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ENZYMATIC CLEAVAGE OF CAROTENOIDS

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SUMMARY

1. Carotene 15,15′-dioxygenase (EC 1.13.11.21) has been isolated from the intestine of guinea pig and rabbit and purified 38- and 30-fold, respectively, by subjecting the intestinal homogenate to protamine sulfate treatment, $(NH_4)_2SO_4$ fractionation and acetone precipitation.

2. The guinea pig enzyme showed a pH optimum at 8.5, an optimum substrate concentration of 200 nmoles of β,β-carotene per 25 ml of reaction mixture, an apparent K_m with β,β-carotene as substrate of $9.5 \cdot 10^{-6}$ M and a V 3.3 nmoles of retinal formation/mg protein per h. The reaction was linear upto 3 h and the reaction rate increased linearly with increase in enzyme protein concentration. The enzyme was activated by GSH and Fe^{2+} and inhibited by sodium dodecylsulfate, sulfhydryl binding and iron chelating agents.

3. The reaction catalysed by guinea pig enzyme was strictly stoichiometric.

4. Rabbit enzyme showed a close similarity with guinea pig enzyme with respect to time course, optimum substrate concentration, activation by Fe^{2+} and GSH, inhibition by sodium dodecylsulfate, iron chelating and sulfhydryl binding agents. However, it showed a slightly lower pH optimum (pH 7.8).

5. The enzyme from guinea pig and rabbit showed remarkable similarity with respect to cleavage of carotenoids. The enzyme from both the species was more specific for β,β-carotene but could also cleave a number of other carotenoids at the 15,15′-double bond.

6. 10′-Apo-β-carotenal and 10′-apo-β-carotenol were readily cleaved compared with other apo-β-carotenals and apo-β-carotenols tested.

7. It has been conclusively shown for the first time that mono-ring substituted carotenoids are also cleaved at the 15,15′-double bond.

INTRODUCTION

The biosynthesis of retinal from β,β-carotene has been shown to be catalysed by a well-characterised enzyme called carotene 15,15′-dioxygenase (EC 1.13.11.21) [1–5]. Our detailed in vivo studies on the metabolism of apo-β-carotenals [6] and apo-β-carotenoic acids (Singh, H. and Cama, H. R., unpublished) clearly demon-

Abbreviation: *p*Cl-HgBzO$^-$, *p*-chloromercuribenzoate.

strate that these apo-carotenoids are biologically active and can be converted into vitamin A. Lakshmanan et al. [4] made an interesting observation that apo-β-carotenals are also cleaved to retinal by the rabbit carotene 15,15′-dioxygenase. The cleavage of apo-β-carotenals by rabbit carotene 15,15′-dioxygenase was reported to be many times higher than with β,β-carotene [4]. However, in vivo studies on apo-β-carotenals and β,β-carotene in the rat have shown that the percentage of vitamin A formation from β,β-carotene is much higher than with apo-β-carotenals [7]. Our in vivo studies on apo-β-carotenals [6] also confirm the above findings and further indicate that, in the rat, the biological activity of 10′-apo-β-carotenal is higher than 12′- and 8′-apo-β-carotenals but the percentage of vitamin A formation from apo-β-carotenals is very low ($< 1.6\%$). In vivo studies carried out in our laboratory on mono-ring-substituted carotenoids suggested that the modification of one β-ionone ring of β,β-carotene or β,ε-carotene either by epoxidation or hydroxylation affects its cleavage at the 15,15′-double bond [8–12]. In the light of the above findings we were prompted to investigate in detail how exactly the modification of the β,β-carotene molecule affects its cleavage by carotene 15,15′-dioxygenase and also to investigate the cleavage of apo-β-carotenals and the related series of compounds.

The present paper deals with the isolation and partial purification of carotene 15,15′-dioxygenase from the intestinal mucosa of guinea pig and rabbit. A comparison of the relative specificity of the enzyme from both sources towards various carotenoids is described.

MATERIALS AND METHODS

Substrates and products

β,β-Carotene was obtained from Hoffmann La Roche Ltd and β,ε-carotene from Sigma Chemical Co. St. Louis. Other substrates, 5,6-epoxy-5,6-dihydro-β,β-carotene, 5,8-epoxy-5,8-dihydro-β,β-carotene, 5,6-epoxy-5,6-dihydro-β,ε-carotene, 5,8-epoxy-5,8-dihydro-β,ε-carotene, β,ε-caroten-3,3′-diol, 3′,4′-didehydro-β,β-caroten-3-ol, apo-β-carotenals, apo-β-carotenoic acids, 5,6-epoxy-5,6-dihydroapo-β-carotenals, 5,8-epoxy-5,8-dihydroapo-β-carotenals, 3-acetoxy- and 3-hydroxy-8′-apo-β-carotenals were prepared and characterised according to the methods reported from our laboratory [8–15]. 5,8,5′,8′-Diepoxy-5,8,5′,8′-tetrahydro-β,β-carotene was prepared from its corresponding 5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene (which was prepared and characterised according to the method of Subbarayan et al. [10]) by treatment with ethanolic HCl and characterised spectroscopically. β,β-Carotene-3,3′-diol was isolated and characterised from Gul Mohr *(Delonix regia)* anthers according to the method of Jungalwala and Cama [16] and β,β-caroten-3′-ol from papaya fruit according to the method of Subbarayan and Cama [17]. Apo-β-carotenols were prepared by $LiAlH_4$ reduction of their corresponding apo-β-carotenals. The substrates were purified by column chromatography over suitably water-deactivated alumina just before use.

