Registry No. ATP, 56-65-5; PKC, 9026-43-1; Mg, 7439-95-4; arachidonic acid, 506-32-1; NADPH-specific oxidase, 9032-22-8; superoxide, 11062-77-4; oleic acid, 112-80-1; elaidic acid, 112-79-8; linoleic acid, 60-33-3; palmitoleic acid, 373-49-9; palmitelaidic acid, 10030-73-6; guanosine 5′-*O*-(3-thiotriphosphate), 37589-80-3; guanosine 5′-(β,γ-imidotriphosphate), 34273-04-6.

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Retinal Is Not Formed in Vitro by Enzymatic Central Cleavage of β-Carotene

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ABSTRACT: Rat intestinal mucosa was prepared and incubated with β-carotene by the procedure of Goodman and Olson [Goodman, DeW. S., & Olson, J. A. (1969) *Methods Enzymol. 15*, 462–475] to determine β-carotene cleavage activity. A new detection system for the reaction products of the described enzyme β-carotene 15,15′-dioxygenase (EC 1.13.11.21) employs solvent extraction of retinoids and carotenoids followed by high-performance liquid chromatography separation and photometric detection of the pigments. It has not detected any newly formed retinal or other retinoids in the intestinal protein preparations from normal or vitamin A deficient rats. The latter were chosen as a possible source of more active enzyme preparations. With corresponding blank samples subjected to identical conditions of incubation but without added protein, small amounts of β-apocarotenals could be detected. They were previously reported as cleavage products of β-carotene [Ganguly, J., & Sastry, P. S. (1985) *World Rev. Nutr. Diet. 45*, 198–220] but are clearly not formed as a result of an enzymatic reaction. The failure to detect in vitro enzymatic central or random cleavage of the β-carotene molecule in extracts of rat intestinal mucosa emphasizes the need to reevaluate the existing theory of conversion of β-carotene into vitamin A.

Ever since it was demonstrated half a century ago by Moore (Moore, 1930) that "carotene" has to be considered a provitamin A, investigators have tried to gain insight into the nature of the conversion of β-carotene to vitamin A (Ganguly & Sastry, 1985). While it was suggested that this conversion of β-carotene takes place predominantly in the small intestine (Sexton et al., 1946), the efficiency of the conversion was always less than expected on the basis of central cleavage of β-carotene into two molecules of vitamin A aldehyde (retinal) (Brubacher & Weiser, 1985). In the late sixties, Goodman and Olson (Goodman & Huang, 1965; Goodman et al., 1966, 1967; Goodman, 1969; Fidge et al., 1969; Olson & Hayaishi,

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1965; Lakshmanan et al., 1968, 1972) reported the existence of a β-carotene 15,15′-dioxygenase (EC 1.13.11.21), catalyzing the central fission of β-carotene into retinal in the small intestine of the rat, rabbit, and hog. Subsequently, β-carotene cleavage activity was detected in the intestinal mucosa of guinea pigs and chicken (Singh & Cama, 1974; Sklan, 1983a) as well as in the corpus luteum (Gawienowski et al., 1974; Stacewicz-Sapuncakis et al., 1975; Sklan, 1983b). The liver has also been described as a site for the conversion of β-carotene into vitamin A (Olson & Hayaishi, 1965; Sklan & Havely, 1984). A central cleavage of β-carotene has recently been challenged by Ganguly and co-workers (Ganguly & Sastry, 1985; Sharma et al., 1977), who provided evidence for a random cleavage of the β-carotene molecule. A similar pathway involving terminal oxidation of β-carotene had been advanced 30 years ago and is referred to as the Glover–Redfearn hypothesis (Glover, 1960). According to the former degradation scheme, three β-apocarotenals [8′, 10′, and 12′] are formed from β-carotene, all of which can be converted to retinal or oxidized to the corresponding β-apocarotenoic acids. The latter are thought to undergo a type of β-oxidation to yield retinal.

In order to decide which of the mechanisms (central versus random cleavage) is operative, we have assayed extracts from rat intestinal mucosa for β-carotene cleavage activity as described (Goodman & Olson, 1969) and analyzed the reaction mixture for possible degradation products of β-carotene by new techniques that are as sensitive as those previously employed but, in addition, are more specific in the characterization of products. These experiments were repeated with vitamin A deficient rats as a possible source of more active enzyme preparations (Villard & Bates, 1986).

