

In vivo biosynthesis of retinoic acid from β -carotene involves an excentric cleavage pathway in ferret intestine

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Abstract This study was done to determine whether retinoic acid can be produced by excentric cleavage of β -carotene in vivo. By using an inhibitor of retinaldehyde oxidation, citral, either retinaldehyde or β -carotene was incorporated in a micellar solution and perfused through the upper portion of small intestine of ferrets. After 2 h perfusion of 1 μ M retinaldehyde, retinoic acid rose in portal blood ($+3.5 \pm 1.3$ nmol/L) and was detected in the intestinal mucosa (30 ± 2 pmol/g). When citral was added at 2 mM along with retinaldehyde, retinoic acid decreased in the portal blood and retinoic acid was not detected in the intestinal mucosa. With or without the presence of citral (2 mM), the perfusion of β -carotene (10 μ M) during 2 h caused a significant rise of retinoic acid in portal blood ($+2.6 \pm 0.6$ nmol/L and $+4.1 \pm 0.6$ nmol/L, respectively) and in liver; moreover, significant amounts of retinoic acid were detected in the intestinal mucosa (19 ± 3 pmol/g and 36 ± 3 pmol/g, respectively). ■ This study demonstrates that after intestinal perfusion of β -carotene in the ferret in vivo, a substantial amount of retinoic acid is formed via an excentric cleavage pathway.—Hébuterne, X., X-D. Wang, D. E. H. Smith, G. Tang, and R. M. Russell. In vivo biosynthesis of retinoic acid from β -carotene involves an excentric cleavage pathway in ferret intestine. *J. Lipid Res.* 1996. **37**: 482–492.

Supplementary key words β -carotene • retinoic acid • vitamin A • β -carotene metabolism • β -carotene absorption • β -carotene cleavage • ferret

The role of β -carotene (β -C) in cancer prevention, which was first proposed by Peto et al. (1), has been supported by both epidemiologic (2) and biologic studies (3). One possible mechanism for this protective effect is attributable to the antioxidant and free radical trapping properties of the intact β -C molecule (4). However, the effects of β -C also may be attributed to one of its metabolites, retinoic acid (RA). Retinoic acid is known to induce cell differentiation (5), and this property has been used in the successful treatment of acute promyelocytic leukemia (6, 7). Several RA receptor pro-

teins with DNA-binding sites have been shown to exist (8, 9).

In 1967, Crain, Lotspeich, and Krause (10) first described the conversion of β -C into RA by the rat intestinal mucosa and the transport of this acidic compound via the portal vein. It has been shown in vitro that β -C is a significant precursor of RA in several mammalian tissues (11). The administration of β -C to rabbits is associated with elevated RA in serum (12), and the intestinal perfusion of β -C in the ferret raises RA level in portal blood (13). Moreover, RA has been shown to be present in human blood (14). Recently, the biosynthesis of 9-*cis* RA from 9-*cis* β -C has been demonstrated in vitro in human intestinal mucosa (15) and in vivo after intestinal perfusion of the ferret (16). All of these findings suggest that β -C may be an important precursor of RA, and the production of RA from β -C may be one explanation for the anticarcinogenic effects of β -C. However, the exact mechanism of formation of RA from β -C is still unclear.

Both central and excentric cleavage pathways have been proposed for the biosynthesis of RA from β -C (17, 18). Central cleavage converts a single β -C molecule to two molecules of retinaldehyde by cleavage at the 15,15' double bond. The retinaldehyde formed can be oxidized into RA by a retinaldehyde dehydrogenase, or the retinaldehyde may be reduced to retinol. Excentric cleavage produces a series of β -apocarotenals of different chain lengths, all of which can be oxidized to the corresponding β -apocarotenoic acids including RA or be converted to retinaldehyde (Fig. 1).

Abbreviations: β -C, β -carotene; RA, retinoic acid; HPLC, high performance liquid chromatography; HNRC, Human Nutrition Research Center on Aging.

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The monoterpene aldehyde, citral (3,7-dimethyl-2,6-octadienal), inhibits the oxidation of retinol to RA in epidermal tissue both in vitro and in vivo (19, 20). When retinaldehyde is incubated in the presence of varying concentrations of citral, RA formation is inhibited. However, after incubation of intestinal homogenates with β -C and citral, it was found that citral does not inhibit the formation of β -apocarotenals and RA (21). These findings, therefore, suggest that, in vitro, RA can be produced from excentric cleavage of β -C via a series of β -apocarotenals. However, until now, the biosynthesis of RA via excentric cleavage of β -C has not been demonstrated in the living animal.

Most commonly used laboratory animals do not absorb β -C; we recently demonstrated that the domesticated ferret can both absorb intact β -C (22) and convert it to RA (13) which is absorbed through the portal blood. Therefore, we used the ferret model in our experiments.

The present study was undertaken to demonstrate in vivo during intestinal perfusion of the ferret that β -C can be metabolized to RA despite the inhibition of retinaldehyde dehydrogenase by citral (i.e., by an excentric cleavage mechanism).

MATERIALS AND METHODS

Chemical products

All-*trans*- β -C, all-*trans*-retinaldehyde, all-*trans*-RA, γ -carotene, retinyl acetate, citral, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), Krebs phosphate buff-

er, α -tocopherol, oleic acid, sodium taurocholate, and other chemicals were purchased from Sigma Co. (St. Louis, MO). β -Carotene was purified by chromatography on a 5% water-weakened alumina column. The purity of β -C achieved was >98%. All solutions were prepared under red light immediately before use. All high performance liquid chromatography (HPLC) solvents were obtained from Baker Chemical Co. (Philipsburg, NJ) and were filtered through a 0.45 μ m membrane filter.

Animals

Male ferrets (*Mustela putorius furo*), from Marshall Farms (North Rose, NY) were housed in an American Association of Accreditation of Laboratory Care-accredited animal facility at the Human Nutrition Research Center on Aging (HNRC) at Tufts University. They were fed dry ferret food (Bil.Jac®, Win-Hy Foods, Tulsa, OK) and water ad libitum. The dry ferret food contained 0.54 μ g/g β -C and 17.7 μ g/g retinyl esters. The diet was extracted following procedures published in the Official Method of Analysis Chemist, Inc. (1984) Chapter 43.020 and analyzed by HPLC. Surgical procedures in the experiments were approved by the Animal Care Committee at Tufts University and surgery was conducted under aseptic conditions in the Division of Comparative Medicine at HNRC.

