa 2,3 maal de activiteit in die met de normale inname (4000 IU per kg voer). D hoge β-caroteen inname had geen effect bij de helft van de groep, terwijl bij d andere helft de splitsingsactiviteit lager was.

Om het belang van dergelijke veranderingen van de *in vitro* gemete splitsingsactiviteit voor de *in vivo* situatie te bepalen werd een studi uitgevoerd met 6 groepen ratten. De ratten kregen 18 weken een voer met ee laag, normaal of hoog vitamine A gehalte (400, 4000 of 40.000 IU per kg voer

De laatste 6 weken van deze periode kreeg de helft van elke groep tever β-caroteen in het voer (50 mg per kg voer). De *in vitro* gemete splitsingsactiviteit aan het einde van de studie in de laag A groep was 1,9 maa de activiteit in de hoog A groep. β-Caroteen in het voer verlaagde d splitsingsactiviteit. De *in vivo* splitsingsactiviteit werd bepaald bij ratten di laag, normaal of hoog A voer zonder β-caroteen hadden gehad door he verzameld lymfevat van de darm te canuleren. Na een eenmalige dosi β-caroteen, toegediend in de darm, werd gedurende 8 uur lymfe verzamel waarin zowel retinylesters als β-caroteen werden gemeten. De verhoudin tussen de hoeveelheid retinylesters en β-caroteen werd gebruikt als maat voo splitsingsactiviteit. Deze verhouding bleek gecorreleerd met de activitei gemeten met de dioxygenase assay in dezelfde dieren. De activiteit gemeten i de dioxygenase assay lijkt dus een goede maat voor de *in viv* splitsingsactiviteit.

De dioxygenase activiteit in de lever werd niet beïnvloed door de vitamine A inname, maar werd verhoogd door de inname van β-caroteen. Op basis van d opslag van vitamine A in de lever van ratten die wel en die geen β-caroteen in het voer hadden werd de β-caroteen conversiefactor berekend (µg β-caroteen inname resulterend in dezelfde vitamine A opslag in de lever als de inname van 1 µg vitamine A). Deze werd geschat op 4 bij de lage en normale vitamine A inname en 9 bij de hoge vitamine A inname.

Omdat er geen diermodel bekend is met een β-caroteen metabolisme vergelijkbaar met dat van de mens is het zinvol om het metabolisme bij de mens te kunnen benaderen. Voor benadering van het darmmetabolisme bij de mens is een methode geëvalueerd waarbij triglyceride-rijke lipoproteïnen (TRL's, voornamelijk rechtstreeks afkomstig uit de darm) werden geïsoleerd uit het bloedplasma op verschillende tijdstippen nadat een hoeveelheid β-caroteen met een maaltijd was gegeten. In deze TRL's kan niet alleen de respons van triglyceriden en retinylesters worden bepaald zoals in plasma, maar ook de respons van β-caroteen die in plasma moeilijk te bepalen is door de hoge basale waarden. De binnenpersoons-variaties in de voor triglyceride gecorrigeerde respons van β-caroteen en retinylpalmitaat werden geschat op respectievelijk 23% en 20%, en de tussenpersoons-variaties op 42% en 36%.

Een lage β-caroteen respons was gerelateerd aan een hoge verhouding van retinylpalmitaat ten opzichte van β-caroteen; de mate van omzetting van geabsorbeerd β-caroteen lijkt dus te verschillen tussen personen.

Tot slot worden in hoofdstuk 9 alle gevonden resultaten bediscussieerd aa
de hand van de in de inleiding gestelde en daaraan gerelateerde vragen, zoa
het belang van de bevindingen voor de voeding van de mens. De conclusies va
het in dit proefschrift beschreven onderzoek zijn:

- De *in vitro* dioxygenase activiteit in darm homogenaten is een geschikt indicator voor de *in vivo* β-caroteen splitsingsactiviteit in de rat.
- Retinal is het voornaamste splitsingsprodukt in de dioxygenase assa terwijl *in vivo* retinal verder wordt omgezet in retinylesters.
- β-caroteen splitsingsactiviteit in de darm is hoger in vitamine A deficiën ratten dan in ratten met een hoge inname van vitamine A of β-caroteen.
- Respons curves van β-caroteen en retinylesters in chylomicronen lijken ee bruikbare relatieve maat voor absorptie en omzetting van β-caroteen bij d mens.

