The bioavailability of α- and β-carotene is affected by gut microflora in the rat

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The present study examined whether the intestinal microflora could affect the bioavailability and vitamin A activity of dietary α- and β-carotene in the rat. In the first set of experiments, we used conventional, germ-free (axenic), and human-flora-associated (heteroxenic) rats. In a second series, conventional rats were treated with either an antibiotic mixture or a potent inhibitor of gastric secretion (Omeprazole). All animals were first depleted of vitamin A over 4 weeks and then were fed on a sterilized diet supplemented with 14 mg β-carotene and 3 mg α-carotene/kg for 2 weeks. In both experiments, a reduction in the intestinal microflora resulted in an increased storage of β-carotene, α-carotene and vitamin A in the liver. Neither the nature of the metabolism of the intestinal microflora (aerobic or anaerobic) nor treatment with omeprazole, to modify intestinal pH, induced a significant effect on the measured variables. When incubated with 15 μmol β-carotene/l for 72 h, neither the anaerobic nor the aerobic sub-fractions obtained from rat or human faeces contributed to β-carotene degradation or to vitamin A synthesis. These findings suggest that reduction in gut microflora results in a better utilization of α- and β-carotene by rats, although bacteria do not have a direct effect on the bioavailability of these pigments.

Carotenoids: Vitamin A: Gut microflora: Bioavailability

Dietary carotenoids exhibit vitamin A activity in animal tissues and are also associated with the prevention of human disease through antioxidant actions, stimulation of the immune system and inhibition of carcinogenic processes (for review, see Krinsky 1994). Both their nutritional function and biological actions could be modulated by factors affecting their bioavailability, and more precisely their absorption by the intestinal mucosa and the enzymic conversion of provitamin A carotenoids to vitamin A in tissues.

Several observations suggested that gut microflora could be one of these factors. McGillivray (1951) reported that sheep excreted more carotene in faeces than the amount they consumed, and demonstrated *in vitro* synthesis of β-carotene by ileal and caecal contents. Yet King *et al.* (1962) reported a prompt degradation of β-carotene in steer and sheep rumen. Several bacteria strains of *Bacillus*, *Staphylococcus* and *Streptococcus*, are known to synthesize yellow carotenoids (Aono & Horikoshi, 1991). Treatment of rats with antibiotics resulted in a decrease in the microbial population. Conversely, this treatment increased liver storage of vitamin A and stimulated additional growth in vitamin A-deficient rats receiving β-carotene (Almquist & Maurer, 1955; Guerrant, 1960). The intestinal absorption of β-carotene is largely dependent on the digestion of fat, and especially on formation of mixed micelles. Since bacteria can hydrolyse conjugated bile salts in the intestine and hydroxylate primary bile acids (Andrieux *et al.* 1989), it could be suggested that they might modulate the absorption rate of fat-soluble compounds.

However, none of these studies has clearly established whether intestinal micro-organisms can affect the bioavailability of β-carotene. The question arises because the composition and the level of microflora have been shown to be altered by antibiotic treatments, intestinal diseases, low-fat and high-fibre diets and aging (Andrieux *et al.* 1989; Gorbach & Goldin, 1992; Mitsuoka, 1992). Thus, the biological activities of β-carotene in human subjects could be modulated as a function of bacterial activity in the intestine.

In the present study we have used germ-free and human-flora-associated (heteroxenic) rats, and administered antibiotics and omeprazole to conventional rats to further investigate the putative effect of the intestinal flora on the bioavailability and the vitamin A activity of α- and β-carotene.

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Materials and methods

Diets

Before feeding the experimental diets, weanling female rats were first depleted of vitamin A for 4 (Expt 1) or 3 weeks (Expt 2). They were fed on a semi-synthetic diet which contained (g/kg): vitamin-free casein 218, maize starch 439, sucrose 219, cellulose 20, rapeseed oil–peanut oil (1 : 1, v/v) 53, DL-methionine 1, mineral and vitamin mixture without vitamin A (Roland *et al.* 1995). Then, rats were fed *ad libitum* the same vitamin A-deficient diet supplemented with a freeze-dried extract from carrots (Arômes de Bretagne, Antrain-sur-Couesnon, France) for 2 weeks (five or six rats per group). All diets were pelleted and sterilized by irradiation of 40 kGy in vacuum-sealed plastic bags. The carotenoid diet contained 3 mg α-carotene/kg and 14 mg β-carotene/kg.