For the characterisation of the reaction products, the following products were prepared: Retinal was prepared by the oxidation of retinol with MnO_2 [18], 3-dehydroretinal from the liver oil of Indian freshwater fish *(Wallago attu)* according to the procedure of Sundaresan and Cama [19] and the 5,6- and 5,8-epoxides of retinal according to the method of Jungalwala and Cama [20].

Isolation and purification of carotene 15,15'-dioxygenase from guinea pig and rabbit intestine

The enzyme carotene 15,15'-dioxygenase was isolated from the intestinal mucosa of guinea pig. All the operations of enzyme purification were done at 0–4 °C unless otherwise described.

The intestinal mucosa was scraped from the small intestine of guinea pig and homogenised in 8 vol. of 0.1 M potassium phosphate buffer (pH 7.8). The homogenate was centrifuged at 20 000 × *g* for 15 min. The pellet was discarded and to every 8 ml of the supernatant (designated as the crude extract) was added slowly 0.1 ml of a 2% (w/v) solution of protamine sulfate. After 10 min the mixture was centrifuged at 20 000 × *g* for 10 min. The precipitate was discarded and the supernatant solution was subjected to $(NH_4)_2SO_4$ fractionation. The precipitate obtained between 25 and 55% saturation with $(NH_4)_2SO_4$ was dissolved in 0.1 M phosphate buffer (pH 7.8) to give a final protein concentration of 13–14 mg/ml. To it was added chilled acetone (−20 °C) to make a final saturation of acetone to 45%. The precipitate was removed by centrifugation at 10 000 × *g* for 1 min at −10 °C. The supernatant was further fractionated by the addition of chilled acetone (−20 °C) so as to give the final concentration of 65% saturation. The mixture was centrifuged at 10 000 × *g* for 1 min at −10 °C. The precipitate obtained at this step was immediately dissolved in 0.1 M Tris–HCl buffer (pH 8.5) and centrifuged at 20 000 × *g* for 10 min and the clear supernatant was used for all the studies. The enzyme can be stored at −20 °C as $(NH_4)_2SO_4$ precipitate (25–55%) for one month without considerable loss in enzyme activity.

The carotene 15,15'-dioxygenase from rabbit intestine was similarly purified about 30-fold with a 200% recovery.

Dispersion of substrates

The substrate used for the enzyme reaction was dissolved in light petroleum or diethyl ether. To this 0.1 ml of Tween-20 was added per 200 nmoles of substrate. The solvent was evaporated under N_2. An adequate volume of glass-distilled water was added to give the final concentration of substrate to 200 nmoles/ml. The substrate was then finely dispersed in water by sonication for 2 min.

Assay of carotene 15,15'-dioxygenase

All the assay conditions were standardised with β,β-carotene as the substrate and the activity of the enzyme against other carotenoids was tested under optimal conditions.

The incubation mixture consisted of 10 ml of 0.1 M Tris–HCl buffer (pH 7.8 for the assay of rabbit enzyme and pH 8.5 for the guinea pig enzyme), 200 nmoles of substrate, 10 mg of GSH, $1.0 \cdot 10^{-3}$ M (final concentration) of ferrous ammonium sulfate, 5–15 mg of enzyme protein and the total volume of incubation mixture was always made up to 25 ml with glass-distilled water. The reaction was initiated by the addition of enzyme. An enzyme blank was taken as the control for each substrate tested. The incubation was carried out in amber-colored flasks (100 ml) at 37 °C for 3 h in the dark. After the incubation period, the reaction was stopped by the addition of 25 ml of acetone. The reaction mixture was extracted thrice with 50-ml aliquots of diethyl ether; the ether extracts were combined, washed with water, evaporated under

reduced pressure, redissolved in a small volume of light petroleum (40–60 °C) and used for further analysis.

Characterisation of products of the enzyme reaction

The light petroleum extract, containing substrate and product(s) of the enzyme reaction, was quantitatively applied on thin-layer chromatographic plates coated with basic alumina (0.5-mm thickness). The reaction products were separated from each other and from their corresponding substrates using 3% (v/v) acetone in light petroleum as the developing solvent. The authentic samples of expected products were also spotted side by side. The area corresponding to the expected products was scraped from the thin-layer chromatographic plates, extracted with acetone, evaporated to dryness, redissolved in light petroleum and their spectra were recorded on a Cary-14 spectrophotometer.