Voor toekomstig onderzoek naar het mechanisme van de β-caroteen splitsing e
de regulering daarvan lijkt de ontwikkeling van een *in vitro* model zinvo
Belangrijke andere aspecten zijn de studie van de conversiefactoren, h
metabolisme van isomeren van β-caroteen en van mogelijke interacties tusse
carotenoïden.

Dankwoord

Tot slot wil ik allen bedanken die aan het tot stand komen van dit proefschrift hebben bijgedragen, te beginnen met Jaap Schrijver, de initiatiefnemer voor het project. Jaap, bedankt voor je enthousiaste begeleiding gedurende de eerste maanden van het onderzoek. Mijn co-promotor, Henk van den Berg, nam het vervolgens over. Henk, ik waardeerde het zeer dat je je zo snel in het onderwerp verdiepte en dat ik altijd 'even' binnen kon vallen. Zolang het niet over het werk ging waren we het eens, maar ook wat betreft het werk zijn we er uiteindelijk altijd uitgekomen. Mijn promotor Wil Schreurs ben ik erkentelijk voor de geboden mogelijkheid om dit onderzoek bij TNO uit te voeren en voor het geschonken vertrouwen.

Frank van Schaik maakte mij wegwijs in de caroteen analyses, en ontwikkelde zich tot dioxygenase expert. We zouden de assay met alle plezier weer op starten, niet waar? Frank, bedankt voor het vele analytische werk dat jij gedaan hebt, maar ook voor je luisterend oor; als kamergenoot was je mijn eerste klankbord en dat kon bij tijd en wijle een drukke bijbaan zijn.

Door de grote variatie in het type werk binnen het project klopte ik in de loop van de jaren steeds bij andere collega's aan voor advies en assistentie. Graag bedank ik Fons Rutten voor de stimulans om het *in vitro* werk uit te breiden met celkweek, waarvoor ik bij de afdeling Toxicologie terecht kon. Winfried Leeman en Nico de Vogel, bedankt voor het op gang helpen en John Groten dank ik voor zijn assistentie en advies in een later stadium. Roeselien Jaspars toonde zich als stagiaire een uitstekend celkweekster, wat helaas niet werd beloond met sprekende resultaten. Geen resultaat is ook een resultaat, maar dit is niet erg stimulerend als eerste onderzoekservaring.

De samenwerking met Toxicologie werd voortgezet met een hamsterstudie. André Wolterbeek bedank ik voor het beschikbaar stellen van materiaal zodat ik een pilot-studie kon uitvoeren.

Aan de hierop volgende uitgebreide rattenstudie hebben zeer velen hun steentje bijgedragen. Gerard van Beek wil ik bedanken voor het mij wegwijs maken in de diervleugel en in de organisatie van een dierproef. Herman en Claudia en hun vervangers wil ik bedanken voor hun goede zorgen voor de ratten en hun betrokkenheid bij de studie. Jasper, bedankt voor het maken van de voeders. Dank ook aan de medewerkers van histologie voor het uitvoeren van de secties en aan Martijn van der Plas voor zijn aandeel in de opwerking van de levers. Voor de lymfe canulering, die heel wat voeten in de aarde

had, ben ik dank verschuldigd aan Henk Hendriks en toenmalige collega's van het IVV
in Leiden die de methode leverden, Wilfried Seifert die de eerste pogingen in Zeist uitvoe
de, Claudia en Trudy, die enthousiast startten, waar het helaas bij moest blijven en Mar
Fentener van Vlissingen die uiteindelijk de canulaties uitvoerde, zelfs op haar vrije dagen

De vele monsters die deze studie opleverde hoefde ik gelukkig niet allemaal zelf
analyseren. Frank, maar ook Jan van Schoonhoven en medewerkers, hartelijk dank vo
jullie hulp.