Experimental Procedures

Materials. β-Carotene (type IV), DL-α-tocopherol, linoleic acid (99%), and glycocholate were purchased from Sigma Chemie, GmbH, Taufkirchen, FRG; retinal, retinoic acid, retinyl palmitate (type IV), and glutathione were from Serva Feinbiochemica, Heidelberg, FRG, and β-apo-8′-carotenal, synthetic lecithin, and BHT[1] were from Fluka, Buchs, Switzerland. Initial studies with β-carotene of lower quality showed a much higher rate of decomposition of β-carotene. β-Apo-10′- and β-apo-12′-carotenal were gifts from F. Hoffman-La Roche & Co. AG, Basel, Switzerland. All other chemicals were of pro analysi grade from Merck, Darmstadt, FRG. Bidistilled water was used throughout.

Rats. One day after birth the offspring (F_1 generation) of Sprague-Dawley rats were divided into two groups: (i) four females and five males and (ii) five females and six males. Each group also contained one female of the F_0 generation. The females, and then after 4 weeks the weanlings, were fed ad libitum either (group i) a standardized control diet (C1000, Altromin International, Lage, FRG) or (group ii) a diet (C1016, Altromin) that had exactly the same composition except for the lack of vitamin A supplement. After 10 weeks the weight of the vitamin A deficient animals was 80% (females) and 60% (males) of that of the control animals. At the same time the two females of the F_0 generation from both the control and vitamin A deficient group did not show a difference in body weight. Vitamin A deficiency of the rats was also evident from (i) shaggy hair, (ii) less motility, and (iii) atrophy of the testes. Before sacrificing the animals, the control group was fasted overnight. When the mucosa was prepared, liver samples of the animals were taken and stored at −28 °C. These samples were later analyzed for their vitamin A content (Olson, 1979). The animals of the control group had mean retinol contents of 53 μg/g of liver (females) and 47 μg/g of liver (males). The retinol content of the vitamin A deficient rats was below the limit of determination by this method.

Preparation of the β-Carotene Cleavage Enzyme from Rat Intestinal Mucosa. The preparation of the enzyme reported to convert *all-trans*-β-carotene into retinal was carried out by following the published procedure as closely as possible (Goodman & Olson, 1969). The F_{20}^{45} fraction, i.e., the precipitated protein obtained by fractionation with ammonium sulfate between 20 and 45% saturation, was stored at −28 °C. F_{20}^{45} samples were thawed in a water bath at room temperature prior to use and dialyzed at 4 °C for 4 h against a large excess of 10 mM potassium phosphate buffer, pH 7.7 (Goodman & Olson, 1969). The yield of protein from one rat was about 7 mg.

Incubation of β-Carotene with the F_{20}^{45} Fraction. The assay for β-carotene conversion was carried out as described (Goodman & Olson, 1969) scaled up 3 times, the only difference to the published procedure being the source of the chemicals. During an incubation time of 1 h, the samples were gently shaken in a water bath at 37 °C in the dark. The β-carotene concentration corresponded to 0.75 μg/mL; the protein concentration as determined by the Lowry method (Lowry et al., 1951) was 3.5 mg/mL. The total volume of the assay was 6 mL. The incubation mixture was a clear yellow solution after the addition of β-carotene. The reaction was quenched by freezing the samples in round-bottom flasks in a methanol bath at −40 °C. The samples were then freeze-dried.

Extraction of the Pigments. Carotenoids and retinoids were extracted from the lyophilized samples (Ito et al., 1974) with anhydrous methanol and *n*-hexane with added BHT (0.005% w/v) at 4 °C. Three extraction steps each comprising 6 mL of solvent with alternating order of solvent addition were used. The solvents containing the pigments were transferred with a Pasteur pipet, collected, and evaporated with a rotary evaporator at 37 °C. The solid residue was taken up in 0.5 mL of acetone containing BHT (0.005% w/v) for HPLC determination or stored up to 6 days at −28 °C when not directly used. During this and all subsequent steps, precautions were taken to protect the pigments against light, heat, and oxygen (Britton, 1985). Stock solutions were always freshly prepared.

