THE ENZYMATIC CLEAVAGE OF β-CAROTENE INTO VITAMIN A BY SOLUBLE ENZYMES OF RAT LIVER AND INTESTINE*

BY JAMES ALLEN OLSON† AND OSAMU HAYAISHI

DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF MEDICINE, UNIVERSITY OF FLORIDA, GAINESVILLE, AND DEPARTMENT OF MEDICAL CHEMISTRY, KYOTO UNIVERSITY FACULTY OF MEDICINE, KYOTO, JAPAN

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That β-carotene can serve as a precursor of vitamin A in mammals was demonstrated almost 40 years ago.[1, 2] In spite of innumerable nutritional and chemical studies on this reaction, however, neither the pathway nor the mechanism of vitamin A formation has been clarified.[3–5] With respect to the pathway of vitamin A formation, two major hypotheses have been suggested: (1) carotene is cleaved at the central 15-15′ double bond to yield two molecules of vitamin A, and (2) carotene is cleaved peripherally to yield one molecule of vitamin A via a series of β-apo-carotenals.[4] Although β-apo-carotenals have been found in nature,[6] are highly effective biologically, and are converted to vitamin A in the mammal,[4] they do not accumulate during the cleavage of β-carotene *in vivo*.[7]

The cleavage of β-carotene to vitamin A seemingly proceeds by an oxidative reaction. In isolated sections of intestine oxygen is required for the conversion of carotene to vitamin A,[8] a requirement which correlates better with the cleavage reaction than with the absorption of carotene into gut slices.[9] Furthermore, oxygen^{18} is incorporated into vitamin A in the liver of carotene-treated animals when O_2^{18} gas is used but not when H_2O^{18} is employed.[10] Purely chemical studies also accord with an oxidative cleavage mechanism.[11, 12] Although the intestine seems to be the major organ in the rat for carotene cleavage into vitamin A,[7, 13] the liver also catalyzes this reaction.[14] Interestingly, bile salts are necessary for the uptake and cleavage of carotene by gut slices,[7] but are not required for cleavage in the liver.[14]

In the present report an enzyme has been found in the supernatant solution of rat liver and intestine which converts β-carotene into retinal and retinol as its sole products. Oxygen is required for the reaction, and the immediate product is retinal. The enzyme is inhibited by sulfhydryl binding reagents and by ferrous-ion chelating agents. The enzyme has been tentatively designated as β-carotene 15-15′ oxygenase.

Materials and Methods.—Preparation of radioactive β-carotene: Sodium acetate 1-C^{14} was added to growing cultures of *Phycomyces blakesleeanus*, and the synthesized β-carotene was extracted and purified as previously described.[15, 16] The three times recrystallized compound was greater than 98% pure, as judged by two-dimensional chromatography on silica gel thin-layer plates, and had a specific activity of 6560 cpm per μg. The labeled β-carotene was stored in hexane in the presence of α-tocopherol in a red glass container in the cold. Just prior to each experiment a suitable quantity was purified through a small column of 6% water-deactivated alumina, dried in the dark under nitrogen, dissolved in 0.05 ml of 20% Tween 40 in distilled acetone, and made up to a suitable volume with 0.15 *M* tris-hydroxymethyl-aminomethane (Tris) buffer, pH 8.0.

Preparation of organ homogenates: Male or female rats of the Wistar strain weighing 150–300 gm were anesthetized with ether, opened by midline incision, and the liver or kidneys were quickly removed and washed in saline. The upper half of the intestine was also removed, cut longitudinally to expose the mucosal surface, and washed in cold saline. Each tissue was minced on a cold Petri plate and homogenized with a loose-fitting, motor-driven glass homogenizer in 5 vol of cold 0.15

