THE SPECIFICITY OF A PARTIALLY PURIFIED CAROTENOID CLEAVAGE ENZYME OF RABBIT INTESTINE

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In the presence of oxygen β-carotene is cleaved at the 15:15' double bond into two molecules of retinal by an enzyme localized in the supernatant fraction of intestinal and liver homogenates (Goodman and Huang, 1965; Olson and Hayaishi, 1965). This oxygenase has been partially purified from intestinal extracts of the rat (Goodman et al., 1966, 1967) and of the rabbit (Pope, 1968). In examining the specificity of this enzyme in vitro, we have become particularly interested in the β-apocarotenals, which occur naturally (Thommen, 1967), have high biological activity, and are efficiently converted into vitamin A in vivo (Glover, 1960) and in epoxy derivatives of β-carotene, which are relatively inert biologically (Subbarayan, et al., 1966). In the present communication we show that the β-apocarotenals, but not epoxy β-carotenes, are readily converted into retinal by the carotene cleavage enzyme from rabbit intestine.

Crystalline β-carotene was purchased from Eastman Organic Chemicals Ltd., Rochester, U.S.A. 5,6-Epoxy β-carotene and 5,6:5',6'-diepoxy β-carotene were synthesized by the method of Tsukida and Zechmeister (1958). Various β-apocarotenals were obtained as gifts from Hoffmann-La Roche and Co., Basel. Each substrate was dissolved in acetone to give a final concentration of 50 mμmoles in 0.5 ml. All other chemicals were of reagent grade.

The carotene cleavage enzyme was prepared as follows. A normal healthy rabbit (2 Kg) of either sex was fasted for 48 hours and killed by a blow on the head. The small intestine from the duodenum to a point about 20 cm from the appendix was quickly removed, washed free of its contents with ice-cold 0.9% (w/v) sodium chloride, cut into segments 15-20 cm long, and placed in an iced beaker. The segments were slit open along the mesenteric line of attachment, and the adhering luminal contents were removed by blotting gently with paper towels. The mucosa was scraped off in the cold with microscope slides and homogenized in a Potter-Elvehjem homogenizer with 8 volumes of 0.1 M potassium phosphate buffer, pH 7.8. After being centrifuged at 43,500 x g for 60 minutes, the cleavage enzyme was precipitated from the supernatant solution between 25% and 50% saturation with $(NH_4)_2SO_4$. The precipitate was stored at -20° C and dissolved just before use in a suitable volume of 0.1 M phosphate buffer, pH 7.8. Protein was determined by the method of Lowry et al., (1951).

The incubation medium consisted of 50 μmoles substrate, 50 mg reduced GSH, 25 μmoles sodium lauryl sulphate and 10-40 mg enzyme protein in 25 ml 0.1 M phosphate buffer, pH 7.8. After incubation for 1 hour at 37° C in the dark, 25 ml of acetone were added and the reaction mixture was extracted thrice with 10 ml portions of diethyl ether. The pooled ether extract was washed with water, dried over anhydrous Na_2SO_4, filtered through a sintered glass funnel containing anhydrous Na_2SO_4, evaporated to dryness under reduced pressure, and finally dissolved in a small quantity of petroleum ether (40-60°C). Control experiments with a substrate blank, with an enzyme blank and with boiled enzyme were conducted under identical conditions.

Retinal was separated from various carotenoids by chromatography on a column of 10 g of 5% (v/w) water deactivated alumina (1 cm x 12 cm). The column was successively developed with the following total volumes of solvent and percentages (v/v) of diethyl ether in petroleum ether (40-60°C):

50 ml of 4%, 50 ml of 6%, 25 ml of 8%, 50 ml of 10% and 50 ml of 25%. β-Carotene, 5,6-epoxy β-carotene and 5,6:5',6'-diepoxy β-carotene were eluted in the 4% ether fraction, retinal was eluted quantitatively in the 10% ether fraction, and the various β-apocarotenals were eluted by 25% ether. Authentic samples were used to establish the elution pattern.