The reaction products were further characterised as their corresponding alcohols. The isolated products were reduced with $LiAlH_4$ at −10 °C for 5 min in diethyl ether solution. The reaction was stopped by the addition of ice-cold water; the ether extract was evaporated to dryness, redissolved in light petroleum and the spectra of the products were recorded.

The reaction products separated by thin-layer chromatography were also characterised as their thiobarbituric acid complex.

When the product formation was very low, 5–10 reaction mixtures were pooled and processed as usual.

Quantitative estimation of reaction products

The product or products formed from various carotenoids were quantitatively separated over 3% (v/w) water-deactivated neutral alumina (National Chemical Laboratory, Poona) column (10 g, 1 cm × 12 cm). The column was standardised using the authentic samples of substrates and their expected products.

In general, retinal and/or α-retinal was separated from the substrates and other products on alumina column by gradient elution. The fraction (25 ml) eluted from the column with 5% (v/v) diethyl ether in light petroleum was collected, evaporated, redissolved in ethanol (2 ml) and the concentration was estimated by the method of thiobarbituric acid as described by Futterman and Saslaw [21]. When 3′,4′-didehydro-β,β-caroten-3-ol was used as the substrate, the reaction product 3-dehydroretinal was separated on an alumina column as described for retinal and estimated like retinal by reading the color of the thiobarbituric acid complex at 550 nm. 5,8,5′,8′-Diepoxy-5,8,5′,8′-tetrahydro-β,β-carotene was separated from the product 5,8-epoxyretinal on an alumina column by elution with 2% (v/v) diethyl ether in light petroleum (25 ml) followed by elution of the product (5,8-epoxyretinal) with 10% (v/v) diethyl ether in light petroleum (25 ml). The fraction containing 5,8-epoxyretinal was evaporated to dryness, redissolved in ethanol and estimated like retinal by reading the color of the thiobarbituric acid complex at 470 nm.

Estimation of retinal

Retinal and/or α-retinal as the reaction product was estimated according to the method of Futterman and Saslaw [21]. To retinal and/or α-retinal dissolved in ethanol (1.0 ml) was added 1.0 ml of thiourea (4%) reagent, 1.0 ml of thiobarbituric

acid (0.6%) reagent and shaken. The color was allowed to develop in the dark at room temperature for 30 min. The color was then read at 530 nm. A standard curve of retinal (using different concentrations of retinal) was drawn to estimate the total amount of enzyme product. An appropriate correction factor was applied when different carotenoids were used as enzyme substrates. The amount of retinal obtained from apo-β-carotenals and apo-β-carotenols was multiplied by the factor 2 (because only one mole of retinal is expected/mole of apo-carotenoids) and then the specific activity of the enzyme towards these substrates was calculated.

Estimation of enzyme protein and unit of activity

Enzyme protein was estimated according to the method of Lowry et al. [22].

One unit of enzyme activity is defined as the amount of protein required to catalyse the formation of 1 nmole of retinal per 3 h under standard assay conditions. Specific activity is expressed as units of activity per protein.

RESULTS

Enzyme purification

Carotene 15,15′-dioxygenase from the intestinal mucosa of guinea pig has been partially purified and the results of the different purification steps are given in Table I. A purification of 38-fold over the crude enzyme activity has been achieved

TABLE I

PURIFICATION OF CAROTENE 15,15′-DIOXYGENASE FROM GUINEA PIG INTESTINE

5.0 ml of enzyme protein from each step of purification was incubated with β,β-carotene under the standard assay conditions and the activity was determined by estimating the amount of retinal formed.

Step No.	Treatments	Total protein (mg)	Total activity	Specific activity	Fold purification
1	Crude	945.0	160.5	0.17	1.0
2	Protamine sulfate supernatant	765.0	251.7	0.33	1.9
3	$(NH_4)_2SO_4$ fraction (25–55%)	571.0	224.4	0.39	2.3
4	Acetone precipitation (45–65%)	68.8	445.6	6.48	37.8

with an about 280% recovery. The final enzyme preparation was found to be devoid of any enzyme activity which can utilise the product (retinal) as tested by the method of Lakshmanan et al. [23]. Retinal disappearance activity (due to the presence of retinal oxidase and retinal reductase) was found to be present in the first three steps of enzyme purification suggesting that the estimation of enzyme activity in the first three steps was not quantitative because a part of the product (retinal) formed by the cleavage of β,β-carotene by carotene 15,15′-dioxygenase was further oxidised to retinoic acid or reduced to retinol. However, the enzyme activity in the fourth step (acetone precipitation, 45–65% saturation) of purification was completely devoid of the retinal disappearance activity as estimated by the method of Lakshmanan et al. [23]. Thus, the increase in total activity at Steps 2 and 4 is due to the removal of

the retinal disappearance activity. The other probable reasons for this increase in total activity may be due to the presence of inactive enzyme in the crude which becomes activated during purification or some endogenous inhibitor may be present in the crude which may be removed during the purification procedure.