Tot slot de studie met vrijwilligers; Jaap Odink, bedankt voor je adviezen, Pet
Zuiderwijk en Theo Hoofwijk voor de medische ondersteuning en Wilfred Sielink en G
Wigbout voor de enthousiaste coördinatie. Theo, de gezellige ritjes tussen Zaandam
Zeist zal ik niet snel vergeten.

Renske Brune maakte de methode voor chylomicron isolatie bruikbaar voor de stud
Renske, wat werd ik moe van je als je werkelijk alles wilde begrijpen, maar je aanstekelij
enthousiasme compenseerde dat ruimschoots. Hans Mocking, bedankt voor h
perfectioneren van de chylomicron isolatie en de uitvoering tijdens de studie. Har
Korthals was de gelukkige die het daarna ook eens mocht proberen. Harm, bedankt!

Herhaaldelijk bezocht ik ook het laboratorium van Jan Catsburg, om even bloed
laten prikken of voor analyses, die zelden volgens de standaard methode konden word
uitgevoerd. Jan en collega's, bedankt voor de soepele medewerking.

Alle medewerkers van voorheen EB, bedankt voor de gezellige tijd. Gelukkig flot
jullie me af en toe even terug als ik dacht dat de hele wereld om β-caroteen draaide. To
het praktische werk afgerond was verhuisde ik dan toch naar de afdeling Voeding. Ook
collega's van deze afdeling en met name van de sectie Fysiologie wil ik bedanken voor
goede sfeer en de belangstelling. Henk Hendriks, als ik vast liep in mijn eigen gedacht
kronkels kon ik altijd even binnen vallen. Jouw directe benadering werkte ze
verhelderend. Erica, jouw positieve kijk op het leven werkte heel relativerend. Overi
AIO's en Marijke, het delen van elkaars frustraties kon me soms verbazend goed doen.

Dirk van der Heij heeft er voor gezorgd dat dit proefschrift ook voor Engels sprekend
leesbaar is. Verder wil ik allen bedanken die ik hier niet met naam heb genoemd, en d
bereid waren mee te denken, even iets voor me te regelen of met een yoghurt-olie meng
te ontbijten.

Als laatste wil ik vrienden en familie bedanken voor de getoonde belangstelling en aa
moedigingen. Marga, jij zorgde dat ik mijn problemen ook eens van de andere kant beke
Pap en mam, jullie steunden mij steeds in mijn keuzes. Dorine, hopelijk blijkt de komen
tijd dat ook voor mij werken niet het enige levensdoel is, en zie je me wat vaker. Alex,
hebt vele stemmingen doorstaan! Bedankt voor de steun, zowel mentaal als op comput
gebied, en voor je relativerende opmerkingen, al was ik er soms een beetje doof voor.

Curriculum vitae

Trinette van Vliet is geboren op 21 april 1965 in Dordrecht. In 1983 haalde zi
haar VWO diploma aan de Christelijke scholengemeenschap "Jan Arentsz" te
Alkmaar. In hetzelfde jaar startte zij haar studie Voeding van de mens aan de
Landbouwuniversiteit te Wageningen. Tijdens haar studie deed zij een
afstudeervak Humane Voeding bij de vakgroep Humane Voeding aan de
Landbouwuniversiteit en een afstudeervak Toxicologie bij het Rijkskwaliteits-
instituut voor Land- en Tuinbouwprodukten (RIKILT) te Wageningen. Van
maart tot september 1989 werd een stage Toxicologie uitgevoerd bij het
Karolinska Instituut in Stockholm. In november 1989 behaalde zij het diploma
van landbouwkundig ingenieur, waarna zij bij de vakgroep Humane Voeding in
dienst trad als onderzoeksassistent. Per 1 maart 1990 trad zij in dienst als
assistent in opleiding (AIO) bij de vakgroep Experimentele Dierkunde van de
Universiteit van Amsterdam. Het onderzoek dat zij als AIO verrichtte en in dit
proefschrift beschreven is, werd uitgevoerd bij TNO-Voeding te Zeist (Dr. H. van
den Berg en Prof. dr. W.H.P. Schreurs).