Biosynthesis of retinoic acid by intestinal enzymes of the rat

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ABSTRACT The incubation of β-carotene-^{14}C with the soluble fraction of the intestinal mucosa resulted in the formation of small amounts of acidic material. The addition of NAD or NADH to the soluble fraction caused a tenfold increase in this material. Incubation of retinal-15-^{14}C with the soluble fraction of the intestinal mucosa plus NAD or NADH resulted in the conversion of 80–90% of the retinal to acidic material, which has been shown to contain retinoic acid.

In vivo studies on the formation of retinoic acid in the intestinal mucosa after the administration of β-carotene-^{14}C revealed that an appreciable amount of β-carotene was converted to acidic compounds. When retinal-15-^{14}C was administered, portal blood contained 30–40% of the absorbed radioactivity. 24% of this radioactivity was found in acidic material, which has been shown to contain retinoic acid.

It is suggested that enzymes in rat intestine cleave β-carotene to retinal and oxidize the latter to retinoic acid, which is then transported via the portal circulation to the liver.

KEY WORDS β-carotene · cleavage · retinal · oxidation · retinoic acid · biosynthesis · intestinal mucosa · portal blood · metabolism · absorption · chromatography · rat

During the past 30 yr numerous studies have been conducted on the metabolism of β-carotene (1–4). It has been well established that certain mammalian tissues are capable of converting β-carotene to retinal. The mechanism of this process has been defined (3–5) but certain other metabolic fates of β-carotene need to be determined.

Recently Goodman and Huang (3) and Olson and Hayaishi (4) have demonstrated the cleavage of β-carotene at the 15, 15′ double bond, with the formation of two molecules of retinal, by enzymes of the soluble fraction of the intestinal mucosa and liver. The retinal formed from β-carotene was thought to be primarily converted to retinol and retinyl ester since these were the major products found in the intestinal mucosa, lymph, and liver of rats after the administration of β-carotene or retinal (6–10). In these tissues only minute amounts of acidic or polar materials were found. At present, no extensive study has been reported on the transport of β-carotene metabolites by the portal blood.

Various workers have demonstrated the conversion of retinal to retinoic acid in vitro by enzymes of the liver (11–14). Dunagin, Zachman, and Olson (15) have recently demonstrated the in vivo conversion of retinal to retinoic acid by the liver. They have shown also that retinoic acid was rapidly eliminated in the bile as retinoyl glucuronide.

Our previous studies on the metabolism of β-carotene suggested that β-carotene was metabolized to products other than retinol (16). In the present investigation evidence is presented to show that one of these products is retinoic acid.

EXPERIMENTAL PROCEDURE

Materials

Uniformly labeled β-carotene-^{14}C was prepared biosynthetically by the fungus *Phycomyces blakesleeanus* grown in the presence of sodium acetate-1-^{14}C according to the method of Lilly, Barnett, Krause, and Lotspeich (17). β-Carotene was isolated and purified from the harvested mycelium according to the procedure of Lotspeich and coworkers (18). The resulting β-carotene had $E^{1\%}_{1Cm}$ values in petroleum ether (bp 33–36°C) higher than 2600 at 448 mμ; the visible, UV and IR spectra were identical with those of a pure synthetic sample obtained from Hoffmann-La Roche, Inc., Nutley, N.J.

Retinal-15-^{14}C was prepared from retinol-15-^{14}C (a generous gift from Hoffmann-La Roche) by oxidation

with MnO_2 (19). This retinal was purified by chromatography over silicic acid that had been treated with NaOH and had an $E^{1\%}_{1cm}$ value in petroleum ether (bp 33–36°C) of 1534 at 370 mμ, which indicated a purity greater than 98%.

NaOH-treated silicic acid was prepared by mixing 200 g of silicic acid (325 mesh) (Bio-Rad Laboratories, Richmond, Calif.) with 1 liter of 0.1 N NaOH for 2 hr. After filtration with suction, the silicic acid was dried at 110°C for 24 hr. A slurry of 10 g of this silicic acid in 100 ml of distilled water had a pH of 9.0–9.5.

NAD, NADP, NADH, NADPH, and GSH were obtained from Sigma Chemical Company, St. Louis, Mo. All organic solvents except chloroform were purified by distillation. Other chemicals were reagent grade commercial products.