By following the same procedure, the enzyme from rabbit intestinal mucosa was purified to 30-fold with a 200% recovery.

Properties of carotene 15,15'-dioxygenase from guinea pig

The reaction has been found to be linear up to 3 h and reaches a plateau stage within 4 h. The reaction increases proportionally with the increase of protein concentration and was found to be linear up to 25 mg protein per reaction mixture. The enzyme has an optimum pH of 8.5 and an optimum substrate concentration of 200 nmoles. The enzyme has an apparent K_m with β,β-carotene of $9.5 \cdot 10^{-6}$ M and a V of 3.3 nmoles of retinal formation/mg protein per h. The reaction is strictly stoichiometric and 2 moles of retinal are formed upon the utilisation of 1 mole of β,β-carotene.

The enzyme is activated by increasing concentrations of GSH (Table II). A concentration of 10 mg of GSH which has been used throughout the studies results in a 4.7-fold increase in basal activity. Iron in the Fe^{3+} state is ineffective but in the Fe^{2+} state exhibits a stimulatory effect. The enzyme shows a maximum activity at $1.0 \cdot 10^{-3}$ M (final concentration) of Fe^{2+} with an activation of 5.8-fold (Table II). However, an inhibition of 60% has been observed when the iron concentration is raised from $1.0 \cdot 10^{-3}$ to $4.0 \cdot 10^{-3}$ M (Table II).

The enzyme is partially inhibited by iron chelating agents like α,α'-dipyridyl and 1,10-phenanthroline (Table II). Sulfhydryl binding reagents like *p*-chloromercuribenzoate (*p* Cl-HgBzO$^-$), iodoacetate and *N*-ethylmaleimide also inhibit the enzyme activity (Table II). The enzyme is inhibited by an increasing concentration of sodium dodecylsulfate and the activity is almost completely inhibited at 40 mg concentration of the detergent per reaction mixture (Table II).

The enzyme isolated during the present studies from rabbit exhibited remarkable similarities with guinea pig enzyme with respect to time course, optimum substrate concentration, activation by Fe^{2+} and GSH, inhibition by sodium dodecylsulfate, iron chelating and sulfhydryl binding reagents. The only distinctive property observed is that the rabbit enzyme has a relatively low pH optimum (pH 7.8).

Identification of reaction products from different carotenoids

When different carotenoids were used as substrates, the products formed were isolated, purified and characterised spectroscopically. The chemical identities of retinal, α-retinal, 3-dehydroretinal, 3-hydroxyretinal, and the 5,6- and 5,8-epoxides of retinal were established by their characteristic chromatographic behavior and identical spectra with those of the synthetic compounds reported in the literature. The λ_{max} of the isolated products and their corresponding alcohols as well as their thiobarbituric acid complexes (products X and Y) are given in Table III. When 12'-, 10'- and 8'-apo-β-carotenals, 12'-, 10'- and 8'-apo-β-carotenols and 8'-apo-β-caroten-8'-yl acetate were used as the substrate, the only product detected was retinal which was identified spectrophotometrically. The absorption spectra of enzyme products in light petroleum are given in Fig. 1.

TABLE II

EFFECT OF GSH, IRON, SODIUM DODECYLSULFATE, IRON CHELATING AND SULFHYDRYL BINDING AGENTS ON THE ACTIVITY OF CAROTENE 15,15'-DIOXYGENASE FROM GUINEA PIG INTESTINE

The enzyme from the final purification step (5.0 mg/reaction mixture) was incubated with different activators and inhibitors prior to the addition of substrate (β,β-carotene). The rest of the conditions were the same as described in the text. Retinal was separated from β,β-carotene by column chromatography over alumina and estimated by the thiobarbituric acid method.

Supplement	Concentration (mg/reaction mixture)	Specific activity	Activity (%)
GSH	0	0.34	100
	10	1.60	471
	20	1.81	532
	30	2.50	735
	40	2.85	838
Sodium dodecylsulfate	0	1.60	100
	5	1.59	99
	10	1.18	70
	20	0.56	35
	30	0.21	13
	40	0.07	4
	(10^{-3} M final concentration of reaction mixture)		
$Fe_2(SO_4)_3$	0	0.57	100
	0.1	0.55	97
	0.5	0.52	91
	1.0	0.56	98
Ferrous ammonium sulfate	0	0.57	100
	0.1	0.84	147
	0.5	2.02	354
	1.0	3.33	584
	2.0	2.86	502
	3.0	2.13	374
	4.0	1.35	237
Control	0	5.65	100
α,α'-Dipyridyl	1.0	2.26	40
1,10-Phenanthroline	1.0	1.41	25
Iodoacetate	1.0	1.98	35
N-Ethylmaleimide	1.0	1.02	18
p-Chloromercuribenzoate	1.0	0.85	15