Study design

We used procedures previously described (22) with certain modifications. Briefly, after an overnight fast,

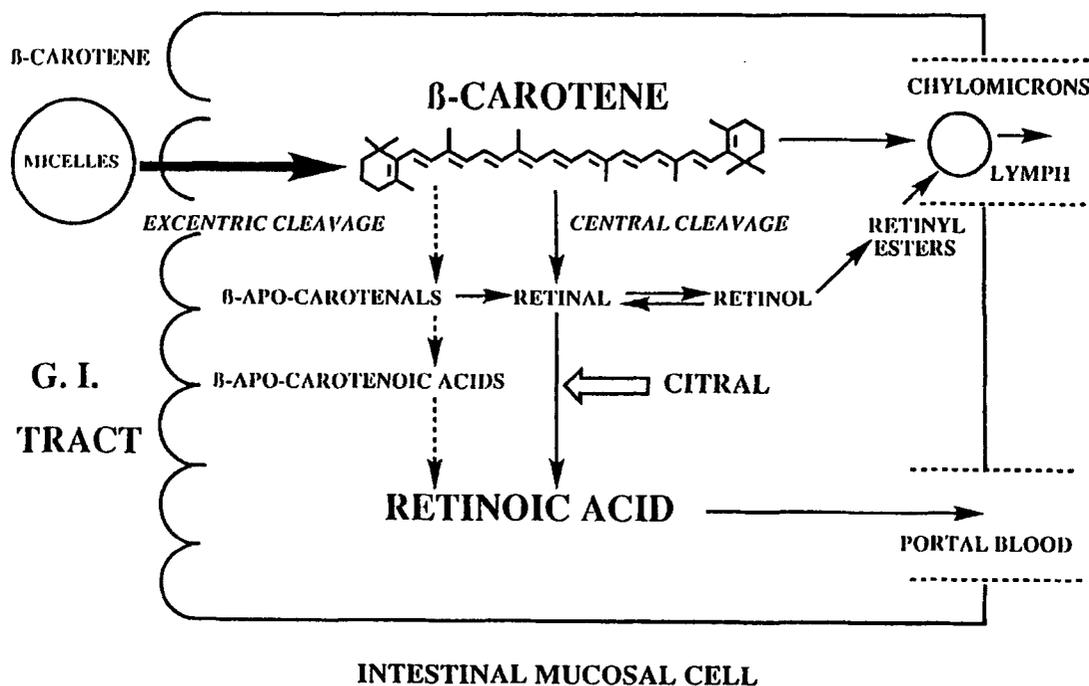


Fig. 1. Intestinal metabolism of β -carotene.

3.0 mL corn oil was administered orally to ferrets to dilate the intestinal lymphatics. Thirty minutes later, ketamine hydrochloride (35 mg/kg) and xylazine (3 mg/kg) were administered intramuscularly to induce anesthesia. Ferrets were intubated with 3.0 mm ID endotracheal tubes, and anesthesia was maintained with 2–3% isoflurane in 100% oxygen. Anesthetized ferrets were kept on circulating hot water blankets at 38°C. Through a midline abdominal incision, the mesenteric lymph duct was cannulated using a polyethylene catheter (1.27 mm OD, 0.86 mm ID; PE-90, Clay Adams, Becton Dickinson, Parsippany, NJ). The portal vein was cannulated using a heparinized polyethylene catheter (1.22 mm OD, 0.76 mm ID; PE-60, Clay Adams). Both lymph and portal tubes were secured with surgical glue. A proximal inflow catheter (0.64 cm OD, 0.32 cm ID; Tygon flexing plastic tubing, Norton, Akron, OH) was inserted into the jejunum 5 cm distally to the ligament of Treitz, the proximal outflow catheter was introduced 50 cm distally. To prevent the perfusate from washing back into the stomach or continuing into the intestine, catheters were secured by encircling ligatures. The intestinal segment was flushed with normal saline to remove intestinal contents.

A micellar solution containing 0.2 mM α -tocopherol, 2.5 mM oleic acid, and 10 mM sodium taurocholate in Krebs phosphate buffer was formed by sonication for 15 min at 80 W of power (Branson, Shelton, CT). All the animals received first this micellar solution for 60 min at 2.0 mL/min. The stability of β -C and retinaldehyde after incorporation into the micellar solution and after 8 h of storage at room temperature was checked by HPLC, and no oxidative products were detected. In a preliminary pilot experiment, two ferrets were studied. One of them received the micellar solution alone at the flow rate of 2.0 mL/min for 2 h. The second animal received the micellar solution containing 2 mM citral at a flow rate of 2.0 mL/min for 2 h. In experiment 1, the micellar solution containing 1 μ M all-*trans*-retinaldehyde (group A: n = 4) or 1 μ M all-*trans*-retinaldehyde plus 2.0 mM citral (group B: n = 3), was perfused at the flow rate of 2.0 mL/min for 2 h. In experiment 2, the micellar solution containing 10 μ M all-*trans* β -C (group C: n = 4) or 10 μ M all-*trans*- β -C plus 2 mM citral (group D: n = 4), was perfused at a flow rate of 2.0 mL/min for 2 h. In all experiments, the lymph drainage was collected by gravity in 30-min collections. The portal vein cannula was sampled every 30 min: a 1.5-mL sample was withdrawn by syringe within a 2-min period and the same volume of normal saline was simultaneously injected into the portal vein. To avoid degradation of β -C, the perfusion experiments were carried out in the dark. In all cases compounds were infused into the jejunum in a single-pass mode. After perfusion, the animals were killed by

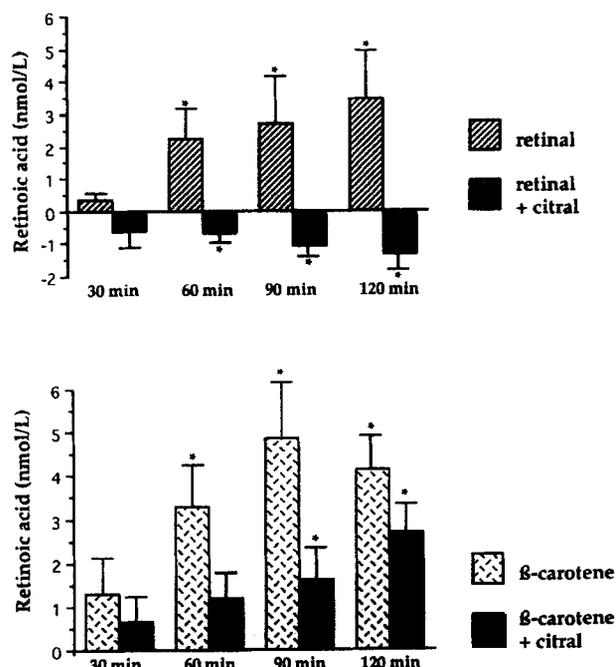


Fig. 2. The net increase of retinoic acid from baseline (mean \pm SEM) in portal vein blood during the intestinal perfusion of (A) 1 μ M retinaldehyde or 1 μ M retinaldehyde + 2 mM citral, (B) 10 μ M β -carotene or 10 μ M β -carotene + 2 mM citral. *, Significantly different from baseline value at $P < 0.05$.

puncturing the abdominal aorta. The perfused intestinal segments were removed and freed of their mesentery and serosal fat. The perfused intestinal mucosa was scraped with a glass slide and 1 g was homogenized in a Brinkmann (Westbury, NY) Polytron homogenizer with 3 mL ice-cold HEPES buffer. In three animals in group C and three in group D, a liver biopsy (150–200 mg of tissue) was performed immediately before and after the perfusion of β -C or β -C plus citral. After the procedure, the liver was removed and weighed.