Animals

All procedures were in accord with the Institute's guide for the care and use of laboratory animals.

Expt 1. Twenty-seven female Fischer 344 rats were used. Germ-free rats, obtained from the INRA (Jouy-en Josas, France; Breeding unit), were housed, three per cage, in isolators fitted with a rapid transfer system (La Calhène, Vélizy, France). Heteroxenic rats were obtained by orally inoculating germ-free rats with 1 ml of a 10^{-2} dilution of a whole-faecal flora obtained from a healthy human subject. Conventional Fisher 344 rats, obtained from the same laboratory, were age-matched to the germ-free animals. Three rats per group were killed before supplementation to determine initial vitamin A levels in the liver. Then, six rats per group were fed on the carotenoid diet.

Expt 2. Twenty-four female Wistar rats were obtained from dams which had been fed previously on a semi-synthetic diet containing low levels (440 μg/kg) of retinyl palmitate (stock colony; INRA, Jouy en Josas). Weanling rats were then assigned during the experiment to one of the three treatments: antibiotics, omeprazole or without drugs. The antibiotics were added daily in the drinking water (g/l): ampicilin 0·6, neomycin 0·6, bacitracin 0·6, streptomycin 0·6, nystatin 0·2. Since omeprazole is unstable in acidic gastric juice, an enteric-coated formulation was used (Mopral, Astra France, Nanterre, France). An omeprazole suspension was prepared (1·65 g/l) and 0·5 ml was intragastrically administered (15 μmol/kg body weight) each day at 18.00 hours. Control rats received the same volume of water. Three rats per group were killed before supplementation to determine stores of liver vitamin A, and five rats per group were used for the carotenoid study.

Animals were maintained at a room temperature of 21° and a 12 h light–12 h dark cycle. At the end of both *in vivo* experiments food was withheld overnight, rats were anesthetized with diethyl ether and killed by exsanguination. Blood was collected for the preparation of serum, and tissues were immediately removed for vitamin A and carotenoid analyses.

Biochemical analyses

Serum retinol and tissue vitamin A and carotenoids were extracted using hexane and assayed using a reverse-phase HPLC apparatus (Kontron series 400; Kontron, St-Quentin-en Yvelines, France) with retinyl laurate and echinenone as internal standards (Duszka *et al.* 1996). Vitamin A and carotenoids from diets, and bacterial incubation media were extracted using tetrahydrofuran and light petroleum (b.p. 40°–60°). Carotenoids were analysed by HPLC using a Vydac TP54 column (250 × 4·6 mm) and a Nucleosil column (150 × 4·6 mm; Interchim, Montluçon, France) in series, and a mixture of acetonitrile–methanol containing 50 mM-ammonium acetate–dichloromethane (75:20:5, by vol.) containing triethylamine (0·5 g/l), as mobile phase (1·5 ml/min; Hart & Scott, 1995).

Bacteriological studies

Freshly-passed faeces from rats were diluted to 10^{-2} in a liquid casein–yeast medium (LCY; Difco, Detroit, MI, USA) and incubated in pre-reduced liquid brain–heart medium containing 5 mg hemin/l (L-BHI; Difco) in a Freter anaerobic cabinet. Appropriate dilutions of the suspensions were plated on brain–heart agar (Difco) under anaerobic and aerobic conditions at 37°. Total anaerobic and aerobic bacteria were determined after 48 h.