Substrate specificity of the carotene 15,15'-dioxygenase of guinea pig and rabbit

The results of the substrate specificity of the enzyme from guinea pig and rabbit with different carotenoids as substrates are given in Table IV. β,β-Carotene is the most active substrate for the enzyme from both the sources. Carotenoids such as β,ε-carotene, epoxides of β,β-carotene and of β,ε-carotene, β,β-caroten-3'-ol, 3',4'-didehydro-β,β-caroten-3-ol, apo-β-carotenals, apo-β-carotenols and 8'-apo-β-caroten-8'-yl acetate are also cleaved at the 15,15'-double bond but the cleavage is slower than with β,β-carotene. Amongst the apo-β-carotenals and apo-β-carotenols tested,

TABLE III

IDENTIFICATION OF REACTION PRODUCTS USING DIFFERENT CAROTENOIDS AS SUBSTRATES FOR CAROTENE 15,15′-DIOXYGENASE FROM GUINEA PIG

The compounds were incubated with the enzyme (from the final purification step) under the standard assay conditions and the products were isolated and processed as described in the text.

Substrate used (λ_{max} (nm) of the authentic samples samples in light petroleum)	λ_{max} (nm) in light petroleum of products formed		λ_{max} (nm) in light petroleum of products after reduction with $LiAlH_4$		λ_{max} (nm) of thiobarbituric acid complexes		Chemical identity	
	X	Y	X	Y	X	Y	X	Y
β,β-Carotene	370	–	328	–	530	–	Retinal (370)	–
β,ε-Carotene	370	245, 360	328	310, 325	530	520	Retinal	α-Retinal*
5,6-Epoxy-5,6-dihydro-β,β-carotene	370	350	328	315	530	470	Retinal	5,6-Epoxy-retinal (352)
5,8-Epoxy-5,8-dihydro-β,β-carotene	370	320, 335	328	280	530	470	Retinal	5,8-Epoxy-retinal (317, 331)
5,8,5′,8′-Diepoxy-5,8-5′,8′-tetrahydro-β,β-carotene	320, 335	–	280	–	470	–	5,8-Epoxy-retinal	–
5,6-Epoxy-5,6-dihydro-β,ε-carotene	245, 360	350	310, 325	315	520	470	α-Retinal	5,6-Epoxy-retinal
5,8-Epoxy-5,8-dihydro-β,ε-carotene	245, 360	320, 335	310, 325	280	520	470	α-Retinal	5,8-Epoxy-retinal
β,β-Caroten-3′-ol	370	365	328	325	530	530	Retinal	3-Hydroxy-retinal**
3′,4′-Didehydro-β,β-caroten-3-ol	383	365	350	325	550	530	3-Dehydro-retinal (385)	3-Hydroxy-retinal

* λ_{max} for α-retinal in ethanol (250, 368 nm) reported by Robeson et al. [26].

** λ_{max} for 4-hydroxyretinal in ethanol (375 nm) reported by Barua and Nair [27].

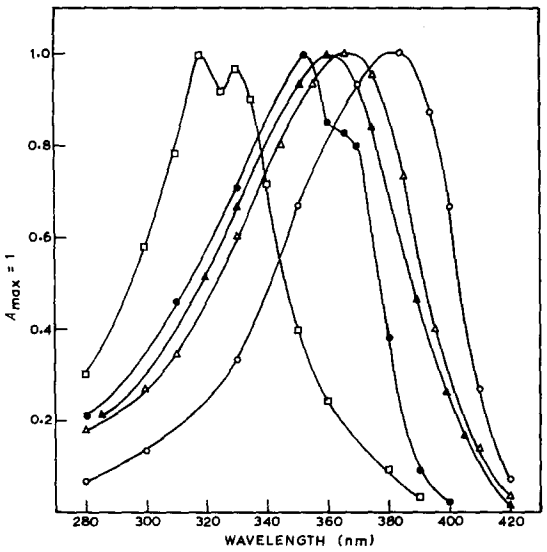

Fig. 1. Absorption spectra of reaction products in light petroleum. ○, 3-dehydroretinal; △, 3-hydroxyretinal and retinal; ▲, α-retinal; ●, 5,6-epoxyretinal; □, 5,8-epoxyretinal.

TABLE IV

SUBSTRATE SPECIFICITY OF CAROTENE 15,15′-DIOXYGENASE FROM GUINEA PIG AND RABBIT INTESTINAL MUCOSA

Different substrates were incubated with the final preparation of the enzyme from guinea pig and rabbit intestine (15.0 mg/reaction mixture) under the standard assay conditions. The activity was estimated by determining the amount of formation of products as described in the text.