Extraction and HPLC assays

All samples were kept on ice and protected from the light. Extraction was performed immediately after the perfusion study under red light. Samples (lymph, serum, intestinal mucosa scrapings, and liver) were extracted without saponification using 5.0 mL of CHCl_3 – CH_3OH 2:1 (vol/vol). Retinyl acetate and γ -carotene were added as internal standards for the analysis of retinoids and carotenoids, respectively. After centrifugation (10 min at 800 g at 4°C), the chloroform layer was saved and the residue was extracted again using 6.0 mL hexane. Chloroform and hexane layers were pooled and evaporated to dryness under N_2 . The residue was redissolved in 100 μ L ethanol. A 50- μ L aliquot of the final extract was injected onto the HPLC system.

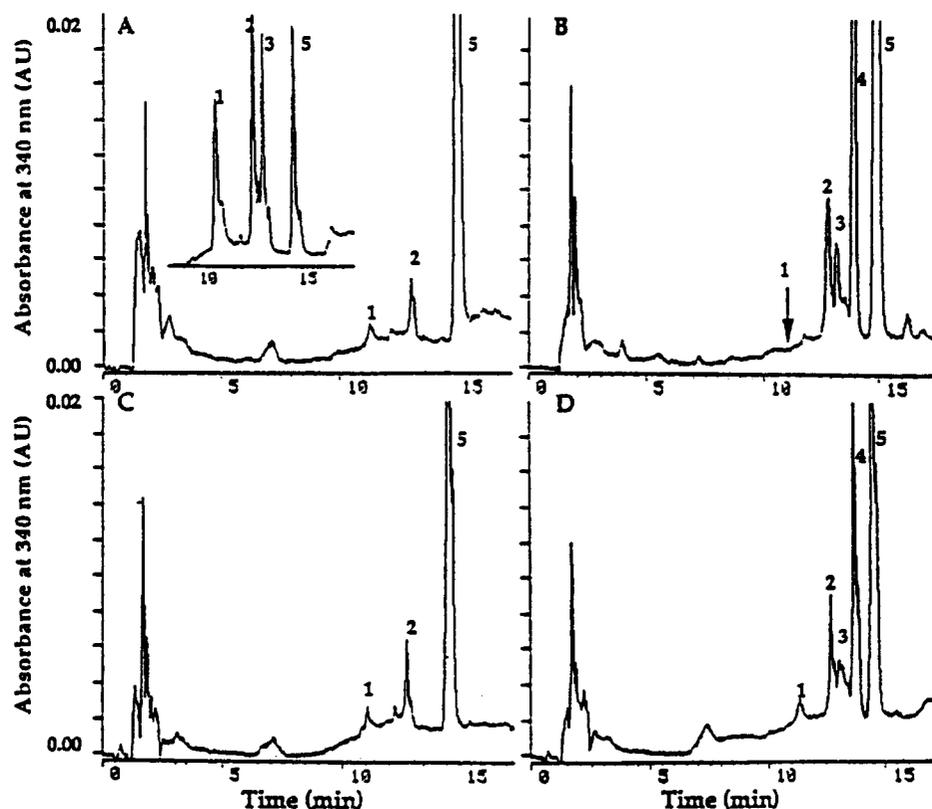


Fig. 3. HPLC chromatography of retinoids in ferret intestinal mucosa; (A) after the intestinal perfusion of 1 μ M retinaldehyde for 2 h; (B) after the intestinal perfusion of 1 μ M retinaldehyde + 2 mM citral for 2 h; (C) after the intestinal perfusion of 10 μ M β -carotene for 2 h; (D) after the intestinal perfusion of 10 μ M β -carotene + 2 mM citral for 2 h. Inserted figure shows the HPLC pattern after injection of standard retinoids. Peak identifications: (1) retinoic acid, (2) retinol, (3) retinaldehyde, (4) citral metabolite, (5) retinyl acetate added as internal standard. Mobile phase was $\text{CH}_3\text{CN}/\text{THF}/\text{H}_2\text{O}$ (Solvent A: 35:5:60, vol/vol/vol, 1% ammonium acetate in H_2O ; Solvent B: 50:44:6, vol/vol/vol, 1% ammonium acetate in H_2O).

β -Carotene, retinol, and retinyl esters (retinyl palmitate and retinyl stearate) were analyzed by reverse-phase HPLC on a Pecosphere-3 C_{18} 0.46 \times 8.3 cm cartridge column (Perkin-Elmer, Norwalk, CT) using CH_3CN -THF-1% ammonium acetate in water (Solvent A: 50:20:30, vol/vol/vol; Solvent B: 50:44:6, vol/vol/vol) as previously described (23, 24). A Waters 490E multi-wavelength spectrophotometer detector was set at 340 nm for retinoids and 450 nm for carotenoids, and an additional Waters 994 programmable photo diode array detector was used for measurement of maximal absorption. For the identification of RA, a modified procedure was used in which the HPLC mobile phase was CH_3CN -THF-1% ammonium acetate in water (Solvent A: 35:5:60, vol/vol/vol; Solvent B: 50:44:6, vol/vol/vol) as described earlier (22).

Individual carotenoids and retinoids were identified by co-elution with their standards, and quantified relative to the recovery of internal standards, retinyl acetate, and γ -carotene, by determining peak areas above observed baseline calibrated against known amounts of

standards. The lowest limit of detection of RA for each assay was 0.2 pmol (24).

Methylation

The positive identification of RA derived from intestinal perfusion with the β -C was accomplished by synthesizing and characterizing methyl retinoate. Three additional ferrets were perfused using the procedure described above. The intestinal mucosa samples were extracted, pooled, and analyzed by HPLC. The HPLC peak that matched with the retention time of standard RA was collected and dried under N_2 . The residue was dissolved in 3 mL peroxide-free diethyl ether at 4°C and derivatized with diazomethane to form the methyl retinoate. After the reaction, the excess diazomethane was removed under nitrogen and the residue was resuspended in ethanol and subjected to HPLC as before.

Statistics

Results are expressed as means \pm SEM. Comparisons among the same group were performed using a one-

TABLE 1. Retinoic acid, retinol, retinaldehyde, retinyl ester, β -apo-12'-carotenal, and β -carotene concentrations in ferret intestinal mucosa after perfusion of 1 μ M of retinaldehyde (n = 4) or 1 μ M of retinaldehyde + 2 mM of citral (n = 3) for 2 h

| | Retinal | Retinal + Citral |
|----------------------------|------------------|-------------------------------|
| β -Carotene | ND | ND |
| β -Apo-12'-carotenal | ND | ND |
| Retinoic acid | 30 \pm 2 | ND ^a |
| Retinaldehyde | ND | 18446 \pm 7149 ^a |
| Retinol | 994 \pm 337 | 2084 \pm 185 ^a |
| Retinyl esters | 10440 \pm 2176 | 5446 \pm 897 |

Values (mean \pm SEM) are expressed in pmol/g mucosa; ND, not detected.

^aSignificantly different ($P < 0.05$) from the result obtained without citral.

tailed paired *t* test while comparisons between groups A and B and groups C and D were performed using a one-tailed unpaired *t* test. Results were considered significantly different at $P < 0.05$.