In vitro *metabolism.* Human stools and rat faeces were diluted in anaerobically pre-reduced L-BHI medium as described previously. Cultures were incubated in the presence of 15 μmol β-carotene/l (water-dispersible beadlets; Hoffmann La Roche, Basel, Switzerland) at 37° either in an anaerobic cabinet or aerobically for up to 72 h (for each atmospheric condition, four tubes/inoculum per period of incubation; forty-eight tubes). Incubations were carried out for 7, 24, 48 and 72 h, after which the cultures were centrifuged (2000 ***g*** for 10 min) and the supernatant fluids analysed for vitamin A and β-carotene as described previously.

Statistics

Results were expressed as means with their standard errors. Comparison between groups was made by one-way ANOVA, with microbial status as a factor. When a significant F value was obtained, differences between group means were assessed by the Fisher's protected least significant difference test. $P < 0{\cdot}05$ was considered statistically significant. The *in vitro* effect of inoculum was analysed by repeated-measures ANOVA followed by a Fisher's test.

Results

As can be seen in Table 1, body weights, body-weight gains and liver weights were similar for all animals within the same experiment. Caecum weight was approximately 5- to 6-fold heavier in the germ-free (axenic) group compared with the conventional and heteroxenic groups. Similarly, treatment with antibiotics, but not with omeprazole, dramatically increased caecal weight. The total bacterial count per g faeces, expressed as $\log_{10}$ of the number of

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Table 1. Final weight and daily weight gain of rats fed for 2 weeks on the diet supplemented with α- and β-carotene*
(Mean values with their standard errors for six rats (Expt 1) and five rats (Expt 2) per group)

	Body wt (g)		Wt gain† (g/d)		Liver wt (g)		Caecum wt (g)	
Treatment	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Expt 1 (df 5)								
Axenic	150	2	1·3	0·1	5·3	0·2	11·3[b]	1·2
Heteroxenic	143	4	1·5	0·2	5·1	0·2	2·4[a]	0·6
Conventional	144	3	1·6	0·1	5·6	0·2	2·0[a]	0·5
ANOVA‡	NS		NS		NS		$P < 0·001$	
Expt 2 (df 4)								
Antibiotics	189	12	2·1	0·2	6·1	0·5	15·8[b]	1·2
Omeprazole	174	9	1·9	0·3	6·3	0·5	3·8[a]	0·4
Control	191	7	2·0	0·2	6.7	0·3	2·7[a]	0·2
ANOVA	NS		NS		NS		$P < 0·001$	

[a,b] Groups means with different superscript letters in the same column and in each experiment were significantly different ($P < 0·05$).
* For details of treatments, see p. 200.
† Measured during the 2 weeks of the supplementation period.
‡ One-way ANOVA and Fisher's protected least significant difference test (NS $P > 0·05$).

colony-forming units, significantly decreased in rats treated with antibiotics (3·51 (SE 0·35) *v.* 10·13 (SE 0·31)), but was not affected after omeprazole dosing.

In Expt 1, no significant level of vitamin A was detected in rat livers after the vitamin A-deficiency period. Food intakes of germ-free, heteroxenic and conventional rats over the supplementation period were 7·1 (SE 0·3), 7·3 (SE 0·8) and 8·6 (SE 0·7)g/d respectively (ANOVA test, $P > 0·05$). Table 2 reports the retinol levels in serum, and the liver contents of vitamin A, and α- and β-carotene. Germ-free rats accumulated approximately twice as much vitamin A, α-carotene and β-carotene in the liver as their heteroxenic and conventional counterparts ($P < 0·001$). The α-carotene : β-carotene value in liver was calculated to indicate the relative absorption of these two carotenoids when combined in food. It was found to be weakly influenced by rat microbial status ($P = 0·029$), but the three groups displayed similar values. These values were 50 % higher than that measured in the diet (dietary α- : β-carotene 0·22), suggesting, when compared with β-carotene, a higher absorption rate of α-carotene or a lower conversion rate of α-carotene to vitamin A. When compared with conventional rats, vitamin A : β-carotene in the liver was only affected in heteroxenic rats (+32 %, $P < 0·05$). Vitamin A concentrations in serum, lungs and kidneys were similar for all the experimental groups.