HPLC Separation and Photometric Detection of Carotenoids and Retinoids. A Du Pont chromatographic system 8800 with a gradient controller was used in combination with a variable-wavelength monitor and recorder from Knauer, West Berlin, and an Integrator Autolab System I from Spectra-Physics. Retinal, retinol, and retinoic acid were separated on a Merck LiChrosorb RP-18 column (5 μm, 0.46 × 25 cm) with water (containing 1% ammonium acetate)/acetonitrile (28:72 v/v) at a flow rate of 2 mL/min. Detection wavelength was 350 nm. For the retinyl esters and β-carotene, the same column was used with a developing system of acetonitrile/methanol/*n*-hexane/methylene chloride (72:15:6.5:6.5 v/v) and a flow rate of 1 mL/min. Detection wavelengths were 330 nm for the retinyl esters and 450 nm for β-carotene. Retinyl palmitate was the only standard available. β-Apocarotenals were separated on a Du Pont Zorbax ODS column (7 μm, 0.46 × 25 cm) with a quaternary system of aceto-

[1] Abbreviations: HPLC, high-performance liquid chromatography; BHT, 2,6-di-*tert*-butyl-*p*-cresol.

nitrile/methanol/*n*-hexane/methylene chloride (80:15:2.5:2.5 v/v) at a flow rate of 0.7 mL/min. Detection wavelength was 440 nm. Standards were run on the same day and after each set of separations performed in triplicate. Injection volumes were 20 μL from 0.5-mL samples containing the pigments (see above). β-Apocarotenals were quantitated by determining peak areas calibrated against known amounts of β-apocarotenals. Absorption spectra were recorded on a Perkin-Elmer Lambda 9 spectrophotometer in 1-cm quartz cells at ambient temperature.

Results

The assay for β-carotene cleavage can be divided into four steps: (i) preparation of the enzyme from intestinal mucosa of rats; (ii) incubation of β-carotene with the partially purified enzyme; (iii) extraction of the pigments from this incubation mixture; (iv) separation and detection of carotenoids and retinoids. Our experimental protocol followed the procedures given for steps i and ii in the literature (Goodman & Olson, 1969) exactly. In the past the following steps were extraction of the pigments with chloroform/methanol, acidification with 5 mM sulfuric acid, chromatography on alumina, and quantitation of the radioactive metabolites (Goodman & Olson, 1969). Hence, the enzymatic assay was only possible with [^{14}C]-β-carotene, which is not available commercially and had to be synthesized by *Phycomyces blakesleeanus* and isolated from this fungus. For several reasons we replaced the latter steps iii and iv by more advanced analytical techniques. Since most carotenoids and retinoids are very sensitive to acids, the acidification with sulfuric acid should be avoided. Due to the presence of traces of hydrochloric acid in chloroform, the extraction with chloroform/methanol is also no longer recommended (Britton, 1985). In addition, the extraction of the pigments as well as the evaporation of solvents was carried out in the present study at temperatures that were lower than those previously employed (Goodman & Olson, 1969). The reported very low specific activity of the cleavage enzyme, i.e., a few nanograms of retinal formed per hour and per milligram of protein at 37 °C (Goodman & Olson, 1969), makes it necessary to handle and detect substances at the lower nanogram level. HPLC separation with photometric detection is presently the method of choice and is employed here at sensitivities at least equal to those obtained by previous radioactivity measurements. A further basis of our concern was the possibility of nonspecific reactions under the conditions of incubation in this range of concentrations. Therefore, three blank samples were subjected to the same conditions of incubation. The assay consisted of the following samples: (A) incubation mixture with F_{20}^{45} and with β-carotene, (B) incubation mixture with F_{20}^{45} and without β-carotene, (C) incubation mixture without F_{20}^{45} and with β-carotene, and (D) incubation mixture without F_{20}^{45} and without β-carotene.

Surprisingly, in our assays we have been unable to detect any retinal, retinol, retinoic acid, or retinyl esters newly formed from the added β-carotene, either in controls or in the vitamin A deficient group of animals. The HPLC profile in Figure 1 illustrates this for retinal, retinol, and retinoic acid. The insert in Figure 1 shows the corresponding separation with a mixture of the pure compounds. In addition, on the basis of the absorbance at 450 nm, an estimation of the β-carotene content of the samples after incubation demonstrated that ≥95% of the β-carotene had been recovered. If at least 1% of the β-carotene in the assay had been converted to retinal, this amount of retinal (about 1.8 ng) would still have been detected in the chromatographic system. The high recovery of β-carotene demonstrates that other retinoids as possible

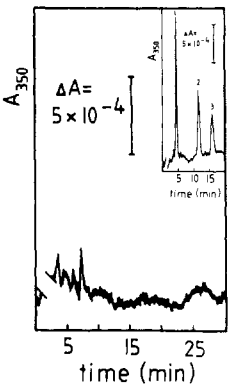

FIGURE 1: HPLC screening for retinoic acid, retinol, and retinal. Vitamin A deficient rats, sample A, i.e., incubation mixture with F_{20}^{45} and with β-carotene. The insert shows the chromatogram of the commercial compounds examined under identical conditions: (1) 12 ng of retinoic acid, (2) 11 ng of retinol, and (3) 11 ng of retinal. Column: Merck LiChrosorb RP-18. Elution: water (containing 1% w/v ammonium acetate)/acetonitrile (28:72 v/v). Flow rate: 2 mL/min. Detection: 350 nm.