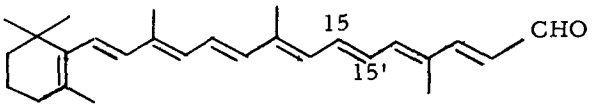
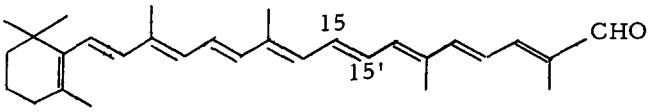
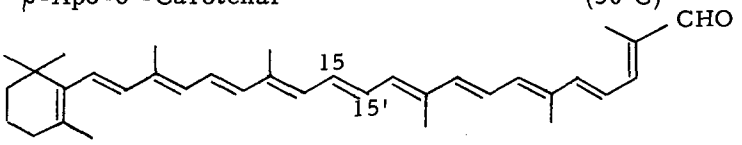
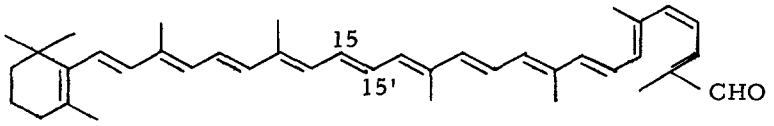
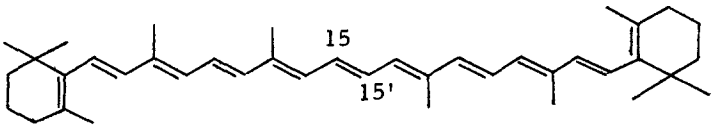
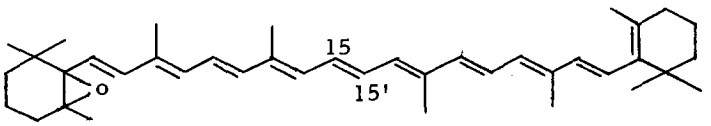
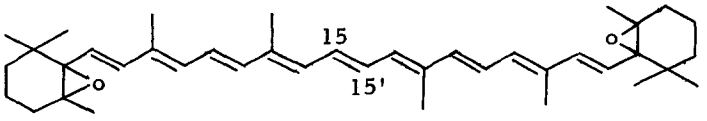
When β-carotene and other active substrates were used, the 10% ether fraction obtained from 4-6 reaction flasks showed the characteristic spectrum of retinal in petroleum ether with a λ max at 370 nm (Ball et al., 1948). The enzymatic product also had the same R_f value (0.53) as all-trans retinal and cochromatographed indistinguishably with it in a Silica gel thin layer system (John et al., 1965). Furthermore, the enzymatic product, unlike any substrate tested, gave a pink color characteristic of retinal with the thiobarbituric acid (TBA) reagent (Futterman and Saslaw, 1961). For estimation of the retinal formed, the 10% ether fraction was evaporated to dryness in an amber colored flask under reduced pressure. Then 1 ml of the TBA reagent was added, the solution was left for 30 minutes in the dark, and the extinction of the complex was measured at 520 nm. All-trans retinal gave a $E^{1\%}_{1\ cm}$ value of 2163 under these conditions. Since β-carotene yields two moles of retinal per mole of substrate whereas each mole of β-apocarotenal gives only one mole of retinal, the specific activity of the enzyme is expressed as mμmoles of the substrate converted into retinal per mg protein per hour.

In control experiments retinal was never detected. Experimentally retinal was the sole product irrespective of the substrate used. As indicated in Table 1 the activity of the enzyme was maximal towards β-apo 10'-carotenal and then decreased as the length of the carbon chain increased. There was virtually no activity towards 5,6-epoxy β-carotene and 5,6:5',6'-diepoxy β-carotene.

From these results some initial guidelines concerning the specificity of the carotene cleavage enzyme of rabbit intestine can be defined: First, the enzyme is seemingly specific for the 15, 15' double bond, in that only

TABLE 1

Substrate Specificity of the Carotene Cleavage Enzyme

Substrate		Specific Activity	Relative Activity
			
β-Apo-10'-Carotenal	(27 C)	0.96	10.7
			
β-Apo-8'-Carotenal	(30 C)	0.91	10.2
			
β-Apo-4'-Carotenal	(35 C)	0.22	2.5
			
3', 4'-Dehydro-18'-Oxo-γ-Carotene	(40 C)	0.18	2.0
			
β-Carotene	(40 C)	0.09	1.0
			
5, 6-Epoxy β-Carotene	(40 C)	0	0
			
5, 6-, 5', 6'-Diepoxy β-Carotene	(40 C)	0	0

retinal was detected as a product irrespective of the substrate used. Second, the enzyme is relatively non-specific towards the length of the carotenoid chain, providing that one unsubstituted β-ionone moiety is present. 5,6-Epoxy β-carotene, which was inert here but possesses 21% of the biological activity of β-carotene (Subbarayan et al., 1966), is an exception. Its conversion to retinal might occur at a rate which is significant in vivo but not in vitro, or possibly by some other mechanism. On the other hand, 5,6:5',6'-diepoxy-β-carotene is inert both enzymatically and physiologically. Third, the enzyme is most active with the shortest β-apocarotenal; β-apo-10'-carotenal, for example is cleaved eleven times faster than β-carotene. Thus the high biological activities of β-apocarotenals and their ready conversion to vitamin A can be simply explained by scission at the 15, 15' position, instead of through the stepwise cleavage pathway postulated by Glover (1960). Two precautions should be noted: 1) although unlikely, the enzyme preparation used here may contain a group of highly specific carotenoid cleavage enzymes rather than a single less specific one, and 2) the limited series of substrates used does not allow full definition of the enzyme's specificity.

Summary: The 15,15' double bond of carotenoids is preferentially cleaved by a soluble enzyme from rabbit intestine to yield retinal as the sole product. The activity of the enzyme is maximal with β-apo 10'-carotenal, and then decreases as the length of the isoprenoid side chain increases. 5,6-Epoxy β-carotene and 5,6:5',6'-diepoxy β-carotene are not cleaved.

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