In Expt 2, all rats consumed equal quantities of the carotene-supplemented diet. When the intestinal microflora was partially destroyed by antibiotic treatment, rats accumulated 33, 135 and 128 % more vitamin A, β- and α-carotene respectively in their livers than did control rats (Table 3). The α-carotene : β-carotene value in liver was similar for the three groups, but was slightly lower than that in the diet (−23 %, $P < 0·05$). After antibiotic treatment, the vitamin A : β-carotene value in the liver was reduced by 48 % ($P < 0·05$) as compared with the control group. No significant effect was associated with the omeprazole treatment.

Table 4 shows that *in vitro* incubation of water-soluble β-carotene with faecal flora in aerobic cabinets resulted

Table 2. Expt 1. Vitamin A, and α- and β-carotene status of germ-free, heteroxenic and conventional Fisher rats fed on the carotenoid-supplemented diet for 2 weeks*
(Mean values with their standard errors for six rats)

	Conventional rats		Heteroxenic rats		Germ-free rats		
	Mean	SE	Mean	SE	Mean	SE	ANOVA†
Serum retinol (μg/ml)	0·5	0·1	0·5	0·1	0·6	0·1	NS
Liver variables:							
Vitamin A (μg RE)	419[a]	22	445[a]	31	780[b]	29	$P < 0·001$
β-Carotene (μg)	11·1[a]	0·5	8·7[a]	0·7	22·4[b]	0·5	$P < 0·001$
α-Carotene (μg)	3·6[a]	0·3	2·9[a]	0·2	6·8[b]	0·4	$P < 0·001$
α- : β-Carotene	0·33[ab]	0·01	0·34[b]	0·01	0·31[a]	0·01	$P = 0·029$
Vitamin A : β-carotene (μg RE/μg)	38[a]	2	50[b]	3	35[a]	2	$P = 0·001$

RE, retinol equivalent.
[a,b] Group means with different superscript letters in the same row were significantly different ($P < 0·05$).
* For details of animals and procedures, see p. 200.
† One-way ANOVA and Fisher's protected least significant difference test.

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Table 3. Expt 2. Vitamin A, and α- and β-carotene status of Wistar rats fed on the carotenoid-supplemented diet for 2 weeks and treated either with antibiotics or omeprazole*

(Mean values with their standard errors for five rats)

Treatment...	Control		Omeprazole		Antibiotics		
	Mean	SE	Mean	SE	Mean	SE	ANOVA†
Serum retinol (μg/ml)	0·4	0·1	0·4	0·1	0·4	0·1	NS
Liver variables:							
Vitamin A (μg RE)	857[a]	43	760[a]	64	1143[b]	39	$P < 0·001$
β-Carotene (μg)	28·6[a]	7·4	30·1[a]	3·4	66·1[b]	8·6	$P = 0·003$
α-Carotene (μg)	4·9[a]	1·2	4·5[a]	0·8	11·2[b]	1·6	$P = 0·002$
α- : β-Carotene	0·17	0·01	0·16	0·02	0·17	0·01	NS
Vitamin A : β-carotene (μg RE/μg)	36[b]	3	28[ab]	3	19[a]	3	$P = 0·038$

RE, retinol equivalent.
[a,b] Group means with different superscript letters in the same row were significantly different ($P < 0·05$).
* For details of animals and procedures, see p. 200.
† One-way ANOVA and Fisher's protected least significant difference test.

in a progressive disappearance of β-carotene (overall time effect $P < 0·001$). The presence of human and rat intestinal microflora in the medium was associated with a lower β-carotene degradation (overall microflora effect $P = 0·016$), but the interaction between inoculum and time effects was not statistically significant. Retinal, retinol and retinyl esters were not detected in the incubation mixture over the time-course of the study. No inoculum effect was observed under anaerobic conditions up to 72 h.