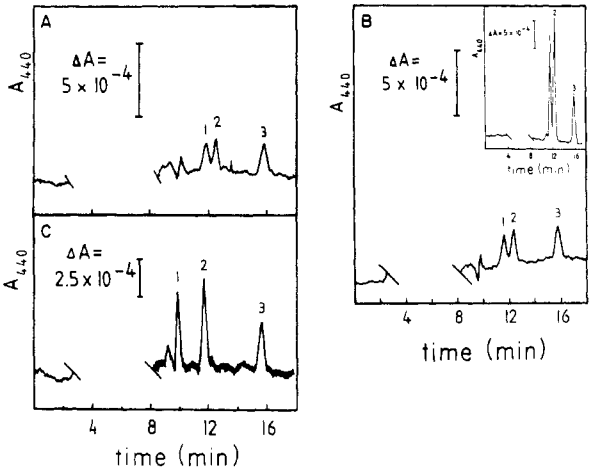

FIGURE 2: HPLC screening for β-apocarotenals. (A) Control rats, sample A, i.e., incubation mixture with F_{20}^{45} and with β-carotene: (1) β-apo-12′-carotenal, (2) β-apo-10′-carotenal, and (3) β-apo-8′-carotenal. (B) Sample C, i.e., incubation mixture without F_{20}^{45} and with β-carotene. The insert shows the chromatogram of the pure compounds examined under identical conditions: (1) 2 ng of β-apo-12′-carotenal, (2) 2 ng of β-apo-10′-carotenal, and (3) 1.5 ng of β-apo-8′-carotenal. (C) Vitamin A deficient rats, sample A, i.e., incubation mixture with F_{20}^{45} and with β-carotene: (1) unidentified compound, (2) β-apo-12′-carotenal, and (3) β-apo-8′-carotenal. Column: Du Pont Zorbax ODS. Elution: acetonitrile/methanol/*n*-hexane/methylene chloride (80:15:2.5:2.5 v/v). Flow rate: 0.7 mL/min. Detection: 440 nm.

cleavage products did not escape our detection system. However, as shown in Figure 2A, small amounts of three compounds were detected and identified as the three β-apocarotenals (8′, 10′, and 12′) by cochromatography with the pure compounds (Figure 2B, insert) and on the basis of optical absorption spectra of the collected fractions. These β-apocarotenals were also formed in the blank samples without added F_{20}^{45} (Figure 2B) and, hence, are not derived from an enzymatic reaction. Only in the vitamin A deficient rats was a compound (peak 1), which was not further characterized, detected (Figure 2C). The retention time of this compound is in the range expected for β-apo-14′-carotenal. However, if this compound were β-apo-14′-carotenal, the amount must be relatively high, since detection at this wavelength is unfavorable for this derivative. It is also noted that the typical double peak of β-apo-10′- and β-apo-12′-carotenal is absent in this sample (see for comparison panels A and B of Figure 2). The retention time of the one compound observed instead

Table I: Amount and Yield of β-Apocarotenals Formed from β-Carotene in the Assay for β-Carotene Cleavage[a]

experiment	peak	β-apo-carotenal	amount (ng)	yield (%)
control rats, β-carotene with F_{20}^{45} (see Figure 2A)	1	12′	6	0.21
	2	10′	5	0.16
	3	8′	7.5	0.21
β-carotene without F_{20}^{45} (see Figure 2B)	1	12′	6	0.21
	2	10′	6	0.20
	3	8′	6.5	0.18
vitamin A deficient rats, β-carotene with F_{20}^{45} (see Figure 2C)	1			
	2	12′	12.5	0.44
	3	8′	11	0.31

[a] A total of 20 μL from 0.5 mL of total sample volume was injected into the HPLC system. The data presented in this table refer to the amount of β-apocarotenals in the original 0.5-mL sample. The yield is expressed on a molar basis of β-carotene in the incubation mixture.