RESULTS

The body weights of the animals (1.2 \pm 0.1 kg, 1.2 \pm 0.1 kg, 1.1 \pm 0.1 kg, and 1.2 \pm 0.1 kg in groups 1 to 4, respectively), weights of the intestinal mucosa after scraping (4.5 \pm 0.2 g, 3.9 \pm 0.4 g, 5.2 \pm 0.2 g, 4.8 \pm 0.5 g in groups 1 to 4, respectively), liver weights (26.8 \pm 0.9 g and 26.3 \pm 2.4 g in groups 3 and 4, respectively) and the length of the perfused intestine (51 \pm 1 cm, 49 \pm 1 cm, 52 \pm 1 cm and 49 \pm 3 cm in groups 1 to 4, respectively) were similar among the different groups.

Preliminary pilot experiment

During the 2-h perfusion of the micellar solution alone (containing no retinoids, carotenoids, or citral), RA levels were stable in the portal blood (1.9 nmol/L before perfusion and 1.8 nmol/L after perfusion). After perfusion, neither RA nor retinaldehyde was detected in the intestinal mucosa. The concentrations of retinol and retinyl esters in the intestinal mucosa were 83 pmol/g and 2483 pmol/g, respectively.

Another ferret received the micellar solution containing 2 mM citral. In the portal blood, RA levels slightly decreased from 1.9 nmol/L before perfusion to 1.2 nmol/L after 2 h of perfusion. In the intestinal mucosa, RA was not detected but retinaldehyde was detected at the concentration of 735 pmol/g. The concentrations of retinol and retinyl esters in the mucosa were 276 pmol/g and 13,187 pmol/g, respectively.

Experiment 1

Synthesis of retinoic acid from retinaldehyde with or without citral. Intestinal perfusion of retinaldehyde raised RA levels significantly ($P < 0.05$) over baseline in portal blood after 60, 90, and 120 min of perfusion (Fig. 2A). Blood levels of RA were 2.2 \pm 0.5 nmol/L before perfusion. In contrast, when citral and retinaldehyde were perfused together, the RA levels decreased significantly from baseline after 60, 90, and 120 min perfusion ($P < 0.05$) (Fig. 2A). In this case, blood levels of RA were 2.4 \pm 0.6 nmol/L before perfusion and 1.1 \pm 0.3 nmol/L after perfusion. The net increase of RA from baseline in portal blood was always significantly ($P < 0.05$) higher during the perfusion of retinaldehyde than during the perfusion of retinaldehyde plus citral under the same experimental conditions (Fig. 2A). After retinaldehyde perfusion, RA was detected in the intestinal mucosa at a concentration of 30 \pm 2 pmol/g of mucosa, but retinaldehyde was not detected (Fig. 3A). After retinaldehyde + citral perfusion, RA was not detected in the intestinal mucosa (Fig. 3B), but large amounts of retinaldehyde were found (Table 1). The retinol concentration in the intestinal mucosa was significantly higher in the presence of citral than in the absence of citral in the micellar solution (Table 1). The retinyl ester concentration in the mucosa was not significantly different between the two groups (Table 1). Retinol and retinyl ester concentrations in the blood were stable and not different between the two groups (Figs. 4A and 4B). Similarly, retinol and retinyl ester absorption in lymph were not significantly different between the two groups (Fig. 5A).

Experiment 2

Synthesis of retinoic acid from β -carotene with or without citral. Intestinal perfusion of β -C raised RA levels in portal blood both in the absence or presence of citral (Fig. 2B). The net increase of RA in portal blood was significant ($P < 0.05$) after 60, 90, and 120 min when β -C was perfused alone (Fig. 2B). Blood levels of RA were 1.4 \pm 0.3 nmol/L before perfusion and 5.5 \pm 1.0 nmol/L after perfusion. When β -C and citral were perfused together, the net increase of RA in portal blood was also significant ($P < 0.05$) after 90 and 120 min (Fig. 2B). Blood levels of RA were 1.1 \pm 0.1 nmol/L before perfusion and 3.7 \pm 0.6 nmol/L after perfusion. The amount of RA in portal blood was consistently higher when β -C was perfused without citral than when it was perfused with citral (Fig. 2B), although the differences did not reach statistical significance ($P = 0.053$ at 90 min and $P = 0.074$ at 120 min). In the intestinal mucosa, with or without the presence of citral in the perfusate, RA was detected after perfusion of β -C (Figs. 3C and 3D). However, the RA concentration was significantly higher ($P < 0.05$) when the β -C was perfused alone than when it was

perfused with citral (Table 2). When citral was added to β -C micellar solution, large amounts of retinaldehyde were detected in the intestinal mucosa (Table 2). Similar to the first experiment (Table 1), the retinol concentration was significantly higher in the presence of citral ($P < 0.05$) than after perfusion of β -C alone (Table 2), whereas the retinyl ester concentrations in the mucosa were not different between the two groups (Table 2).

In the liver, RA concentrations increased after perfusion of β -C in the absence or in the presence of citral in all animals (Fig. 6). The concentration of RA in the liver increased significantly from 116 ± 36 pmol/g to 292 ± 34 pmol/g when β -C was perfused alone ($P < 0.05$) and from 147 ± 30 pmol/g to 194 ± 23 pmol/g when β -C was perfused with citral ($P < 0.05$). However, the net increase of RA in the liver was significantly higher ($P < 0.05$) after the perfusion of β -C alone than after the perfusion of both β -C and citral. Retinol and retinyl ester concentrations in the blood were stable and did not differ between the two groups (Figs. 4C and D). Retinol and retinyl ester absorption in the lymph were not significantly different between the two groups (Fig. 5B).

Methylation

In order to identify RA positively, the HPLC peak that matched the retention time of standard RA (Fig. 7A) was derivatized. Derivatization of the RA peak resulted in a 75% loss of the original peak and the appearance of a new peak with a slower retention time (Fig. 7B). Authentic all-*trans*-RA also produced this peak on derivatization (Fig. 7C). The UV spectrum of the derivatized peak exactly matched authentic methyl retinoate, confirming the identity of this metabolite as all-*trans*-RA.

DISCUSSION

In 1931, Karrer, Morf, and Schöpp (25) proposed that β -C is cleaved symmetrically by the addition of two molecules of water at the central 15,15'-double bond to yield two molecules of retinol. In 1946, Hunter (26) suggested that the first step of the conversion of β -C into retinol should be an oxidative attack of the central double bond to yield retinaldehyde. In 1965, two different groups (27, 28) demonstrated the enzymatic syn-