Discussion

In the present study, we demonstrate that in rat the bioavailability of both α- and β-carotene is improved when the intestinal microflora in the intestine is partially destroyed. This effect cannot be explained by higher food intakes. As a consequence, these animals accumulated much more α- and β-carotene, and vitamin A in their livers than did control rats. No significant differences in liver carotene and vitamin A were observed between conventional and heteroxenic (inoculated with a human whole-faecal flora) rats, suggesting that the microflora composition does not affect carotene bioavailability. Vitamin A : β-carotene in the liver was decreased by the antibiotic treatment. This may be due to a higher vitamin A metabolism in this group or to saturation of the enzyme which converts α- and β-carotene to vitamin A. Such a reaction would result in an accumulation of carotenes in liver and a lower vitamin A : β-carotene. The α- : β-carotene value was not modified by the different treatments, but it was two-fold higher in the first experiment than in the second experiment. Since α-carotene was reported to be a poor substrate of the β-carotene

Table 4. *In vitro* effect of inoculum from human and rat faeces on β-carotene levels (μmol/l) under aerobic and anaerobic conditions*

(Mean values with their standard errors for four determinations)

Incubation period (h)	7		24		48		72	
Inoculum	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Aerobiosis								
None[a]	13·6	0·5	12·6	1·0	8·1	0·8	6·2	0·5
Rat[ab]	14·2	0·3	12·0	0·7	10·7	1·2	8·8	1·7
Human[b]	14·3	0·3	14·5	0·6	11·3	1·0	9·2	1·4
ANOVA†								
Inoculum (I)				$P = 0·016$				
Time (T)				$P < 0·001$				
I×T				$P = 0·532$				
Anaerobiosis								
None	14·5	0·7	14·8	0·6	13·3	0·3	13·4	1·5
Rat	14·3	0·9	13·5	0·9	12·7	1·0	12·5	1·4
Human	15·0	0·5	12·4	1·2	11·3	0·4	11·3	0·8
ANOVA†								
I				$P = 0·306$				
T				$P = 0·001$				
I×T				$P = 0·453$				

[a,b] Inoculum not sharing a similar superscript produced a significantly different effect ($P < 0·05$).
* Water-soluble beadlets of β-carotene were used and the initial concentration was 15 μmol/l. For details of procedures, see p. 200.
† ANOVA for repeated measures. When the overall inoculum effect was significant a Fisher's least square difference post hoc test was performed.

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15,15′-dioxygenase (*EC* 1.13.11.21) enzyme as compared with β-carotene (Grolier *et al.* 1997), α-carotene accumulation in tissues would be less sensitive to variations in dioxygenase activity than that of β-carotene. This activity was shown to be different in tissues from Norwegian hooded and Sprague-Dawley rats (Villard & Bates, 1986). Hence, the high α-:β-carotene value we observed in Fisher rats may be the result of efficient dioxygenase activity, and the low ratio in Wistar rats may be the result of a low dioxygenase activity in these animals.

Previous studies indicated that penicillin and chlorotetracycline treatment increased liver storage of vitamin A in chicks (Almquist & Maurer, 1955) and rats (Guerrant, 1960) fed on β-carotene, but there were no data concerning β-carotene accumulation. It was also not clearly established whether this effect was related to the bacterial activity of antibiotics or to a direct action of antibiotics on absorption and/or metabolism of carotenoids. Since results for germ-free rats and for antibiotic-treated rats were similar, we suggest that the greater bioavailability of β-carotene observed in these animals was primarily related to the decrease in the intestinal flora.

Several hypotheses can be proposed to explain the effect of microflora. First, intestinal micro-organisms were reported to metabolize a wide range of nutrients (Eggum *et al.* 1982; Cobb *et al.* 1991; Roland *et al.* 1995). It might be postulated that they also partially catabolize α- and β-carotene in the gut and thus reduce the amounts of carotene available for absorption. When β-carotene was incubated in culture media under O_2 (200 ml/l) and at 37°, its level progressively declined, probably due to oxidation. The final β-carotene concentration was higher when incubated with an inoculum from rat and human faeces, suggesting that microflora elicited a protection against β-carotene oxidation. These results and the absence of vitamin A metabolites in the different incubation mixtures suggest that intestinal bacteria are unlikely to play a direct role in β-carotene metabolism. It could be argued that the micro-organisms which colonize the jejunum, where β-carotene is absorbed, differ from those present in the inoculum we tested in our *in vitro* assay. However, it was reported in a recent study that when bacteria from human jejunum were incubated with labelled β-carotene no biological product were detected (Tang *et al.* 1996).