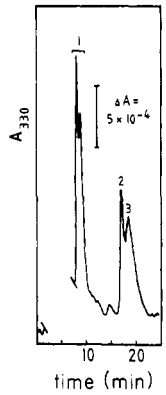

FIGURE 3: HPLC screening for carotenoids. Sample C, i.e., incubation mixture without F_{20}^{45} and with β-carotene. Identical chromatograms were obtained for samples A of control or vitamin A deficient rats. (1) DL-α-Tocopherol, (2) *all-trans*-β-carotene, and (3) unidentified compound. Column: Merck LiChrosorb RP-18. Elution: acetonitrile/methanol/*n*-hexane/methylene chloride (72:15:6.5:6.5 v/v). Flow rate: 1 mL/min. Detection: 330 nm.

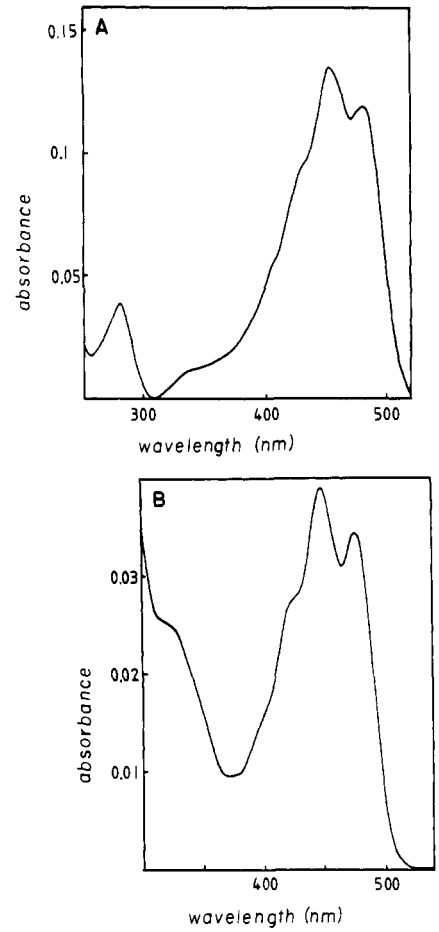

FIGURE 4: Absorption spectra of an unidentified derivative of β-carotene. Collected fractions (peak 3) of separations as described in the legend of Figure 3 from samples A of control (A) or vitamin A deficient rats (B). Solvent for the sample in (A) was the elution solvent described in the legend of Figure 3. For the sample in (B), the solvent was first evaporated and then the residue taken up in *n*-hexane.

is in agreement with β-apo-12′-carotenal. In Table I the amounts of β-apocarotenals formed are summarized together with the yields of β-apocarotenals formed from β-carotene calculated on a molar basis.

Furthermore, during the HPLC screening for carotenoids and retinoids at 330 nm, a derivative of β-carotene formed nonenzymatically in controls and vitamin A deficient rats was detected (compound **3** in Figure 3). Retinyl palmitate elutes in this system at 25 min. The addition of 0.45 mg of linoleic acid to the incubation mixtures A–D during the assays yielded samples in which a peak in a chromatographic system as described in the legend of Figure 3 could no longer be assigned to β-carotene. Absorption spectra of collected fractions of compound **3** (Figure 3) from either controls (Figure 4A) or vitamin A deficient rats (Figure 4B) show the characteristic spectrum of a C_{40}-carotenoid with additional absorption in the ultraviolet indicative of either a secocarotenoid or cis–trans isomerization at the 15,15′ double bond.

Taken together, the experiments with vitamin A deficient rats indicate an influence of the animals' nutritional status on the type of β-apocarotenal formed in the mucosal extract (Figure 2C) and possibly on the modification of the β-carotene molecule (Figure 4B).

DISCUSSION

The question how is β-carotene transformed into vitamin A has great nutritional importance in view of new knowledge of the function of vitamin A and β-carotene (Wolf, 1984; Burton & Ingold, 1984; Hemken & Bremel, 1982; Peto et al., 1980). The complete absence of retinal or its metabolites as possible cleavage products of β-carotene during our attempts to demonstrate β-carotene cleavage activity in extracts from rat intestinal mucosa is striking, particularly in view of the fact that this is the only organ in which β-carotene cleavage had been demonstrated with some confidence (Goodman & Olson, 1969). The results presented above strongly support the absence of any enzymatic cleavage of the β-carotene molecule in rat intestinal mucosa extracts and are in agreement with previous reports that could not demonstrate the enzymatic formation of vitamin A from β-carotene in vitro as reviewed by Glover (Glover, 1960). However, the experiments reported here show that nonenzymatic modification of the β-carotene molecule occurs under the conditions of the assay system described by Goodman and Olson (Goodman & Olson, 1969). The following discussion tries to rationalize our results by summarizing arguments against a central cleavage of the β-carotene molecule.