Techniques for In Vitro Studies

The techniques employed in these studies were essentially the same as those described by Goodman and Huang (3). The mucosa of the upper half of the small intestine of male albino rats of the Wistar strain weighing 175–250 g was homogenized with a loose fitting glass pestle in a Dounce homogenizer with 8 volumes of cold 0.1 M phosphate buffer (8 ml of buffer per g of mucosa), pH 7.7, which was 30 mM in nicotinamide and 4 mM in $MgCl_2$. The particulate and soluble fractions were separated as described by Shelton, Krause, and Gross (20). The resulting soluble fraction contained approximately 100 mg of protein per 12 ml. 72 μmoles of sodium taurocholate and 60 μmoles of reduced glutathione were added to 12 ml of the soluble fraction. This solution made up the basic incubation medium and will be referred to hereafter as the soluble fraction.

12 ml of the soluble fraction was distributed equally into six 25-ml flasks and 1 μg of β-carotene or retinal in 50 μl of acetone was added to each flask. These samples were incubated at 37°C for 2 hr in an atmosphere of 95% O_2–5% CO_2. At the end of the experimental period the samples were combined and extracted with chloroform–methanol containing 10 mg of α-tocopherol as described by Goodman and Huang (3). In our hands the addition of α-tocopherol was as effective as the addition of unlabeled carrier compounds in protecting various retinol derivatives against oxidation.

Techniques for In Vivo Studies

Male albino rats of the Wistar strain weighing 175–250 g were anesthetized with ether and a midline abdominal incision was made. An intestinal loop was formed by ligation of the small intestine at the pyloris and 12 inches below the ligament of Treitz. β-Carotene, solubilized in either 0.5 ml of corn oil or 1.5 ml of 5% Tween 80 (polyoxyethylene Sorbitan monooleate), was injected into this intestinal loop. The abdominal cavity was closed and the rat was allowed to recover from anesthesia. At the end of the experimental period the rat was again anesthetized with ether and the intestinal loop and liver were removed. The intestinal lumen was flushed twice with 10-ml portions of isotonic saline and then cut lengthwise, and the mucosal surface was rinsed with another 10 ml portion of isotonic saline.

Portal blood was collected continuously for 1–2 hr after the administration of retinal by an exchange transfusion technique, as follows. An untied suture was placed around the portal vein about 1 cm distal to its entrance into the liver and another untied suture was placed 1 cm distal to the first suture. The inferior vena cava was then exposed and a 20 gauge needle (from a pediatric scalp vein intravenous set) was inserted into the inferior vena cava. The first suture around the portal vein was tied and a second 20 gauge needle was inserted into the portal vein (directed toward the small intestine) and through the second suture around the portal vein. This suture was tightly tied around the needle. Blood was collected from the portal vein at a rate of 2 ml/min and freshly drawn heparinized blood from a rat donor was infused into the inferior vena cava at a similar rate.

From all objective signs such as rate of respiration, heart rate, peristalsis, and gross appearance of the intestines, the animals appeared to be tolerating the experimental procedures very well. Of course it must be acknowledged that the animals were not in a perfectly normal physiological state.

The liver, intestinal mucosa, intestinal rinsings, and portal blood were extracted in a Waring Blendor with chloroform–methanol that contained 10 mg of α-tocopherol, according to the procedure of Bligh and Dyer (21).

Chromatography of Lipid Extracts

The lipid extracts were chromatographed over 18 g of NaOH-treated silicic acid by the method of Hirsch and Ahrens (22). The solvents used to elute the various chromatographic fractions, and the carotenoid derivative eluted in each fraction, were as follows: I, β-carotene (75 ml of 1% diethyl ether–hexane 1:99); II, retinyl ester (275 ml of diethyl ether–hexane 1:99); III, retinal (300 ml of diethyl ether–hexane 4:96); IV, retinol (300 ml of diethyl ether); and V, retinoic acid (200 ml of methanol).

The efficiency of the separation of these compounds was determined by placing a mixture of intestinal lipids (50–100 mg) and the ^{14}C-labeled carotenoid derivative (10–500 μg) on the column and eluting as described. More than 90% of the ^{14}C-labeled compound recovered from the column was in the expected fraction. The total recovery of radioactivity from the column was in all cases greater than 90%, except for retinoic acid where the recovery was about 60%.

The absolute recovery of radioactivity in each incubation listed in Tables 1–4 (compared to total activity added as substrate) after extraction and chromatography was >90%, except when retinoic acid was formed. The recovery was then about 80%.

Radioactivity Analysis

Samples were dissolved in 10 ml of toluene scintillation fluid (4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxalolyl)]benzene per liter of toluene) and were counted in the Packard liquid scintillation spectrometer model 3324. Quenching due to the yellow color of the carotenoids was corrected for by the use of internal standards.