Second, in germ-free rats the villi were reported to be one-third longer in the proximal intestine, where the absorption of β-carotene occurs, as compared with control rats (Meslin *et al.* 1974). This may bring mixed micelles into contact with the mucosa where carotenes could diffuse from the micelles through the enterocyte membranes. Unfortunately, using electron microscopy, we failed to find any significant differences in mucosa thickness or jejunal villus height between germ-free and conventional rats (data not shown).

Alternatively, the absorption of β-carotene is dependent on bile salts which promote the formation of mixed micelles. On the other hand, bacteria in the small intestine and colon can convert glycine- and taurine-conjugated bile acids to free bile acids which are less soluble in the acidic conditions which follow fibre fermentation (Moundras *et al.* 1994). Moreover, caecal bacteria have been shown to dehydroxylate cholic and β-murocholic acids (Andrieux *et al.* 1989). These features may affect the caecal re-absorption of bile acids, and in turn reduce β-carotene absorption. Consistent with this hypothesis is the observation that faecal excretion of bile acids decreases in cats fed on antibiotics (Kim *et al.* 1996) and in germ-free rats (Gustafsson & Norman, 1969) compared with control animals. However, faecal loss of bile acids represents less than 2 % of the 24 h biliary bile acid flux in conventional rats (Moundras *et al.* 1997). Thus, it seems unlikely that microflora significantly affect β-carotene bioavailability through a bile salt-mediated mechanism.

Finally, because the absorption of carotenes occurs through passive diffusion, factors increasing the carotene concentration in the lumen of the jejunum would increase their absorption. The intestinal transit time was found to be slower in germ-free rats (Riottot *et al.* 1980) and could explain the two-fold increase in the small intestinal lumen concentration of bile salts in these animals as compared with controls (Sacquet *et al.* 1976). Thus, we suggest that the absence of intestinal microflora results in a more efficient solubilization of β-carotene in mixed micelles and a longer interaction between these particles and the jejunal mucosa. Both these factors are capable of promoting the absorption of α- and β-carotene.

Bacterial overgrowth may occur in the small intestine when intraluminal pH increases, as in the case of gastric hypochlorhydria (Haboubi & Montgomery, 1992). Omeprazole inhibits gastric acid secretion and induces bacterial overgrowth in the human upper intestine (Tang *et al.* 1996). In the rat, an oral dose of 15 μmol/body weight inhibited gastric acid secretion by 50–60 % (Larsson *et al.* 1983). Thus, we administered the same dose of this compound daily for 2 weeks, to examine whether bacterial overgrowth could modify carotene bioavailability in rats. Unfortunately, we failed to detect any effect related to omeprazole treatment. Tang *et al.* (1996) demonstrated that the blood response to a single dose of β-carotene was significantly lower in volunteers dosed with omeprazole than in control subjects, and they have suggested that this result may have been primarily due to a high lumen pH rather than to bacterial overgrowth. In our study, rats ingested β-carotene for 2 weeks and repeated doses of β-carotene may not be sensitive to an omeprazole effect.

In conclusion, the bioavailability of both α- and β-carotene was improved when the intestinal microflora was absent or decreased in the rat. We suggest that intestinal microflora do not act directly on carotenes, but rather diminish their intestinal absorption by decreasing the intestinal transit time and the pool of bile salts in the jejunum. As a consequence, treatment with antibiotics and nutritional factors which could modify the intestinal bacterial activity may indirectly affect the tissue accumulation of carotenoids, and their vitamin A activity. Moreover, these findings suggest that antibiotics, which can be administered with vitamin A or β-carotene supplements in areas where vitamin A deficiency occurs, have no adverse effects on the bioavailability of these micronutrients.

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