M Tris buffer pH 8.0 which contained 0.1 *M* nicotinamide and 0.1 *M* cysteine. The resulting homogenate was centrifuged at 10,000 × *g* for 10 min in a Lourdes centrifuge in the cold, and the supernatant solution was subsequently centrifuged at 105,000 × *g* in a Spinco preparative ultracentrifuge or a Hitachi ultracentrifuge for 60 min at 0°. The resultant supernatant solution, which contained the carotene cleavage enzyme, was stored in the cold until used. Rat liver homogenate in 0.25 *M* sucrose was separated into fractions of nuclei, mitochondria, microsomes, and supernatant solution by the method of Schneider and Hogeboom.[17]

Chemicals: Hexane was distilled over potassium hydroxide, and reagent grade acetone was refluxed with permanganate and potassium carbonate and then distilled. para-Chloromercuribenzoate was crystallized before use. Other compounds were reagent grade products of the Nakarai Chemical Company in Kyoto, Japan.

Incubation procedure: The micellar solution of radioactive β-carotene, which usually contained 0.5–3.0 μg in 0.2 ml of Tris buffer, pH 8.0, was added to 5 ml of the supernatant solution. Flasks were gently rocked in the dark at 36° in air for 60 min.

Extraction and analysis of products: At the conclusion of the incubation period, 20 ml of distilled acetone, which contained 30 μg each of carrier β-carotene, retinal, and retinol, plus 50 μg of α-tocopherol, were added, followed by 20 ml of ether and 20 ml of water. Solutions were swirled, separated in a separatory funnel, and the aqueous layer was extracted again with 20 ml of acetone-ether (1:1). The combined lipid extract was dried over anhydrous sodium sulfate, reduced to a small volume in a roto-evaporator under nitrogen at less than 55°, and transferred to a small tube with ether. The aqueous emulsion was broken with anhydrous sodium sulfate. The ether extract, after drying over sodium sulfate, was chromatographed on thin-layer plates of silica gel G. Silica gel was buffered at pH 8.0 and the extracts were spotted under red light by use of a nitrogen stream. One-dimensional chromatography was carried out with ether-hexane (1:1) as a solvent, whereas two-dimensional chromatography was conducted initially with 5% acetone in hexane, followed by drying under nitrogen and then development in ether-hexane (1:1). All procedures from the initial incubation to the final separation on thin-layer chromatographic plates were conducted in the dark or under red light. Carotene, retinal, retinol, and retinyl ester were localized by brief exposure to a UV light (360 mμ), and appropriate areas of the silica gel plate were scraped into 1.5 ml of 10% ethanol in hexane, which contained 10 μg of α-tocopherol and a small drop of ammonia. After the silica gel was centrifuged down, aliquots of the supernatant solution were placed on aluminum planchets and counted in a Nuclear-Chicago thin-window counter under Geiger-Müller operation. When the given precautions were carefully observed, the over-all yield of radioactivity exceeded 85%. Under less stringent conditions the yield was 20–40%, and many artifacts appeared.

Reduction of retinol to retinal and the preparation of anhydroretinol: In order to characterize the products of β-carotene cleavage more effectively, retinal which was recovered from the thin-layer chromatographic plate was dried in the dark under nitrogen, dissolved in a small volume of absolute ethanol, and treated with several milligrams of solid $NABH_4$. After 1–2 hr in the dark at room temperature, the ethanolic solution was spotted on a thin-layer plate of silica gel and developed in ether-hexane (1:1). The retinol formed was eluted and analyzed. Retinol, which was formed biologically, was dehydrated to anhydroretinol by treatment with ethanolic 0.01 *N* HCl. When the optical density at 371 mμ reached a maximum (15–20 min), the solution was neutralized with a drop of ammonia and separated on a thin-layer plate in the dark. Anhydroretinol was visualized with UV light and eluted into 10% ethanol in hexane. Each solution was analyzed spectrophotometrically, and an aliquot was withdrawn for radioactivity measurements. The R_F values of various components in ether-hexane (1:1) are: β-carotene, 0.8; anhydroretinol, 0.75; natural retinyl ester, 0.65; all-*trans* retinal, 0.5; and all-*trans* retinol, 0.25. In 2% acetone in hexane the R_F values are: β-carotene, 0.75; natural retinyl ester, 0.57; retinal,0 .25; and retinol, 0.05.