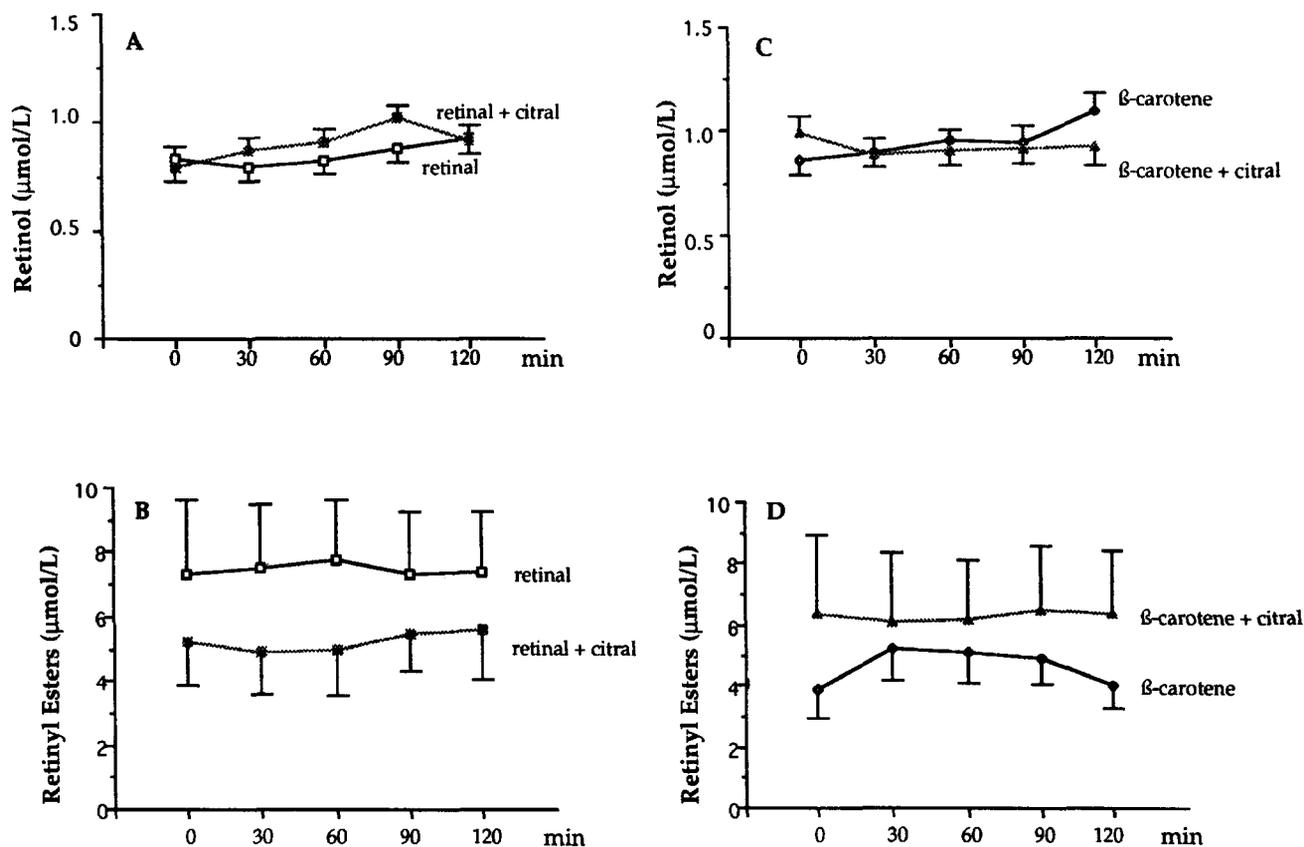


Fig. 4. The concentrations of retinol and retinyl esters (mean \pm SEM) in portal vein blood before and during the intestinal perfusion of either 1 μ M retinaldehyde or 1 μ M retinaldehyde + 2 mM citral (A for retinol and B for retinyl esters), 10 μ M β -carotene or 10 μ M β -carotene + 2 mM citral (C for retinol and D for retinyl esters).

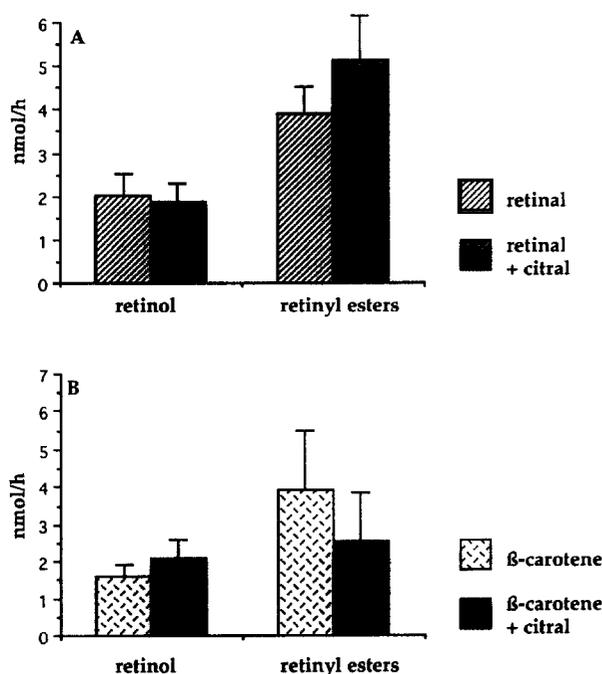


Fig. 5. The absorption of retinol and retinyl esters (mean \pm SEM) into ferret mesenteric lymph during the intestinal perfusion of (A) 1 μ M retinaldehyde or 1 μ M retinaldehyde + 2 mM citral or (B) 10 μ M β -carotene or 10 μ M β -carotene + 2 mM citral for 2 h.

thesis of retinol from β -C in the rat intestine. They both suggested that the process took place by means of a central cleavage mechanism forming retinaldehyde, which would be subsequently reduced to retinol. However, in 1960, Glover proposed the hypothesis that β -C could undergo both central and excentric cleavage (29). This hypothesis was supported by observations in rats, that β -apo-carotenals are converted into retinol or oxidized into their corresponding carotenoic acids (30, 31). Such apocarotenals have been identified in the intestine of chicken after administration of β -C (32).

Until recently, the existence of central or excentric pathways of β -C has been a matter of debate (17, 18, 33). Some authors claimed that central cleavage does not exist (34), whereas others (35) demonstrated enzymatic conversion of β -C to retinaldehyde by a cytosolic enzyme from rabbit and rat intestinal mucosa, supporting a central cleavage pathway mechanism.

Recent work by our group showed that excentric cleavage of β -C does take place in vitro. The incubation of homogenates of human, monkey, ferret, and rat intestinal mucosa with β -C resulted in the formation of retinaldehyde, RA, and the β -apo-12', 10', and 8'-carotenals (24). The formation of β -apo-13-carotenone and β -apo-14'-carotenal was demonstrated in the same species (23). We also demonstrated that when intestinal homogenates are incubated with β -C, RA can be produced by excentric cleavage (21) and that intestinal

perfusion of β -C in the ferret raises RA level in portal blood (13). In light of these results, the question arose whether the biosynthesis of RA via excentric cleavage of β -C can take place in vivo. This question is of importance because RA is a ligand for nuclear receptors (i.e., RAR, RXR) (8, 9) and is involved in cell regulation (5). Moreover, if excentric cleavage exists in vivo, the biological activity of intermediate compounds (i.e., β -apo-carotenals and β -apo-carotenoic acids) should be investigated.

In the preliminary pilot experiment we demonstrated that the perfusion of the micellar solution had no effect on the RA concentration in portal blood. Neither RA nor retinaldehyde was detected in the intestinal mucosa. When citral alone was added to the micellar solution, it resulted in a slight decrease of the RA levels in portal blood. After perfusion of citral, a significant amount of retinaldehyde was detected in the intestinal mucosa, which may result from an inhibition of oxidation of endogenous vitamin A. The high concentration of retinol and retinyl esters detected in the intestinal mucosa after the perfusion of citral alone may be the result of increased production of retinol from retinaldehyde and of high retinyl ester status in the ferret that was used (36).

