Mechanism of the Biosynthesis of Vitamin A from β-Carotene*

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SUMMARY

Experiments were conducted to determine whether the hydrogen atoms attached to the 2 central carbon atoms of β-carotene are retained or are lost during the conversion of β-carotene to vitamin A. Doubly labeled β-carotene, uniformly labeled with 14C throughout the molecule but specifically labeled with 3H only at the central carbon atoms (C-15 and C-15'), was fed to each of two lymph fistula rats. Lymph was collected and the retinyl esters isolated. The ratio of 3H to 14C in the retinyl esters from lymph, and in the retinol obtained from these esters by saponification, was identical with the ratio of 3H to 14C in the dietary β-carotene. This indicated that there was no loss of 3H relative to 14C during the conversion of β-carotene to vitamin A. Complete retention of 3H relative to 14C was also found during the conversion in vitro of doubly labeled β-carotene to retinal with homogenates of rat intestinal mucosa. The hydrogen atoms attached to the central carbon atoms of β-carotene must therefore be retained completely during the biosynthesis of vitamin A.

In order to verify that the 3H label was attached only to the central carbon atoms of β-carotene, the retinol (from lymph) was oxidized to retinoic acid by a two-step procedure. This oxidation was accompanied by a complete loss of 3H label from the molecule.

The biosynthesis of vitamin A from β-carotene is most likely a dioxygenase reaction, in which molecular oxygen reacts with the 2 central carbon atoms of β-carotene, followed by the cleavage of the central double bond of β-carotene to yield 2 molecules of retinal.

The biosynthesis of vitamin A from β-carotene was first conclusively shown by Moore by means of experiments in which β-carotene was fed to vitamin A-deficient rats (2). Subsequent examination of these rats showed that the feeding of β-carotene had produced a definite increase in the vitamin A content of their livers.

Since then, the conversion of β-carotene to vitamin A has been shown with a variety of preparations, including lymph fistula rats and pigs (3, 4), rat intestinal loops (5), rat intestinal slices (6), and isolated perfused rat livers (7). Despite these studies, however, detailed information about the mechanism of the conversion of β-carotene to vitamin A has not been available.

We have recently reported the active conversion of β-carotene to retinal with cell-free homogenate fractions from rat intestinal mucosa (4). Molecular oxygen and bile salts were required for the reaction to take place. Calculations based on the stoichiometry of the results indicated that the reaction mechanism involved the central cleavage of β-carotene into 2 molecules of retinal.

We now report the results of experiments designed to examine the question of whether the hydrogen atoms attached to the 2 central carbon atoms of β-carotene are retained or are lost during the conversion of β-carotene to vitamin A.

EXPERIMENTAL PROCEDURE

Labeled β-Carotene—Uniformly labeled 14C-β-carotene was produced biosynthetically from a mixture of 1-14C-acetate and 2-14C-acetate by the fungus Phycomyces blakesleeanus according to the method of Lilly et al. (8). The β-carotene was isolated and purified as described previously (9), including chromatography on alumina (grade III) followed by recrystallization from hexane. The specific radioactivity was 0.107 μC per μg.

β-Carotene-15, 15'-3H2 was the generous gift of Dr. R. Blomstrand of Stockholm, Sweden, who had previously received it as a gift from Dr. U. Gloor of Hoffmann La Roche of Basle, Switzerland. The β-carotene-15, 15'-3H2 had been prepared by partial hydrogenation with tritium gas of carotinin, the 15, 15'-acetylenic derivative of β-carotene. The specific radioactivity of this preparation was 1.09 μC per μg. It was chromatographed on a column of alumina just prior to its use.

Doubly labeled β-carotene, uniformly labeled with 14C and specifically labeled with 3H only at the center of the molecule (C-15 and C-15'), was prepared by mixing together approxi-
mately $3 \times 10^6$ cpm of the above $^{14}$C-$\beta$-carotene, 1.1 $\times 10^6$ cpm of the above $^{3}$H-$\beta$-carotene, and 200 $\mu$g of purov, unlabeled, recently recrystallized $\beta$-carotene. The carotene was then serially recrystallized twice without change in its specific radioactivity or in its ratio of $^{3}$H to $^{14}$C counts per min.

A second preparation of doubly labeled $\beta$-carotene, for use in incubations in vitro, was subsequently prepared in an identical manner. This second preparation had a slightly lower ratio of $^{3}$H to $^{14}$C counts per min.

**Experiment in Vivo**—Doubly labeled $\beta$-carotene (first preparation). 220 $\mu$g, and 2 mg of $\alpha$-tocopherol were dissolved in 0.7 ml of olive oil, and 0.3 ml of the resultant mixture was fed by gastric intubation to each of two rats whose cisternae chyli had been cannulated the previous day. Lymph was collected on ice for 9 hours. A carrier mixture of nonradioactive retinol, retinal, retinoic acid, $\beta$-carotene, and a mixture of retinyl esters (see Reference 9) was added to each sample, and the entire lymph sample was extracted with chloroform-methanol (2:1, v/v) as described previously (9). The total lipid extract contained 3 to 4% of the fed radioactivity in each case.