Biochemical Considerations. It is noteworthy that conditions under which the assay of β-carotene is performed, i.e., elevated temperature of 37 °C and periods of time of up to 1 h (Goodman & Olson, 1969) and occasionally the addition of ferrous ions (Singh & Cama, 1974), are those used to study lipid peroxidation. It is well established that cyclic endoperoxides, which decompose to aldehydes such as malonaldehyde, are formed in these lipid peroxidations (Bird & Draper, 1984). Therefore, the detection of a nonenzymatic

transformation of β-carotene into β-apocarotenals in the assay system for β-carotene cleavage (Goodman & Olson, 1969) in our study (Figure 2 and Table I) is not surprising. These three β-apocarotenals [8′, 10′, and 12′] have also been isolated from intestines of chicken (Sharma et al., 1977), a fact that has been taken as strong support for the enzymatic random cleavage theory (Ganguly & Sastry, 1985). A lipid peroxidation mechanism is corroborated by the finding that the presence of linoleate in the assay mixture (see Results) increased the extent of β-carotene decomposition in the absence as well as in the presence of the intestinal protein fraction.

Furthermore, an enzymatic cooxidation mechanism is known for soybean lipoxygenase, which can oxidize β-carotene in the presence of linoleate (Sumner & Sumner, 1940). Hence, lipoxygenase is often referred to as carotenase (EC 1.13.11.12). The mechanism of cooxidation by lipoxygenase has not been elucidated. However, it is known that the bleaching of β-carotene by lipoxygenase in the presence of linoleate is very fast and leads to colorless products, which must have less than five conjugated double bonds (Klein et al., 1984). The observations that the aldehyde xanthoxin is formed from violaxanthin and β-apocarotenals are formed from β-carotene (Firn & Friend, 1972), indeed, provide evidence for chain cleavage in this reaction. To our knowledge, a lipoxygenase has not been described in the intestine but was reported to occur in the stomach (Howe, 1943). Three types of lipoxygenases have also been located in leukocytes, one of which gives rise to an aldehydic product in arachidonic acid metabolism (Glasgow et al., 1986).

Other enzymes capable of similar cooxidations contain heme, e.g., cytochrome P-450, cyclooxygenase, and peroxidase (Ullrich, 1979; Nastainczyk, 1985; Yamazaki, 1974). A peroxidase as well as cytochrome P-450, including a hexadecane hydroxylation system, has been isolated from the intestine (Lindeskog et al., 1986; Ichihara et al., 1981; Stelmaszyńska & Zgliczyński, 1971; Kimura & Jellinck, 1982). The intestine has also been described as an organ of very high peroxidatic activity (Stelmaszyńska & Zgliczyński, 1971). β-Carotene oxidation has been demonstrated for lactoperoxidase (Ekstrand & Björck, 1986). A role of these enzymes in the metabolism of β-carotene seems plausible and awaits further assays of microsomal and particulate fractions for β-carotene cleavage.

In this context it should be noted that the putative β-carotene 15,15′-dioxygenase at its highest purification never exceeded a specific activity of 1 nmol of retinal formed per hour per milligram of protein at 37 °C (Goodman et al., 1967), corresponding to a turnover number of 3.3×10^{-3} min^{-1}. [This calculation is based on a M_r of 200 000 estimated for β-carotene dioxygenase (Goodman et al., 1967).] For comparison, the turnover of a dioxygenase purified to homogeneity, protocatechuate 3,4-dioxygenase, is 2.5×10^5 min^{-1} at 23 °C (Whittaker et al., 1984). Without taking the difference in assay temperature into account, this turnover number is 10^8-fold higher as compared to that of the β-carotene dioxygenase. Interestingly, a 100% yield of retinal has never been reported and is given as close to 50% under optimal conditions of the assay (Goodman & Olson, 1969). In fact, yields as low as 2% have been observed (Gronowska-Senger & Wolf, 1970).

Methodological Considerations. The occurrence of retinal as a detectable cleavage product in the reported assay for β-carotene cleavage activity (Goodman & Olson, 1969) is highly questionable. A related study (Hansen and Maret, unpublished results) showed that retinal is oxidized to retinoic acid by aldehyde dehydrogenase and aldehyde oxidase from intestinal mucosa of rabbits. In the small intestine of rats we have determined the oxidizing activity toward retinal in the absence of added NAD^+ to be about 20 nmol of retinal oxidized per minute per milligram of protein at 37 °C. This corresponds to an activity 20-fold higher than that of the β-carotene dioxygenase (see above). Retinal oxidizing activity will be present in the F_{20}^{45} fraction used in the β-carotene cleavage assay, since the first step in the isolation of this activity from intestines involves an ammonium sulfate precipitation (Crain et al., 1967; Moffa et al., 1970). In view of these findings the analysis of possible reaction products of β-carotene cleavage was extended to retinol, retinoic acid, and retinyl esters in the present study.