Substrate	Guinea pig enzyme		Rabbit enzyme	
	Specific activity	Relative activity	Specific activity	Relative activity
β,β-Carotene	5.93	1.00	8.41	1.00
β,ε-Carotene	4.23	0.72	4.67	0.56
5,6-Epoxy-5,6-dihydro-β,β-carotene	1.92	0.32	2.12	0.25
5,8-Epoxy-5,8-dihydro-β,β-carotene	1.11	0.19	0.91	0.11
5,8,5′,8′-Diepoxy-5,8,5′,8′-tetrahydro-β,β-carotene	0.10	0.02	0.12	0.02
5,6-Epoxy-5,6-dihydro-β,ε-carotene	1.46	0.25	1.81	0.22
5,8-Epoxy-5,8-dihydro-β,ε-carotene	1.55	0.09	0.71	0.09
β,β-Caroten-3′-ol	0.22	0.04	0.22	0.03
3′,4′-Didehydro-β,β-caroten-3-ol	0.18	0.03	0.16	0.02
8′-Apo-β-carotenal	0.56	0.09	0.28	0.03
10′-Apo-β-carotenal	3.37	0.57	4.65	0.55
12′-Apo-β-carotenal	0.68	0.12	0.57	0.07
8′-Apo-β-caroten- 8′-yl acetate	0.96	0.16	0.80	0.10
8′-Apo-β-carotenol	0.41	0.07	0.15	0.02
10′-Apo-β-carotenol	2.98	0.50	3.60	0.43
12′-Apo-β-carotenol	0.59	0.08	0.51	0.07

the enzyme shows a maximum activity towards 10′-apo-β-carotenal and 10′-apo-β-carotenol. The enzyme is unable to cleave other apo-β-carotenoids such as 12′-, 10′- and 8′-apo-β-carotenoic acids, 5,6-epoxy-5,6-dihydro- and 5,8-epoxy-5,8-dihydro-12′-, and 8′-apo-β-carotenals, 5,6-epoxy-5,6-dihydro- and 5,8-epoxy-5,8-dihydro-8′-apo-β-caroten-8′-yl acetate, 3-acetoxy- and 3-hydroxy-8′-apo-β-carotenals. The dihydroxycarotenoids, β,β-caroten-3,3′-diol and β,ε-caroten-3,3′-diol are also not cleaved by the enzyme. The enzyme from guinea pig as well as from rabbit shows a close similarity with respect to cleavage of various carotenoids. The enzyme from guinea pig and rabbit shows relative activities with 10′-apo-β-carotenal of 57 and 55% and with 10′-apo-β-carotenol of 50 and 43%, respectively, compared with β,β-carotene. Other apo-β-carotenals and apo-β-carotenols tested show a much lower activity.

A change of one β-ionone ring of β,β-carotene to an ε-ionone ring lowers the activity in both the species. Any other modification like epoxidation at one of the β-ionone rings of β,β-carotene and β,ε-carotene results in a tremendous decrease in enzyme activity. The introduction of an hydroxyl group at one of the β-ionone rings of β,β-carotene drastically lowers the activity. 3′,4′-Didehydro-β,β-caroten-3-ol, which has an additional double bond in the ring than β,β-caroten-3′-ol, is less active compared with β,β-caroten-3′-ol. Modification of both the rings of β,β-carotene by epoxidation results in an about 2.0% activity compared with β,β-carotene. However, modification of both the rings of β,β-carotene and β,ε-carotene molecules by hydroxylation makes the molecules inert towards the enzyme.

DISCUSSION

The carotene 15,15′-dioxygenase system isolated from the intestinal mucosa of guinea pig and rabbit has been partially purified by 38- and 30-fold, respectively. The enzyme at the final step of purification was found to be free of other enzyme activities responsible for further degradation of the product (retinal) of carotene 15,15′-dioxygenase. The enzyme from both the sources resembles hog enzyme [5] with regard to sodium dodecylsulfate inhibition but is distinct from rat enzyme [3] where sodium dodecylsulfate is reported to have a stimulatory effect. The findings that the rabbit and guinea pig enzymes are inhibited by iron chelating agents are consistent with the observations made earlier in rat [1, 3] and hog [5] as well as in rabbit [24, 25]. Interestingly, the enzyme from guinea pig and rabbit is markedly stimulated by the increasing concentration of Fe^{2+} and the maximum activity has been observed with the final concentration of $1.0 \cdot 10^{-3}$ M of Fe^{2+}. However, iron in the Fe^{3+} state has no effect on the activity of the enzyme isolated from guinea pig as well as from rabbit. These findings, therefore, suggest that iron, most probably in the ferrous state, may be involved in the catalytic activity of the enzyme, however, the exact role of iron in this enzyme remains to be established. The inhibition of the enzyme with sulfhydryl binding agents and activation by GSH suggests that the enzyme may require free –SH groups for activity. Similar observations have been reported in the case of carotene 15,15′-dioxygenase from different animal species [1, 3, 5, 24, 25].