Results.—Fractionation of rat liver: When a homogenate of rat liver was separated into conventional particulate fractions, the majority of the β-carotene cleavage activity was found in the supernatant solution, as seen in Table 1. The addition of microsomes to the supernatant solution did not enhance the activity of the soluble cleavage enzymes, and on occasion slightly inhibited it.

TABLE 1

DISTRIBUTION OF β-CAROTENE CLEAVAGE ENZYME IN PARTICULATE FRACTIONS OF RAT LIVER

Fraction	Amount of retinal + retinol formed (nanograms/0.5 gm wet liver)
Homogenate	124
Nuclei	20
Mitochondria	21
Microsomes	14
Supernatant	40, 71
Microsomes + supernatant	57

TABLE 2

FORMATION OF PRODUCTS FROM β-CAROTENE (1.2 μg) BY RAT LIVER SUPERNATANT ENZYME

Compound	Amount formed (nanograms)
Retinal	48
Retinol	22
Retinyl ester	5
Origin	<8
Acidic extract	2

Identification of products of β-carotene cleavage: When the supernatant solution of rat liver homogenates was employed as the enzyme, the major products obtained by two-dimensional chromatography on thin-layer plates were retinal and retinol, as shown in Table 2. Neither retinyl ester nor more polar derivatives at the origin contained appreciable quantities of radioactivity. Furthermore, other areas of the thin-layer chromatographic plate contained little or no radioactive products. Acidic materials, which were extracted from the aqueous solution after removal of nonpolar ones, were also devoid of radioactivity (Table 2). The major products, retinal and retinol, were characterized in several ways. Upon treatment of retinal with sodium borohydride and rechromatography on thin-layer plates, over 70 per cent of the radioactivity migrated with a newly formed retinol. In addition, retinol with a specific activity of 0.193 cpm per μmole could be converted to anhydroretinol with a specific activity of 0.229 cpm per μmole. If correction was made for a small amount of radioactive material which migrated with retinol but was not dehydrated by acid, the initial specific activity of the retinol was calculated to be 0.215 cpm per μmole. In the above experiments carrier retinal and retinol were used to isolate the radioactive products. When 20 μg β-carotene was incubated with the supernatant solution, however, the net synthesis of retinol from carotene could be demonstrated (Fig. 1). The difference spectrum corresponds closely with that of all-*trans* retinol, and little absorption occurs above 350 mμ. Thus, intermediates or contaminants with longer conjugated chains, such as the β-apo-carotenols, are either absent, or are present only in minute amounts.

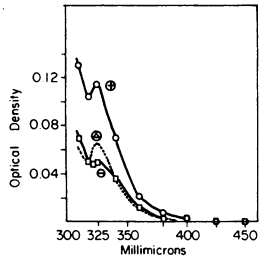

FIG. 1.—Spectrum of the retinol fractions eluted from thin-layer plates of silica gel G after incubation of the enzyme with (+) and without (−) 20 μg of β-carotene. The difference spectrum is indicated with a dotted line.

Evidence for the formation of retinal as a primary product of carotene cleavage: Invariably both retinal and retinol were formed as products of β-carotene cleavage in the rat liver supernatant system. Since the rat liver supernatant solution contains alcohol dehydrogenase and NAD, the interconversion of retinal and retinol was expected. That retinal is the initial product of carotene cleavage was favored by a number of observations. For example, when the enzyme solution was diluted, which would also dilute the concentration of NAD and alcohol dehydrogenase, the ratio of retinal to retinol increased markedly (Fig. 2). The retinal to retinol ratio was also increased by omitting nicotinamide from the homogenizing solution, by use

of a 20–70 per cent ammonium sulfate fraction of the supernatant solution, and by the addition of 0.017 *M* hydroxylamine to the medium. At this concentration hydroxylamine inhibits alcohol dehydrogenase, but has no effect on the total synthesis of retinal plus retinol from β-carotene. In addition, when semicarbazide was added to the medium, 90 per cent of the radioactivity in synthesized products appeared in the semicarbazone of retinal.