The separation of retinoids and carotenoids by HPLC with subsequent photometric detection has advantages concerning sensitivity and specificity as compared to the analytical procedures of Goodman and Olson (Goodman & Olson, 1969). Sensitivity: A specific radioactivity of [^{14}C]-β-carotene of 2.4×10^5 dpm/μg was previously used (Goodman & Olson, 1969); 0.8 μg of this material was then incubated in an assay for β-carotene cleavage activity (Goodman et al., 1967) and after the assay diluted with 8 μg of carrier β-carotene, corresponding to a final specific radioactivity of 1.9×10^4 dpm/μg or 19 dpm/ng. Since the HPLC system used in the present study detects a few nanograms of retinoids or carotenoids, it has to be considered approximately 10 times more sensitive than previous assays based on radioactivity measurements. Specificity: The HPLC technique provides a more direct means to identify products. Previous methods (Goodman & Olson, 1969) are able to detect an aldehyde, but do not discriminate between the β-apocarotenals and retinal. For instance, it has been shown that thin-layer chromatography on silica gel does not separate β-apocarotenals, including retinal, in the order of their chain length (Stahl, 1967).

Physiological Considerations. It is not well understood how the absorption of β-carotene and vitamin A is regulated. If the supply of vitamin A in the diet is too high, signs of vitamin A poisoning become evident. The absorption of β-carotene, on the other hand, is very different among species (Moore, 1957), and even when levels of β-carotene are high, signs of toxicity have not been observed (Heywood et al., 1985). If there were an enzyme in rat intestinal mucosa which converts β-carotene to vitamin A, then its activity must be very efficiently regulated in view of the potential toxicity of vitamin A and the beneficial role of (Burton & Ingold, 1984; Peto et al., 1980) and possible need for β-carotene (Hemken & Bremel, 1982). Several factors have been reported to control the conversion of β-carotene to vitamin A. Among those are thyroid hormones or zinc, protein, and lipid content of the diet (Moore, 1957; Simpson & Chichester, 1981). A role of thyroxine in the ontogeny of intestinal enzymes has recently been postulated (Henning, 1984). Thyroxine as well as the retinol/retinol binding protein complex binds to transthyretin. On this level a possible influence of thyroxine could exist, though an interdependence of binding of the two ligands could not be demonstrated in vitro (van Jaarsveld et al., 1973).

In this study a search for β-carotene cleavage activity in vitamin A deficient rats has been initiated, since a decrease of the liver vitamin A content was expected to be a possible feedback mechanism of regulation for the cleavage enzyme (Villard & Bates, 1986). The absence of newly formed retinoids in the experiment with vitamin A deficient rats, however, clearly demonstrates that low levels of retinol in the liver do not enhance the activity of a β-carotene cleavage enzyme.

At present it is not clear why different β-apocarotenals are formed in assays with protein from the intestines of vitamin A deficient rats (Figure 2C).

It has never been possible to show that 1 mol of β-carotene has the same biological activity as 2 mol of vitamin A (Brubacher & Weiser, 1985). The closest conversion factor is found in the rat (about 2 μg of β-carotene corresponds to 1 μg of retinol), which is still far from the theoretical value of 1:1. The wide-spread values of conversion factors in humans (on the average 6:1) are thought to reflect the availability and conversion efficiency of β-carotene. The latter has been shown to decrease with increasing intake of β-carotene (Brubacher & Weiser, 1985). The inability to demonstrate a biopotency of β-carotene that corresponds to the expected stoichiometry according to central cleavage corroborates the theory of random cleavage (Ganguly & Sastry, 1985) and is in agreement with a cooxidation mechanism dependent on the nutritional and hormonal status of the species.