Portions of the total lipid extract of each sample were chromatographed on columns of alumina as described previously (9). Five fractions were collected from each column, namely: Fraction 1, $\beta$-carotene; Fraction 2, retinyl esters; Fraction 3, retinal; Fraction 4, retinol; Fraction 5 (+6), more polar compounds including retinoic acid. Portions of each fraction were assayed for radioactivity.

Part of the retinyl ester fraction of one of the samples was saponified under nitrogen at 90$^\circ$ for 1½ hours in 2.2% KOH in 80% ethanol. After addition of an equal volume of water and of 0.5 mg of pure carrier retinol, the nonsaponifiable fraction was extracted with hexane followed by purification of retinol by chromatography on a column of alumina.

Retinol was oxidized to retinal with MnO$_2$ (10) and the retinal was purified by chromatography on a column of alumina.

Retinal was enzymically oxidized to retinoic acid with rat liver aldehyde oxidase with the use of a modification of the method described by Mahadevan, Murthy, and Ganguly (11). The enzyme preparation consisted of that portion of the soluble protein fraction (104,000 $\times g$ supernatant) of a rat liver homogenate which precipitated with ammonium sulfate between 0 and 45% saturation. In order to oxidize retinal, an incubation was carried out containing 48 mg of enzyme protein (dialyzed extensively against 0.01 M potassium phosphate buffer, pH 7.4, just before use), 300 moles of potassium phosphate buffer, pH 7.4, 90 moles of nicotinamide, 12 moles of MgCl$_2$, 200 moles of glutathione, and 3 moles of NADH. Substrate (labeled retinal, 0.4 mg) was added in solution in 100 $\mu$l of acetone. The final volume was 3 ml. After incubation at 37$^\circ$ for 2 hours in the dark, with room air as gas phase, the incubation mixture was extracted with the solvent mixture described by Dole (12), but containing n-hexane instead of heptane. Retinoic acid was separated from unreacted aldehyde by extracting the hexane solution with 0.1 $\times$ NaOH in 50% ethanol. The retinoic acid was subsequently recovered by acidification of the ethanol-NaOH solution followed by extraction of acids therefrom with light petroleum ether.

**Experiment in Vitro**—Incubations were carried out as described elsewhere (1) in order to effect the conversion of doubly labeled $\beta$-carotene to retinal. Each incubation flask contained, as enzyme, 10 mg of protein of a 10,000 $\times g$ supernatant of a homogenate of rat intestinal mucosa plus 200 $\mu$moles of potassium phosphate buffer, pH 7.7, 30 $\mu$moles of nicotinamide, 12 $\mu$moles of sodium taurocholate, 10 $\mu$moles of glutathione, and 1 mg of $\alpha$-tocopherol added in solution in 50 $\mu$l of acetone. The final volume was 2 ml. Substrate doubly labeled $\beta$-carotene (1 $\mu$g of the second preparation) was added in solution in 50 $\mu$l of acetone, and the mixture was incubated at 37$^\circ$ in the dark with room air as gas phase. After 1 hour, the incubation mixture was extracted (see Reference 1) and the lipid extract chromatographed on alumina.

**Radioassay**—Samples were dissolved in 15 ml of 0.5% diphenyloxazole in toluene and assayed with a Packard Tri-Carb liquid scintillation counter. All of the samples were reassayed twice, after the addition of $^{14}$C-toluene, and then after addition of $^{3}$H-toluene, as internal standards, in order to obtain a quantitatively accurate simultaneous assay for $^{14}$C and for $^{3}$H. The observed counting efficiency was 50% for $^{14}$C and 18% for $^{3}$H.

The sources of all of the materials and compounds used in this study were described previously (9).

**RESULTS**

**Experiment in Vivo**—The retinyl ester fractions (Fraction 2) contained 91% and 87%, respectively, of the radioactivity recovered in the two lymph extracts. Radioassay of portions of each retinyl ester fraction showed that in both cases the ratio of $^{3}$H to $^{14}$C was almost the same as that found in the dietary doubly labeled $\beta$-carotene (see Table I).

Part of the retinyl ester fraction from one of the lymph samples was saponified, and retinol was subsequently isolated from the nonsaponifiable fraction. This retinol contained the same ratio of $^{3}$H to $^{14}$C as was seen in the dietary $\beta$-carotene (see Table I).

These results indicate that there was no loss of $^{3}$H relative to $^{14}$C during the conversion of doubly labeled $\beta$-carotene into vitamin A.