RESULTS

In Vitro Experiments

Formation of Retinoic Acid by Enzymes of the Soluble Fraction of Intestinal Mucosa. The distribution of radioactivity in the various chromatographic fractions after a 2 hr incubation of 6 μg of β-carotene-^{14}C either with the soluble fraction alone or with the addition of reduced NAD or NADP is shown in Table 1. When β-carotene was incubated with the soluble fraction, 65.5% of the total radioactivity recovered from the column was found in the retinal fraction (III) while only small amounts of radioactivity were present in the retinyl ester (II), retinol (IV), and retinoic acid fractions (V). From these data one can calculate that 90% of the radioactivity that was *not* in carotene was in the retinal fraction (III). The enzymatic activity of the soluble fraction was destroyed when the fraction was heated at 65°C for 1 min. The addition of the particulate fraction to the soluble fraction did not increase the enzymatic activity of the soluble fraction nor did it change the distribution of radioactivity in any of the chromatographic fractions.

Since the soluble fraction contains alcohol dehydrogenases, one would expect it to reduce retinal to retinol. In an attempt to demonstrate this reduction, we added 1 μmole of either NADH or NADPH to the soluble fraction. The addition of reduced NAD showed no increase in the amount of radioactivity in the retinol fraction (IV); instead, there was tenfold increase in the amount of radioactivity in the retinoic acid fraction (V) with an equivalent decrease in the amount of radioactivity in the retinal fraction (III). The addition of reduced NADP to the soluble fraction gave similar results, but of a much lesser magnitude.

TABLE 1 CONVERSION OF β-CAROTENE TO RETINAL AND RETINOIC ACID BY ENZYMES OF THE SOLUBLE FRACTION OF INTESTINAL MUCOSA

6 μg of β-carotene (6500 cpm) was incubated with the soluble fraction (S.F.) as described in Experimental Procedure. Where indicated, 1 μmole of NADH or NADPH was added to the soluble fraction.

Chromatographic Fraction*	Incubation Media			
	S.F.	S.F. (Heated) †	S.F. Plus NADH	S.F. Plus NADPH
	% of total ^{14}C eluted from column			
I	23.6	94.5	20.5	28.3
II	1.0	2.4	3.1	0.9
III	65.5	0.8	27.5	54.4
IV	5.4	1.3	6.6	6.9
V	4.5	1.0	42.3	9.5

* Chromatographic fractions I, II, III, IV, and V have been shown to contain β-carotene, retinyl ester, retinal, retinol, and retinoic acid, respectively.

† Soluble fraction heated at 65 °C for 1 min.

Identification of Material in the Retinoic Acid Fraction. The lipid material eluted from silicic acid with methanol was dissolved in *n*-hexane, and extracted with 0.1 N NaOH. The NaOH solution was acidified and extracted with *n*-hexane. 90% of the radioactivity present in the methanol eluate was extractable by base. This indicated the acidic nature of this radioactive material. The absorption spectrum of the acidic material was obtained in a Beckman DB recording spectrophotometer with an extract of the control as a blank. Presumably the control extract (which contained no added retinoic acid) corrected for all substances in the acidic material that were not derived from carotene. Since the spectrum of the acidic material and that of pure retinoic acid are superimposable it seems that the acidic material contains retinoic acid.

Additional evidence indicating that the acidic material contained retinoic acid derived from β-carotene was obtained from gas–liquid chromatography. The techniques used to detect retinoic acid were those described by Dunagin and Olson (23). A comparison between retention times of a standard methyl retinoate and the methyl ester derivatives of the acidic material demonstrated that retinoic acid was present. The acidic fraction was collected in vials containing siliconized anthracene and counted in a Packard Tri-Carb scintillation spectrometer. 40% of the total count was present as radioactive methyl retinoate.

Cofactor Requirements for Conversion of Retinal to Retinoic Acid. The cofactor requirements for the conversion of retinal to retinoic acid were studied further with retinal-15-^{14}C as the substrate. Typical results are shown in Table 2. Both the reduced and oxidized forms of NAD stimulated the formation of retinoic acid to the same degree. The incubation of the soluble fraction with retinal and reduced NAD in the absence of molecular oxygen, reduced glutathione, or sodium taurocholate did not impair the formation of retinoic acid. Thus, there was no apparent requirement for these factors in this conversion process. The enzymatic activity of the soluble fraction

TABLE 2 COFACTOR REQUIREMENTS FOR CONVERSION OF RETINAL TO RETINOIC ACID BY ENZYMES OF THE SOLUBLE FRACTION OF INTESTINAL MUCOSA

12 μg of retinal-15-^{14}C (8400 cpm) was incubated with the soluble fraction (S.F.) as described in Experimental Procedure. Where indicated, 1 μmole of either NAD or NADH was added to the soluble fraction.