The enzyme from guinea pig catalyses the scission of β,β-carotene at the 15,15′-double bond to retinal. The oxygenative cleavage at the 15,15′-double bond in

a number of other carotenoids has been unequivocally shown to be catalysed by carotene 15,15′-dioxygenase as well.

Though 10′-apo-β-carotenal and 10′-apo-β-carotenol are readily cleaved by the enzyme from both the species compared with other apo-β-carotenals and apo-β-carotenols tested, the activity with β,β-carotene is about two times higher than 10′-apo-β-carotenal and 10′-apo-β-carotenol. This is in contrast to the observations of Lakshmanan et al. [25] where 10′-apo-β-carotenol has been reported to be 12.5 times more active with rabbit enzyme and 5.4 times more active with guinea pig enzyme than β,β-carotene. Furthermore, the activity was shown to decrease with the increase in the chain length of apo-β-carotenals [4] (10′ > 8′ > 4′) and it was suggested that the increase in chain length results in a decrease in its susceptibility to the enzyme. However, from our studies no such correlation between the enzyme activity with respect to the chain length of apo-β-carotenals can be drawn. The high relative activity with short chain apo-β-carotenals compared with the long chain apo-β-carotenal and β,β-carotene observed by Lakshmanan et al. [4, 25] may be due to the solubility differences of these compounds under the experimental conditions. 10′-Apo-β-carotenal and 10′-apo-β-carotenol are relatively more soluble than β,β-carotene and other apo-β-carotenals tested by Lakshmanan et al. [4] and the differences in the enzyme activity observed by them may be due to differences in solubility and not due to the actual high specificity of the enzyme towards these substrates. It is important to note that during the present studies the solubility factor has been taken into account and a suitable method has been developed by which almost all the carotenoids tested as substrates for the carotene 15,15′-dioxygenase can be solubilised properly before they were used for the enzyme assay. Moreover, the solubility factor has been kept constant during the present studies. It is also interesting to note that during the present studies using a suitable method of solubilisation of the substrate, the cleavage of 5,6- and 5,8-epoxides of β,β-carotene and of β,ε-carotene as well as of 5,8,5′,8′-diepoxy-5,8,5′,8′-tetrahydro-β,β-carotene could be shown, whereas Lakshmanan et al [4] failed to show the cleavage of 5,6-epoxy-5,6-dihydro-β,β-carotene and 5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene. Moreover, enzymatic cleavage of β,β-caroten-3′-ol, 3′,4′-didehydro-β,β-caroten-3-ol as well as the epoxides of β,β-carotene and of β,ε-carotene has been shown here for the first time. The much higher activity of carotene 15,15′-dioxygenase towards apo-β-carotenals and 10′-apo-β-carotenol compared with that of β,β-carotene (natural substrate for the enzyme) reported by Lakshmanan et al. [4, 25] seems to be quite unlikely. However, our enzymatic studies with apo-β-carotenals are further supported by in vivo findings in the rat where the percentage of vitamin A formation from apo-β-carotenals is reported to be very low compared with β,β-carotene [6, 7].

The enzymatic studies with apo-β-carotenals indicate that they are cleaved at the 15,15′-double bond to retinal and thus this supports our in vivo findings where they were found to be biologically active [6]. On the other hand, 3-hydroxy-8′-apo-β-carotenal [15] and epoxides of apo-β-carotenals [6, 14] which are not cleaved at the 15,15′-double bond in vivo, are also resistant to attack by the enzyme. Though apo-β-carotenoic acids have vitamin A activity as tested by growth experiments and are converted to vitamin A in the intestine in vivo (Singh, H. and Cama, H. R., unpublished), the isolated carotene 15,15′-dioxygenase failed to carry out oxygenative cleavage in these compounds at the 15,15′-double bond. This anomaly may be

explained: Apo-β-carotenoic acids are very slowly converted to vitamin A in vivo (Singh, H. and Cama, H. R., unpublished) and it is quite probable that they may be very slowly converted to retinal by the isolated carotene 15,15′-dioxygenase as well. The trace amounts of retinal if formed may escape detection. It is quite likely that the apo-β-carotenoic acids may exist, at the pH 7.8 or 8.5 (alkaline pH) which is employed during the present studies, in the form of the anion (COO^-). The presence of a negative charge on the substrate may affect its binding with the enzyme. It is also possible that apo-β-carotenoic acids may be degraded slowly to vitamin A in vivo by an enzyme system which is different from carotene 15,15′-dioxygenase.