In the first experiment we demonstrated that intestinal perfusion of retinaldehyde significantly increases RA levels in the portal blood. After retinaldehyde perfusion, a significant amount of RA was also detected in the intestinal mucosa, but retinaldehyde was not detected, presumably due to its rapid conversion into either retinol or RA. When citral was added to the retinaldehyde perfusate, RA levels decreased significantly in the portal blood and were not detected in the intestinal mucosa even though a large amount of retinaldehyde was present, reflecting blockage of the oxidation of retinaldehyde to RA by citral. The intestinal concentration of retinol was more than 2-fold higher

TABLE 2. Retinoic acid, retinol, retinaldehyde, retinyl ester, β -apo-12'-carotenal, and β -carotene concentrations in ferret intestinal mucosa after perfusion of 10 μ M of β -carotene (n = 4) or 10 μ M of β -carotene + 2 mM of citral (n = 4), for 2 h

| | β -Carotene | β -Carotene + Citral |
|----------------------------|-------------------|-------------------------------|
| β -Carotene | 1636 \pm 1213 | 12351 \pm 1480 |
| β -Apo-12'-carotenal | 79 \pm 10 | 68 \pm 19 |
| Retinoic acid | 36 \pm 3 | 9 \pm 5 ^a |
| Retinaldehyde | ND | 10110 \pm 3431 ^a |
| Retinol | 251 \pm 36 | 437 \pm 68 ^a |
| Retinyl esters | 7310 \pm 2882 | 6847 \pm 2978 |

Values (mean \pm SEM) are expressed in pmol/g mucosa; ND, not detected.

^aSignificantly different ($P < 0.05$) from the result obtained without citral.

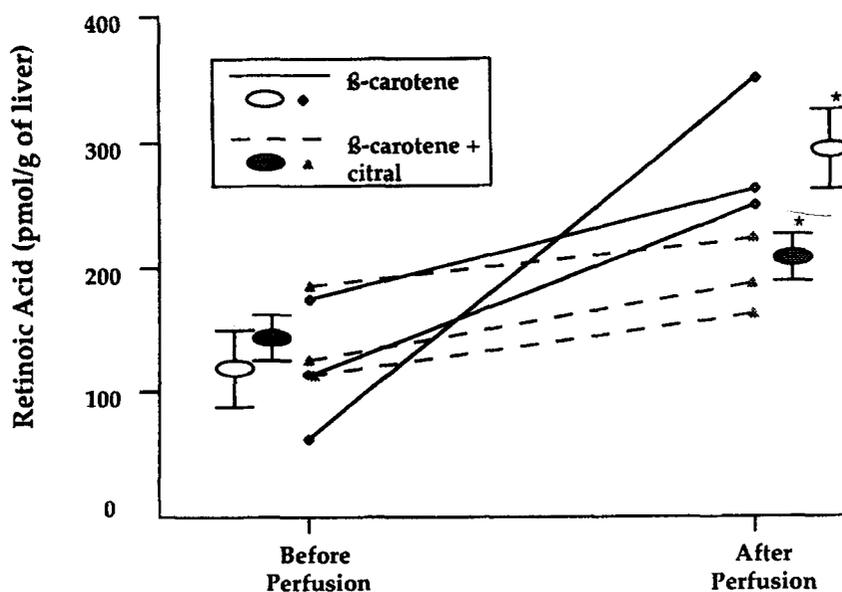


Fig. 6. Concentrations of retinoic acid in the ferret liver (individuals and means \pm SEM) before and after the intestinal perfusion of $10 \mu\text{M}$ β -carotene or $10 \mu\text{M}$ β -carotene + 2 mM citral for 2 h. *Significantly different from baseline value at $P < 0.05$.

after perfusion of retinaldehyde and citral than after perfusion of retinaldehyde alone (Table 1), suggesting an increase in the reduction of the accumulated retinaldehyde to retinol.

In this model, where the lymph duct is cannulated, it is not surprising to see that portal concentrations of retinyl esters did not change during perfusion as most are absorbed in the lymph. There was no effect of citral on retinyl ester absorption in lymph due to slow absorption and presence of endogenous retinol and retinyl esters at the beginning of the study. Thus, our first experiment clearly demonstrates that after intestinal perfusion in the ferret, citral blocks the oxidation of retinaldehyde to RA, which results in an accumulation of retinaldehyde and retinol in the intestinal mucosa.

In the second experiment, we proceeded with the perfusion of β -C under the same conditions to confirm that RA can arise from excentric cleavage of β -C in vivo. From the work of Napoli and Race (11) we chose a concentration of $10 \mu\text{M}$ of β -C to study the effect of citral on RA formation from β -C. Retinoic acid levels obtained in the portal blood and in the intestinal mucosa after perfusion of $1 \mu\text{M}$ retinaldehyde or $10 \mu\text{M}$ β -C without citral were quite similar (Tables 1 and 2). As observed in the perfusion of retinaldehyde plus citral, the perfusion of a mixture of β -C plus citral resulted in the accumulation of retinaldehyde and an increase of the concentration of retinol in the intestinal mucosa of the ferret (Fig. 3, Tables 1 and 2). This result demonstrates once again that at this concentration, citral blocks the

oxidation of retinaldehyde to RA and increases the reduction of retinaldehyde to retinol.

After β -C perfusion, if all the RA were being produced via oxidation of retinaldehyde resulting from the central cleavage mechanism, we would not have expected to find any RA in the mucosa or in the portal blood after the perfusion of a mixture of β -C plus citral. However, contrary to the first experiment, RA was still detected in the mucosa (Fig. 3D), and RA levels increased significantly in portal blood (Fig. 2B) after the perfusion of β -C plus citral. Moreover, the RA concentration in the liver increased in all the animals perfused with β -C (Fig. 6), regardless of the presence of citral, and β -apo-12'-carotenol was detected in the intestinal mucosa. These findings clearly demonstrate for the first time in the living animal that RA can be formed by a pathway other than oxidation of retinaldehyde (i.e., via excentric cleavage).