	Incubation Media			
Chromatographic Fraction*	S.F. Plus NADH (Heated)†	S.F.	S.F. Plus NADH	S.F. Plus NAD
	% of total ^{14}C eluted from column			
II	1.8	2.1	2.9	2.5
III	86.6	70.9	29.7	34.6
IV	6.4	9.4	6.0	6.8
V	5.2	17.6	61.4	56.1

* See footnote* to Table 1.
† Soluble fraction heated at 65 °C for 1 min.

TABLE 3 DISTRIBUTION OF RADIOACTIVITY IN INTESTINAL MUCOSA AND LIVER AFTER INTRADUODENAL ADMINISTRATION OF β-CAROTENE TO RATS

β-Carotene (1.94 × 10^5 cpm in 200 μg) was injected into a ligated intestinal loop in 0.5 ml of corn oil. Lipid extracts of the intestinal mucosa and liver contained 12,800 and 4150 cpm respectively. Experimental period, 2 hr.

Chromatographic Fraction*	Intestinal Mucosa	Liver
	% of total ^{14}C eluted from column	
I	28.6	3.1
II	44.5	80.5
III	0.8	0.9
IV	9.4	11.2
V	16.7	4.3

* See footnote* to Table 1.

was destroyed when the fraction was heated at 65 °C for 1 min.

In Vivo Experiments

Appearance of Products Formed from β-Carotene in the Intestinal Mucosa. The distribution of radioactivity in the various chromatographic fractions in the intestinal mucosa and liver 2 hr after the intraduodenal injection of 200 μg of β-carotene-^{14}C is shown in Table 3. The retinyl ester fraction (II) contained the majority of radioactivity in both the intestinal mucosa and liver. However, appreciable amounts of radioactivity were found in the acidic fraction (V) of the intestinal mucosa. Subcellular fractionation of the intestinal mucosa showed that this radioactive material was associated with the soluble fraction. The radioactive material in the acidic fraction (V) was not extractable from *n*-hexane with 0.1 N NaOH. The radioactive material was soluble in dilute NaOH after saponification with alcoholic KOH. At the present time, this material has not been identified.

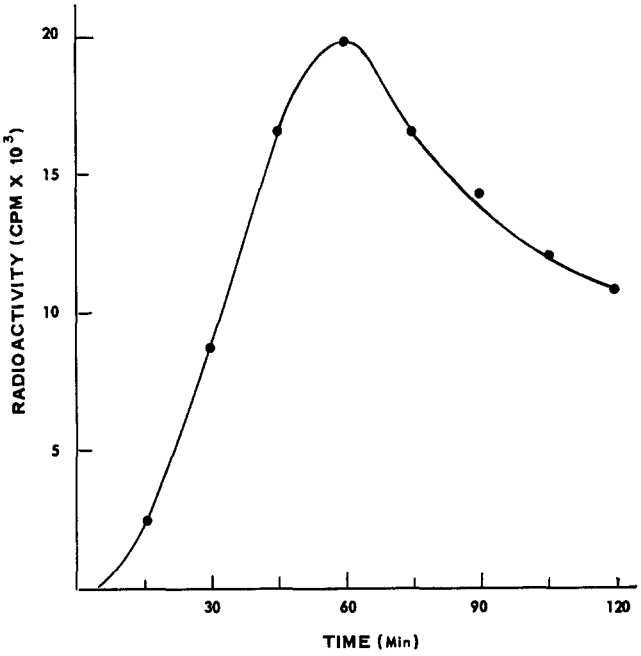

FIG. 1. Appearance of radioactivity in the portal blood at various times after the intraduodenal administration of 1 mg of retinal-15-^{14}C (7.0 × 10^6 cpm) to a rat. 40 ml of blood was extracted for each point on the graph.