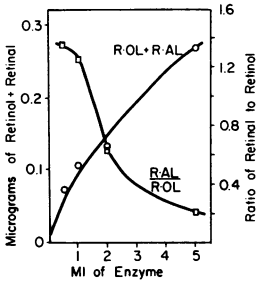

FIG. 2.—Effect of enzyme concentration on the formation of retinol + retinal from β-carotene and on the ratio of retinal to retinol.

The influence of the β-carotene concentration on the formation of retinal plus retinol: When the concentration of β-carotene was varied from 1 to 30 μg per 5 ml of incubation solution, the rate of product synthesis increased and then leveled off, as shown in Figure 3.

Requirement for molecular oxygen: In keeping with earlier studies with intestinal slices,[8, 9] oxygen was found to be essential for β-carotene cleavage (Table 3). Multiple evacuation alone caused a slight decrease in the formation of retinal plus retinol, perhaps due to foaming, but essentially no cleavage took place in the absence of oxygen. The addition of methylene blue to the anaerobic incubation mixture did not stimulate appreciably the synthesis of retinal.

Effective inhibitors of β-carotene cleavage: para-Chloromercuribenzoate and silver ions strongly inhibited the enzyme in the absence of cysteine, but had no effect when 0.1 *M* cysteine was present (Table 4). After preincubation with the enzyme for 20–100 min, the chelating agents ethylenediaminetetraacetate, ortho-phenanthroline, and α,α′-dipyridyl inhibited the formation of products by about 50 per cent. Cyanide, on the other hand, stimulated the activity slightly at 10^{-4} *M* and inhibited by about 40 per cent at 0.013 *M* (Table 4).

Stability of the liver enzyme: The enzyme in the supernatant solution of rat liver may be frozen and stored at −20°, or frozen and thawed three to five times in rapid succession, without extensive loss of activity. Although ammonium sulfate itself is innocuous, freezing and thawing in its presence causes inactivation. The enzyme is also inactivated by incubation with aqueous acetone, conditions which stabilize metapyrocatechase.[18]

Effect of glycocholic acid on the activity of enzymes from the intestine and kidney: Because of the known dependence of carotene cleavage on glycocholic acid in intestinal sections,[8, 15] supernatant solutions of homogenates prepared from the intestine and kidney were studied in the presence and absence of glycocholic acid. As seen in Table 5, glycocholic acid had no effect on the cleavage of β-carotene into vitamin A by either of these tissues. At a given β-carotene concentration the overall activities of supernatant solutions of gut and liver were roughly comparable, whereas the kidney enzyme was much less active.

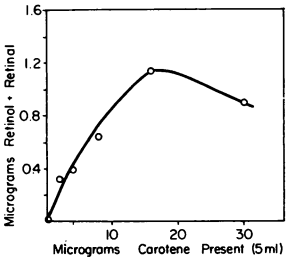

FIG. 3.—Effect of β-carotene concentration on the synthesis of retinal + retinol.

Discussion.—The cleavage of β-carotene is catalyzed by enzymes in the cytoplasmic fraction of rat liver and intestine. The primary product of this reaction

TABLE 3

REQUIREMENT FOR OXYGEN IN β-CAROTENE CLEAVAGE

Evacuation	Gas phase	Amount of retinol + retinal (nanograms)
None	Air	70
10 ×	Air	47
10 ×	Nitrogen	8

Enzyme solutions were evacuated 10 times in Thunberg tubes and then refilled 9 times with nitrogen. After the 10th evacuation, one tube was filled with air, and the other with nitrogen. The substrate was added and the solutions were incubated under normal conditions.