The results of this study are consistent with the "β-oxidation mechanism" proposed by Glover (29), which involves cleavage at several double bonds of the conjugated system. This cleavage continues with successive removal of two carbon units until the 15,15' double bond is reached. At this point further β-oxidation is blocked by the methyl group located on C13. The recent isolation of apocarotenals and particularly β-apo-13-carotenone and β-apo-14'-carotenol from the intestine of different species (23, 24) strongly supports this hypothesis. In order to provide some information about the presence of intermediates, e.g., β-apo-carotenoic acids, we perfused additional ferrets ($n = 3$) with β -C

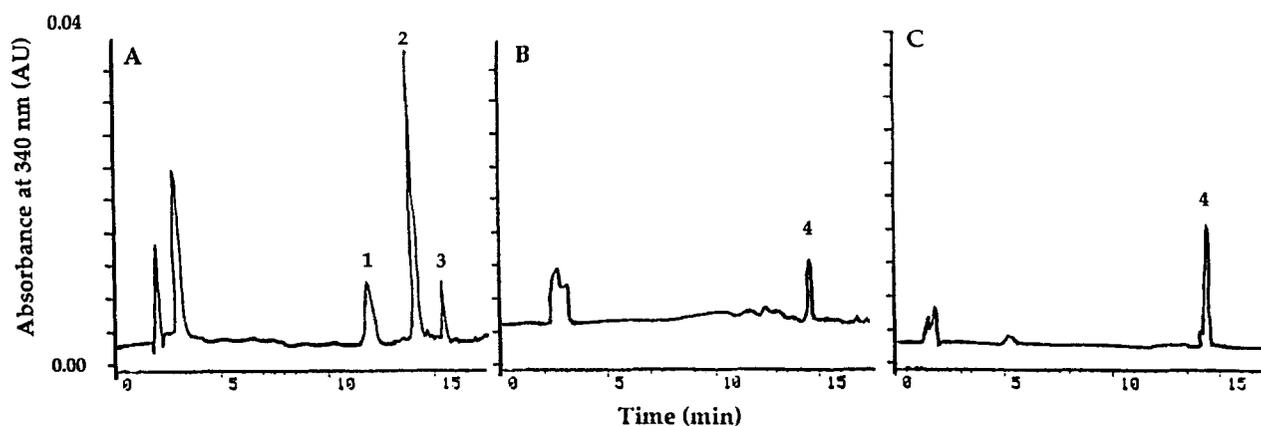


Fig. 7. HPLC chromatography of ferret intestinal mucosa (A) after the perfusion of 10 μM of β -carotene for 2 h (B) after derivatization with ethereal diazomethane and an additional HPLC separation. (C) HPLC chromatography of standard methyl retinoate. Peak identifications: (1) retinoic acid, (2) retinol, (3) retinyl acetate added as internal standard, (4) methyl retinoate. Mobile phase was $\text{CH}_3\text{CN}/\text{THF}/\text{H}_2\text{O}$ (Solvent A: 35:5:60, vol/vol/vol, 1% ammonium acetate in H_2O ; Solvent B: 50:44:6, vol/vol/vol, 1% ammonium acetate in H_2O).

using the same protocol. We still could not detect any additional intermediate besides β -apo-12'-carotenal. We then perfused 5 μM β -apo-14'-carotenal (at a concentration which is much higher than a physiologic level) through the ferret intestine ($n = 5$; X-D. Wang, C. S. Jean, and R. M. Russell, unpublished data). The results showed that β -apo-14'-carotenal disappeared rapidly and only retinol and small amounts of retinoic acid and β -apo-14'-carotenol were detected in the intestinal mucosa, which suggests that *in vivo* these intermediates may be converted rapidly to retinoids and other polar metabolites.

An interesting aspect of this study is the demonstration that the addition of citral to the β -C micellar solution resulted in a 47% decrease in the RA concentration in the intestine and a 36% decrease in the RA concentration in the portal blood after 2 h perfusion, as compared to the perfusion of β -C alone. This finding suggests that after perfusion of β -C, less than 50% of the RA is formed via oxidation of retinaldehyde (i.e., via central cleavage). However, after perfusion, the net increase from baseline in RA in the liver was 73% lower when citral was added to the β -C micellar solution versus when citral was not added to the β -C micellar solution; in the portal blood, after 60 and 90 min perfusion, the net increase of RA was about 60% lower with the perfusion of citral versus without the perfusion of citral, suggesting that more than 60% of the RA is formed directly via oxidation of retinaldehyde (i.e., via central cleavage). Because the data in the pilot study indicate that less than 10% of the decrease in RA concentration in the portal blood was due to citral alone, the decrease in RA concentration in the portal blood may overestimate the inhibition of RA formation from β -C when citral is added. It is not surprising that the results

obtained in the last blood sample and intestine taken just before the end of the experiment (after 120 min perfusion) are in close agreement, as the newly formed RA leaves the intestine via the portal route. On the other hand, the net increase of RA in the liver is a reflection of the flow of RA coming via the portal vein during the entire 2-h perfusion. Discrepancies between intestine and liver results can also be explained by limits on the capacity of absorption of RA from the intestine into blood and/or redistribution of the RA produced to other organs than the liver. Retinoic acid can also be further oxidized to degradation products or undergo glucuronidation.

Although the results of the present study do not allow us to determine with accuracy the exact importance of central versus excentric pathways in the metabolism of β -C, this study demonstrates for the first time *in vivo* that a substantial amount of RA is formed via a pathway that bypasses retinaldehyde oxidation, i.e., by excentric cleavage. It is possible that some retinaldehyde results from the degradation of the β -apocarotenals formed from an excentric pathway (Fig. 1) resulting in an underestimate of excentric cleavage. It has been shown that the incubation of β -apo-8'-carotenals or β -apo-12'-carotenals with human intestinal homogenates results in the production of retinaldehyde, but this pathway seems to be less important than the formation of RA *in vitro*, as the β -apo-carotenals produced 2.7- to 6.6-times more RA than retinaldehyde (21). Moreover, the addition of citral to β -apo-carotenol incubations decreased the formation of RA by only 14% (21). It is interesting to note the results of the work of Sharma, Mathur, and Ganguly (31), who evaluated the relative biopotencies of β -apo-carotenals compared with β -C. In a curative growth assay in the rat, the biopotencies were 72, 78,

and 72% of that for β -C on a molar basis for β -apo-8', 10', and 12'-carotenal, respectively. However, the relative amount of vitamin A in the liver for the β -apo-12'-carotenal was only 20% of that for β -C, suggesting that a large fraction of this β -apo-carotenal may be transformed into RA, while only a small fraction is ultimately reduced to the corresponding alcohol.

In summary, this study demonstrates unambiguously for the first time in vivo that an excentric cleavage mechanism of β -C metabolism is involved in the biosynthesis of RA in the ferret intestine. This excentric cleavage mechanism represents a quantitatively important pathway in the metabolism of β -C into RA. ■■

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REFERENCES

- Peto, R., R. Doll, J. D. Buckley, and M. B. Sporn. 1981. Can dietary β -carotene materially reduce human cancer rates? *Nature*. **290**: 201-208.
- Ziegler, R. G. 1991. Vegetables, fruits, and carotenoids and the risk of cancer. *Am. J. Clin. Nutr.* **53**: 251S-259S.
- Krinsky, N. I. 1991. Effects of carotenoids in cellular and animal systems. *Am. J. Clin. Nutr.* **53**: 238S-246S.
- Burton, G. W., and K. U. Ingold. 1984. β -Carotene: an unusual type of lipid antioxidant. *Science*. **244**: 569-573.
- Strickland, S., and V. Mahdavi. 1978. The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell*. **15**: 393-403.
- Chen, Z. X., Y. Q. Xue, R. F. Tao, X. M. Xia, C. Li, W. Y. Zu, X. Z. Yao, and B. J. Ling. 1991. A clinical and experimental study of all-trans retinoic acid treated acute promyelocytic leukemia patients. *Blood*. **78**: 1413-1419.
- Warrell, R. P., S. R. Frankel, W. H. Miller, D. A. Scheinberg, L. M. Itri, W. N. Hittelman, R. Vyas, M. Andreeff, A. Tafuri, A. Jakubowski, J. Gabrilove, M. S. Gordon, and E. Dmitrovsky. 1991. Differentiation therapy of acute