Transport of Products from Retinal by Portal Blood. The amount of radioactivity appearing in the portal blood over a 2 hr period after the intraduodenal injection of 1 mg of retinal-15-^{14}C is shown graphically in Fig. 1. Appreciable amounts of radioactivity were found in the portal blood within 15 min and maximum intestinal absorption was obtained 1 hr after the administration of retinal. The total radioactivity transported by the portal blood accounted for 39% of the absorbed dose (total dose administered minus amount recovered in intestine). The percentage distribution of radioactivity in the portal blood is shown in Table 4. It may be noted that all of the chromatographic fractions contained appreciable amounts of radioactivity, the retinal fraction (III) having the most. 88% of the radioactivity in the acidic fraction

TABLE 4 DISTRIBUTION OF RADIOACTIVITY IN PORTAL BLOOD AFTER INTRADUODENAL ADMINISTRATION OF RETINAL TO A RAT

1 mg of retinal-15-^{14}C (7.0 × 10^6 cpm) was injected into a ligated intestinal loop in 1.5 ml of 5% Tween 80. Portal blood was collected over a period of 2 hr as described in Experimental Procedure. The lipid extract of the portal blood contained 1.0 x 10^6 cpm.

Chromatographic Fraction*	Percentage of Total ^{14}C Eluted from Column
II	19.3
III	34.6
IV	21.7
V	24.4

* See footnote* to Table 1.

(V) was extractable from *n*-hexane with 0.1 N NaOH. An absorption curve was obtained on this acidic material as previously described for the identification of retinoic acid isolated in the in vitro studies. The spectrum of this acidic material and that of pure retinoic acid closely resembled each other; however, the isolated acidic material had a peak at 355 mμ while that of pure retinoic acid was at 361 mμ. In view of the limitations of this technique, it would appear reasonable to conclude that this acidic material is retinoic acid.

DISCUSSION

Our previous studies on the metabolism of β-carotene suggested that β-carotene was metabolized to products other than retinol (16). The results of the present investigation demonstrate the presence in the intestinal mucosa of the rat of an enzyme system or systems capable of the degradation of β-carotene to retinoic acid.

When β-carotene was incubated with the soluble fraction of the intestinal mucosa, 90% of the β-carotene degraded was found in the retinal fraction (III). Only small amounts were found in the retinol (IV) and retinoic acid fractions (V). These findings agree with the in vitro observations of Goodman and coworkers (3, 5) and Olson and Hayaishi (4) that β-carotene is first cleaved at the 15, 15′ double bond to yield two molecules of retinal. Glover, Goodwin, and Morton (6) have shown in vivo that retinal is rapidly reduced to retinol by the intestinal mucosa. In our attempt to demonstrate this reduction by our in vitro system we used reduced NAD since Bliss (24) has shown that this reduction is mediated by an NAD-dependent alcohol dehydrogenase in the soluble fraction of the liver. We were surprised to find that the addition of either the oxidized or the reduced form of NAD to the soluble fraction of the intestinal mucosa did not increase conversion of retinal to retinol but instead produced a tenfold increase in the conversion of retinal to retinoic acid. This conversion of retinal to retinoic acid apparently has no requirement for molecular oxygen. The enzyme may be similar to the retinal oxidase isolated from liver by Mahadevan, Murthy, and Ganguly (11). Further purification will be necessary before this enzyme can be characterized.

The conversion of retinal to retinoic acid in vivo may be questioned since only small amounts of retinoic acid are found in tissues and lymph after the administration of β-carotene or retinal (7, 8, 10). Evidence that the conversion of retinal to retinoic acid does occur in vivo was obtained in this study by collection of portal blood after the administration of retinal-15-^{14}C to a rat. Approximately 39% of the absorbed radioactivity was found in the portal blood and 24% of this radioactivity was found in the retinoic acid fraction (V). Additional support for this conversion was the finding of an appreciable amount of radioactivity in the retinoic acid fraction (V) of the intestinal mucosa after the administration of β-carotene-^{14}C to rats. This radioactive material was not extractable with base. However, saponification of this fraction resulted in the release of an acidic compound(s). We have been unable to identify this polar material since only small quantities can be isolated from the intestinal mucosa and also since the material is contaminated with relatively large quantities of phospholipids. However, this polar material may be related in some manner to several metabolic products of retinoic acid reported in the literature. Zile and DeLuca (25) have isolated a metabolite of retinoic acid from livers of rats fed retinoic acid which supports growth in rats as well as retinoic acid. This metabolite released an acidic group on saponification, which suggested that it may be an ester of retinoic acid. Lippel and Olson (26) have recently demonstrated the biosynthesis of retinoyl glucuronide by the microsomes of the intestine and liver.

The results presented in this report suggest that β-carotene is cleaved by intestinal enzymes at the 15, 15′ double bond with the formation of two molecules of retinal. Some of the retinal in the intestinal mucosa may then be oxidized to retinoic acid, which is transported to the liver via portal blood and conjugated with glucuronic acid and rapidly eliminated in the bile (27).

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