Chemical Considerations. From the calculated electron distribution in the β-carotene molecule it has been concluded that the initial site of attack of oxygen is at the β-ionone rings and not at the central double bond (El-Tinay & Chichester, 1970). While it is possible that an enzyme exists that provides the specificity for a central cleavage of the β-carotene molecule by oxygen, there is no precedence for a dioxygenase that cleaves a double bond with concomitant formation of two aldehyde molecules. Enzymatic cleavage has only been observed when activating groups are attached to the double bond, e.g., catechol dioxygenases. Benzene and toluene dioxygenases serve as an example for dioxygenation of nonactivated double bonds. However, these enzyme systems from *Pseudomonas putida* with three protein components catalyze the cis hydroxylation of the benzene ring (Geary et al., 1984; Subramanian et al., 1985), and a cleavage of the benzene ring is not observed. In this regard the putative enzymatic central or random cleavage of β-carotene poses a fascinating mechanistic problem. The postulated dioxetane adduct of the 15,15′ double bond of β-carotene with dioxygen (Goodman et al., 1966; Vartapetian et al., 1966) is certainly not favored (Hamilton, 1974). Its decomposition would be highly exothermic (about 100 kcal/mol), as exemplified by the firefly luciferase reaction, where the energy from the decomposition of the dioxetane intermediate is released as bioluminescence.

The present investigation does not challenge the conversion of β-carotene to vitamin A in vivo. Because of the great significance of carotenoids and retinoids for human health, establishing the mode of conversion remains the most important step toward understanding the multiple functions of these substances.

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Registry No. β-Carotene, 7235-40-7; retinal, 116-31-4; vitamin A, 68-26-8.

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Inhibition of Phosphatase and Sulfatase by Transition-State Analogues†

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ABSTRACT: The inhibition constants for vanadate, chromate, molybdate, and tungstate have been determined with *Escherichia coli* alkaline phosphatase, potato acid phosphatase, and *Helix pomatia* aryl sulfatase. Vanadate was a potent inhibitor of all three enzymes. Inhibition of both phosphatases followed the order $WO_4^{2-} > MoO_4^{2-} > CrO_4^{2-}$. The K_i values for potato acid phosphatase were about 3 orders of magnitude lower than those for alkaline phosphatase. Aryl sulfatase followed the reverse order of inhibition by group VI oxyanions. Phenol enhanced inhibition of alkaline phosphatase by vanadate and chromate but did not affect inhibition of acid phosphatase. Phenol enhanced inhibition of aryl sulfatase by metal oxyanions in all cases following the order $H_2VO_4^- > CrO_4^{2-} > MoO_4^{2-} > WO_4^{2-}$, and *N*-acetyltyrosine ethyl ester enhanced inhibition of aryl sulfatase by $H_2VO_4^-$ and CrO_4^{2-} more strongly than did phenol. It is apparent that the effectiveness of metal oxyanions as inhibitors of phosphatases and sulfatases can be selectively enhanced in the presence of other solutes. The relevance of these observations to the effects of transition metal oxyanions on protein phosphatases in vivo is discussed.

Phosphotyrosine protein kinase activity has been linked to the action of insulin (Rosen et al., 1983), growth factors (Ushiro & Cohen, 1980), and oncogenes (Sefton et al., 1982). Control of phosphotyrosine protein phosphatases, which inactivate the kinases and oppose their action, is very likely important in these biological processes (Gresser et al., 1987). Both mammalian alkaline (Swarup et al., 1981) and acid phosphatases (Lau et al., 1985) have been implicated in the control of protein phosphotyrosine content, but the specificity of cellular protein phosphatases is poorly understood (Sparks & Brautigan, 1985, 1986).

Vanadate mimics the effects of insulin (Heiliger et al., 1985; Tamura et al., 1984) and epidermal growth factor (Dubyak & Kleinzeller, 1980; Smith, 1983; Carpenter, 1981) and affects various other physiological processes in which phosphorylation of tyrosine is thought to be important (Nechay et al., 1986). There have been some reports that vanadate selectively inhibits phosphotyrosine phosphatase relative to phosphoserine or phosphothreonine phosphatases (Leis & Kaplan, 1982; Leis et al., 1985; Swarup et al., 1982a,b; Nelson & Branton, 1984; Klarlund, 1985), but is also appears that vanadate is not an equally potent inhibitor for all phosphotyrosine phosphatases (Brunati & Pinna, 1985). The mechanism for the selective inhibition of protein phosphatases by vanadate is not clear, and no explanation for it has been advanced. The work reported here was undertaken in an effort to test one hypothesis which provides a rationalization of this behavior. The hypothesis being tested is that a vanadate ester which resembles the substrate should, in some cases, be a more potent inhibitor of the phosphatase than vanadate alone. In cases for which this is true, inhibition of a given phosphatase by vanadate will be stronger in the presence of the dephosphorylated substrate of the phosphatase if it forms a vanadate ester. Proteins which, when phosphorylated, are the best substrates for a phosphatase

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