- promyelocytic leukemia with tretinoin (all-trans-retinoic acid). *N. Engl. J. Med.* **324**: 1385-1393.
- Heyman, R. A., D. J. Mangelsdorf, J. A. Dyck, R. B. Stein, G. Eichele, R. M. Evans, and C. Thaller. 1992. 9-Cis-retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell*. **68**: 397-406.
- Petkovich, M., N. J. Brand, A. Krust, and P. Chambon. 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature*. **330**: 444-450.
- Crain, F. D., F. J. Lotspeich, and R. F. Krause. 1967. Biosynthesis of retinoic acid by intestinal enzymes of the rat. *J. Lipid Res.* **8**: 249-254.
- Napoli, J. L., and K. R. Race. 1988. Biogenesis of retinoic acid from β -carotene. Differences between the metabolism of β -carotene and retinal. *J. Biol. Chem.* **263**: 17372-17377.
- Folman, Y., R. M. Russell, G. Tang, and G. Wolf. 1989. Rabbits fed on β -carotene have higher serum levels of all-trans retinoic acid than those receiving no β -carotene. *Br. J. Nutr.* **62**: 195-201.
- Wang, X. D., R. M. Russell, R. P. Marini, G. Tang, G. G. Dolnikowski, J. G. Fox, and N. I. Krinsky. 1993. Intestinal perfusion of β -carotene in the ferret raises retinoic acid level in portal blood. *Biochim. Biophys. Acta.* **1167**: 159-164.
- De Leenheer, A. P., W. E. Lambert, and I. Claeys. 1982. All-trans retinoic acid: measurement of reference value in human serum by high performance liquid chromatography. *J. Lipid Res.* **23**: 1362-1367.
- Wang, X. D., N. I. Krinsky, P. N. Benotti, and R. M. Russell. 1994. Biosynthesis of 9-cis-retinoic acid from 9-cis- β -carotene in human intestinal mucosa in vitro. *Arch. Biochem. Biophys.* **313**: 152-155.
- Hébuterne, X., X. D. Wang, E. J. Johnson, N. I. Krinsky, and R. M. Russell. 1995. Intestinal absorption and metabolism of 9-cis- β -carotene in vivo: biosynthesis of 9-cis-retinoic acid. *J. Lipid Res.* **36**: 1264-1273.
- Ganguly, J., and P. S. Sastry. 1985. Mechanism of conversion of β -carotene into vitamin A: central cleavage versus random cleavage. *World Rev. Nutr. Diet.* **45**: 198-220.
- Krinsky, N. I., X. D. Wang, G. Tang, and R. M. Russell. 1993. Conversion of carotenoids to retinoids. In *Retinoids: Progress in Research and Clinical Applications*. M. Dekker, M. A. Livrea and L. Packer, editors. New York, Basel, Hong-Kong, 1-16.
- Connor, M. J., and M. H. Smit. 1987. Terminal group oxidation of retinol by mouse epidermis. Inhibition in vitro and in vivo. *Biochem. J.* **244**: 489-492.
- Connor, M. J. 1988. Oxidation of retinol to retinoic acid as a requirement for biological activity in mouse epidermis. *Cancer Res.* **48**: 7038-7040.
- Wang, X. D., N. I. Krinsky, G. Tang, and R. M. Russell. 1992. Retinoic acid can be produced from excentric cleavage of β -carotene in human intestinal mucosa. *Arch. Biochem. Biophys.* **293**: 298-304.
- Wang, X. D., N. I. Krinsky, R. P. Marini, G. Tang, J. Yu, R. Hurley, J. G. Fox, and R. M. Russell. 1992. Intestinal uptake and lymphatic absorption of β -carotene in ferrets: a model for human β -carotene metabolism. *Am. J. Physiol.* **263**: G480-G486.
- Tang, G., X. D. Wang, R. M. Russell, and N. I. Krinsky. 1991. Characterization of β -apo-13-carotenone and β -apo-14'-carotenal as enzymatic products of the excentric cleavage of β -carotene. *Biochemistry.* **30**: 9829-9834.

24. Wang, X. D., G. Tang, J. G. Fox, N. I. Krinsky, and R. M. Russell. 1991. Enzymatic conversion of β -carotene into β -apocarotenals and retinoids by human, monkey, ferret, and rat tissues. *Arch. Biochem. Biophys.* **285**: 8-16.
25. Karrer, P., R. Morf, and K. Schöpp. 1931. Zur kenntnis des vitamins-A aus fishtranen II. *Helv. Chim. Acta.* **14**: 1431-1436.
26. Hunter, R. F. 1946. The conversion of carotene into vitamin A. *Nature.* **158**: 257-260.
27. Goodman, D. S., and H. S. Huang. 1965. Biosynthesis of vitamin A with rat intestinal enzymes. *Science.* **149**: 879-880.
28. Olson, J. A., and O. Hayaishi. 1965. The enzymatic cleavage of β -carotene into vitamin A by soluble enzymes of rat liver and intestine. *Proc. Natl. Acad. Sci. USA.* **54**: 1364-1370.
29. Glover, J. 1960. The conversion of β -carotene to vitamin A. In *Vitamins and Hormones*. R. S. Harris and D. J. Ingle, editors. Academic Press, New York and London. **18**: 371-386.
30. Glover, J., and E. R. Redfearn. 1954. The mechanism of the transformation of β -carotene into vitamin A in vivo. *Biochem. J.* **158**: XV-XV.
31. Sharma, R. V., S. N. Mathur, and J. Ganguly. 1976. Studies of the relative biopotencies and intestinal absorption of different apo- β -carotenoids in rats and chickens. *Biochem. J.* **158**: 377-383.
32. Sharma, R. V., S. N. Mathur, A. A. Dimitrovskii, R. Das, and J. Ganguly. 1977. Studies on the metabolism of β -carotene and apo- β -carotenoids in rats and chickens. *Biochem. Biophys. Acta.* **486**: 183-194.
33. Olson, J. A. 1993. The irresistible fascination of carotenoids and vitamin A. *Am. J. Clin. Nutr.* **57**: 833-839.
34. Hansen, S., and W. Maret. 1988. Retinal is not formed in vitro by enzymatic central cleavage of β -carotene. *Biochemistry.* **27**: 200-206.
35. Lakshman, M. R., I. Mychkovsky, and M. Attlesey. 1989. Enzymatic conversion of all-*trans*- β -carotene to retinal by a cytosolic enzyme from rabbit and rat intestinal mucosa. *Proc. Natl. Acad. Sci. USA.* **86**: 9124-9128.
36. Schweigert, F. J., O. A. Ryder, W. A. Rambeck, and H. Zucker. 1990. The majority of vitamin A is transported as retinyl esters in the blood of most carnivores. *Comp. Biochem. Physiol.* **95A**: 573-578.