It is important to note that carotene 15,15′-dioxygenase cleaves 5,8,5′,8′-diepoxy-5,8,5′,8′-tetrahydro-β,β-carotene but is inert towards 5,6-epoxy-5,6-dihydro- and 5,8-epoxy-5,8-dihydro-12′- and 8′-apo-β-carotenals. This anomaly may be attributed to the fact that 5,8,5′,8′-diepoxy-5,8,5′,8′-tetrahydro-β,β-carotene is a symmetrical molecule whereas the 5,6- and 5,8-epoxides of apo-β-carotenals are not which may, therefore, affect the binding of the enzyme with the substrates (epoxides of apo-β-carotenals).

Nutritional studies with 5,8-epoxy-5,8-dihydro-β,β-carotene [8], 5,6-epoxy-5,6-dihydro-β,β-carotene [10], 5,6-epoxy-5,6-dihydro-β,ε-carotene [11], β,β-caroten-3′-ol [12] and 3′,4′-didehydro-β,β-caroten-3-ol [9] reported from our laboratory suggested that these compounds may be cleaved excentrically in the small intestine of the rat. However, the detection of the ring-substituted part of these carotenoid molecules as the ring-substituted retinal derivative during the present studies unequivocally show for the first time that these compounds are also cleaved at the 15,15′ double bond by carotene 15,15′-dioxygenase from guinea pig and rabbit. It may be pointed out that it is the first time that 3-dehydroretinal formation has been shown from 3′,4′-didehydro-β,β-caroten-3-ol using an isolated enzyme system.

It may be concluded from these studies that the enzyme has a very high specificity for the chemical environment of the β-ionone ring as well as the polyene chain of β,β-carotene and the structural modification at either the β-ionone ring or the polyene chain of the β,β-carotene molecule affects the cleavage by carotene 15,15′-dioxygenase.

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REFERENCES

1 Olson, J. A. and Hayaishi, O. (1965) Proc. Natl. Acad. Sci. U.S. 54, 1364–1370
2 Goodman, D. S. and Huang, H. S. (1965) Science 149, 879–880
3 Goodman, D. S., Huang, H. S., Kanai, M. and Shiratori, T. (1967) J. Biol. Chem. 242, 3543–3554
4 Lakshmanan, M. R., Pope, J. L. and Olson, J. A. (1968) Biochem. Biophys. Res. Commun. 33, 347–352
5 Fidge, N. H., Smith, F. R. and Goodman, D. S. (1969) Biochem. J. 114, 689–694
6 Singh, H., Krishna Mallia, A. and Cama, H. R. (1972) in Current Trends in the Biochemistry of Lipids (Ganguly, J. and Smellie, R. M. S., eds), pp. 219–232, Academic Press, New York

7 Glover, J. (1960) in Vitamins and Hormones (Harris, R. S. and Ingle, D. J., eds), Vol. 18, pp 371–386, Academic Press, New York
8 John, J. and Cama, H. R. (1972) Int. J. Vitam. Nutr. Res. 42, 278–284
9 Savithry, K. N., Krishna Mallia, A. and Cama, H. R. (1972) Indian J. Biochem. Biophys. 9, 325–327
10 Subbarayan, C., Lakshmanan, M. R. and Cama, H. R. (1966) Biochem. J. 99, 308–311
11 Kishore, G. S., Subbarayan, C. and Cama, H. R. (1970) Indian. J. Biochem. 7, 98–101
12 John, J., Kishore, G. S., Subbarayan, C. and Cama, H. R. (1970) Indian J. Biochem. 7, 222–225
13 Singh, H., John, J. and Cama, H. R. (1973) Int. J. Vitam. Nutr. Res. 43, 147–149
14 Singh, H. and Cama, H. R. (1972) Int. J. Vitam. Nutr. Res. 42, 379–388
15 John, J., Singh, H. and Cama, H. R. (1973) Int. J. Vitam. Nutr. Res. 43, 70–80
16 Jungalwala, F. B. and Cama, H. R. (1962) Biochem. J. 85, 1–8
17 Subbarayan, C. and Cama, H. R. (1964) Indian J. Chem. 2, 451–454

18 Ball, S., Goodwin, T. W. and Morton, R. A. (1948) Biochem. J. 42, 516–523
19 Sundaresan, P. R. and Cama, H. R. (1961) Br. J. Nutr. 15, 225–230
20 Jungalwala, F. B. and Cama, H. R. (1961) Biochem. J. 95, 17–26
21 Futterman, S. and Saslaw, L. D. (1961) J. Biol. Chem. 236, 1652–1657
22 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
23 Lakshmanan, M. R., Vaidyanathan, C. S. and Cama, H. R. (1964) Biochem. J. 90, 569–573
24 Olson, J. A. (1969) Am. J. Clin. Nutr. 22, 953–958
25 Lakshmanan, M. R., Chansang, H. and Olson, J. A. (1972) J. Lipid Res. 13, 477–482
26 Robeson, C. D., Blum, W. P., Dieterle, J. M., Cawley, J. D. and Baxter, J. G. (1955) J. Am. Chem. Soc. 77, 4120–4125
27 Barua, R. K. and Nair, M. G. R. (1962) Nature 193, 165