TABLE 4

EFFECT OF INHIBITORS ON THE FORMATION OF VITAMIN A FROM β-CAROTENE

Expt.	Compounds added	Preincubation time (min)	Amount of retinol + retinal (nanograms)
I	None	20	66
	8×10^{-4} *M* p-Chloromercuribenzoate	20	17
	1.7×10^{-3} *M* $AgNO_3$	20	<10
	0.017 *M* Ethylenediaminetetraacetate	20	47
II	None	110	107
	10^{-3} *M* o-Phenanthroline	110	54
	10^{-3} *M* α,α'-Dipyridyl	110	61
III	None	—	184
	10^{-4} *M* Cyanide	—	226
	10^{-3} *M* Cyanide	—	132
	0.013 *M* Cyanide	—	107

seems to be retinal, which is converted in part to retinol in the presence of endogenous alcohol dehydrogenase and NADH. Retinyl ester is not formed in appreciable quantities by the supernatant enzyme, in keeping with Futterman's observation that the esterification of vitamin A in liver occurs largely in the microsomal fraction.[19] Furthermore, few acidic and polar products are formed, in spite of the fact that aldehyde dehydrogenase, an enzyme in liver which may convert retinal to retinoic acid, is present in the supernatant solution.[20] Other possible breakdown products of β-carotene, such as the β-apo-carotenols and carotenals, were not detected in the present study. Although these results do not exclude the possibility that stepwise cleavage of carotene may occur in rat liver, the central cleavage of β-carotene to yield two molecules of retinal is clearly the major reaction.

The enzyme, although not yet purified and characterized in detail, behaves like an oxygenase.[21] The enzyme requires molecular oxygen and is inhibited by agents which bind ferrous iron. Cyanide, which strongly inhibits ferric protoporphyrin enzymes at low concentrations, is at best a mild inhibitor. Although the stoichiometry of oxygen utilization has not yet been established, this reaction may well proceed by a mechanism similar to that postulated for metapyrocatechase and pyrocatechase[21] (Fig. 4).

After the completion of the work described in this paper, Goodman and Huang reported the presence of an enzyme in rat intestine which converts β-carotene into vitamin A.[22] Our major findings with the liver enzyme are in close agreement with

TABLE 5

EFFECT OF GLYCOCHOLATE ON THE CLEAVAGE OF β-CAROTENE

Tissue	Initial carotene concentration (μg)	Amount of Retinol + Retinal Formed (nanograms)	
		No glycocholate	0.007 *M* glycocholate
Intestine	0.46	22.4	24.7, 17.4
Kidney	0.46	3.7	3.7

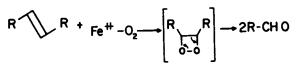

FIG. 4.—Suggested mechanism for β-carotene cleavage. *R* denotes the C-19 substituent adjacent to the central double bond.

theirs, namely, that retinal is the primary product of carotene cleavage, that molecular oxygen is required, that the enzyme is present in the high-speed supernatant solution, and finally that the enzyme contains sulfhydryl groups essential for activity. On the other hand, Goodman and Huang also reported that both bile salts and a lipid fraction of microsomes were required for the activity of the intestinal enzyme. In the present investigation, neither particulate fractions nor bile salts were essential for retinal formation, nor indeed did they stimulate it. These minor discrepancies might well be due to the manner in which carotene was added to the enzyme system. In the present investigation micellar solutions of carotene in Tween 40 were employed, whereas Goodman and Huang added acetone solutions of carotene directly to their enzyme preparations. If indeed a micellar solution is required for enzyme action, bile salts and particulate lipid might serve to disperse the carotene in a more suitable form for enzyme action.

Summary.—Radioactive β-carotene suspended in a micellar solution in Tween 40 is converted to retinal and retinol by a high-speed supernatant solution of rat liver and intestine. The first product of the reaction seems to be retinal, which was characterized by reduction to retinol and then dehydration to anhydroretinol. β-Apo-carotenols and carotenals were not detected in the incubation solution. The enzyme is inhibited by sulfhydryl binding agents, protected by cysteine, and inhibited after preincubation with α,α′-dipyridyl, ortho-phenanthroline, and ethylenediaminetetraacetate. Cyanide did not inhibit appreciably at concentrations which completely inactivate ferric protoporphyrin enzymes. Oxygen is required for the reaction. Bile salts, although essential for the uptake and cleavage of β-carotene into retinyl ester by intestinal slices *in vivo* and *in vitro*, are not required for the cleavage reaction in intestinal homogenates. In view of the properties of the enzyme, it has tentatively been termed β-carotene 15-15′ oxygenase.

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*INCREASED TEMPLATE ACTIVITY OF LIVER CHROMATIN, A RESULT OF HYDROCORTISONE ADMINISTRATION**

BY MICHAEL E. DAHMUS AND JAMES BONNER

DIVISION OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY

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We have found that the administration of hydrocortisone to adrenalectomized rats increases the template activity of their liver chromatin for RNA synthesis. Such administration is known to cause a two- to threefold increase in rate of nuclear RNA synthesis in the liver.[1, 2] This increase is followed by an increase in the activities of a series of liver enzymes.[3–5] Since the induction of these enzymes by hydrocortisone is abolished by simultaneous treatment with actinomycin D, it is clear that new RNA synthesis is required to support their formation.[4, 5] The increased rate of liver RNA synthesis caused by administration of hydrocortisone might in principle be due to changes in the template activity of the liver genetic material such as would accompany derepression of genes previously repressed. We shall show below that the administration of hydrocortisone does result in an increased availability of the genetic material for transcription.

Materials and Methods.—Treatment of rats: Male albino Sprague-Dawley rats weighing 150–250 gm each were obtained in the bilaterally adrenalectomized condition from Berkeley Pacific Laboratories. Hydrocortisone A grade was obtained from Calbiochem.

Rats were fasted 18 hr prior to the beginning of the experiment. Hydrocortisone-treated rats were given an intraperitoneal injection of hydrocortisone, 5 mg/100 gm body weight, suspended in physiological saline. Control rats were injected with an equal volume of saline. Four hours after treatment the rats were sacrificed, their livers immediately removed and washed with cold saline. The livers were then frozen in dry ice and chipped into small pieces. Tissue from identical treatments (24 rats per treatment) was pooled and stored at −80°C.

Preparation of purified chromatin: Crude chromatin was prepared from 10-gm samples of frozen tissue by the procedures of Marushige and Bonner[6] with the modification that the tissue was homogenized in 0.05 *M* NaCl plus 0.016 *M* Na_2 EDTA (pH 8.0). The chromatin was purified by centrifugation through 1.7 *M* sucrose and dialyzed against two changes of 0.01 *M* tris, pH 8.0.

Preparation of deproteinized DNA: Protein was removed from the purified chromatin by centrifugation in 4 *M* CsCl according to the method of Huang and Bonner.[7] Samples were centrifuged at 35,000 rpm for 22 hr in a Spinco SW-39 rotor. The gelatinous DNA pellet was dissolved in 0.01 *M* tris, pH 8.0.

Preparation of RNA polymerase: RNA polymerase was prepared from early log phase cells of *E. coli* strain B (General Biochemicals) by the methods of Chamberlin and Berg[8] to the stage of their fraction 3, hereafter referred to as F_3.

Assay of template activity: The complete incubation mixture for RNA synthesis contained in a final volume of 0.25 ml: 10 μmoles tris buffer (pH 8.0), 1 μmole $MgCl_2$, 0.25 μmole $MnCl_2$, 3 μmoles β-mercaptoethanol, 0.05 μmole spermidine phosphate, 0.10 μmole each of CTP, UTP, and GTP, 0.10 μmole 8-C^{14}-ATP (spec. act. 1 μc/μmole), DNA or chromatin, and F_3. Samples were incubated at 37°C for 10 min. The reaction was then stopped by the addition of cold 10%