In order to verify that the $^{3}$H label was in fact originally attached entirely to the 2 central carbons of $\beta$-carotene (C-15 and C-15'), part of the doubly labeled retinol, obtained by saponification of the lymph retinyl esters, was oxidized to retinoic acid. This oxidation removes the hydrogen atoms attached to the terminal carbon (C-15) of retinol, and hence should remove all of the $^{3}$H originally present at C-15 and C-15' of $\beta$-carotene. Because of the very small amount of retinol involved, oxidation to retinoic acid was carried out in a two-step procedure: (a) chemo-

<table>
<thead>
<tr>
<th>Table I</th>
<th>Isotope ratios in experiment in vivo</th>
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<tr>
<td>Compound and source</td>
<td>Observed ratio of $^{3}$H to $^{14}$C</td>
</tr>
<tr>
<td>$\beta$-Carotene</td>
<td>$^{3}$H to $^{14}$C</td>
</tr>
<tr>
<td>Experimental diet</td>
<td>3.70</td>
</tr>
<tr>
<td>Retinyl ester fraction</td>
<td>3.48</td>
</tr>
<tr>
<td>Lymph of rat No. 1</td>
<td>3.51</td>
</tr>
<tr>
<td>Lymph of rat No. 2</td>
<td>3.74</td>
</tr>
<tr>
<td>Retinol</td>
<td>0.00</td>
</tr>
<tr>
<td>Saponification of retinyl esters</td>
<td></td>
</tr>
<tr>
<td>Lymph (rat No 1)</td>
<td></td>
</tr>
<tr>
<td>Retinoic acid</td>
<td></td>
</tr>
<tr>
<td>Oxidation (two-step) of retinol</td>
<td></td>
</tr>
</tbody>
</table>

The sources of all of the materials and compounds used in this study were described previously (9).
ical oxidation to retinal, and (b) enzymic oxidation of retinal to retinonic acid. The yields for the two steps were 87% and 73%, respectively, as calculated from the recovery of 14C in the desired product. Radioassay of the retinonic acid showed the presence of only 14C in the molecule (3H to 14C ratio of 0.00, see Table I). This established that the 3H in retinol had indeed been attached to the terminal carbon (C-15), and hence had originally been attached entirely to the 2 central carbon atoms (C-15 and C-15') of β-carotene. It should be noted that this conclusion would not be valid if there had occurred an absolute discrimination against 3H-containing molecules during the conversion of retinol to retinonic acid. Some isotopic discrimination did occur during both steps in the conversion of retinol to retinonic acid. Thus, after chemical oxidation of retinol to retinal, the ratio of 3H to 14C counts per min in the product retinal was 2.7 instead of the theoretical 1.87; in addition, after enzymic oxidation of retinol to retinonic acid the ratio of 3H to 14C in the small amount of retinol remaining at the end of the incubation had risen to approximately 4. These data indicate that during the conversion of retinol to retinal the oxidation reaction preferentially removed normal hydrogen atoms, rather than tritium atoms, attached to C-15. This resulted in 3H enrichment in the product retinal. The alternative conclusion that the 3H enrichment resulted from the presence of some 3H at positions other than C-15 is excluded by the finding that no 3H remained in the subsequently formed retinonic acid. During the enzymic oxidation of retinol to retinonic acid some isotopic discrimination against 3H-labeled molecules occurred. The extent of this discrimination, however, was not sufficient to account for the complete absence of 3H in the retinonic acid, in view of the yield obtained. Furthermore, the occurrence of isotopic discrimination in each of these reactions is itself evidence supporting the location of the tritium label on the terminal carbon (C-15) of retinol, since both reactions involved oxidations affecting only the terminal carbon atom.

**Experiment in Vitro**—The results listed in Table II show that there was no loss of 3H relative to 14C during the conversion of doubly labeled β-carotene to retinal with an enzymatically active homogenate fraction from rat intestinal mucosa.

### Discussion

The results presented here show that the hydrogen atoms attached to the 2 central carbon atoms of β-carotene are entirely retained during the conversion of β-carotene into vitamin A. This conclusion derives from the finding that there was no loss of tritium, relative to 14C, during the conversion of doubly labeled β-carotene to vitamin A. Since the β-carotene was uniformly labeled with 14C, but was specifically labeled with 3H at the 2 central carbon atoms, removal of one or both of the hydrogen atoms attached to the central carbon atoms of β-carotene during the biosynthesis of vitamin A would have been reflected in a corresponding loss of tritium, relative to 14C, in the vitamin A. Complete retention of 3H, relative to 14C, was, however, found, both during the conversion in *vivo* of β-carotene to vitamin A in the lymph fistula rat, and during the conversion in *vivo* of β-carotene to retinal. Furthermore, the fact that the 3H was specifically attached to the 2 central carbon atoms of β-carotene was verified by oxidation of some of the newly synthesized vitamin A to retinonic acid; as anticipated, this resulted in the complete loss of 3H from the molecule.

We have recently shown that the over-all reaction of vitamin A biosynthesis consists of the central cleavage of β-carotene into 2 molecules of retinal (1). Retinal is then reduced to retinol, which in turn is esterified prior to its absorption and transport via the intestinal lymphatics (1, 9). The results presented here indicate that the hydrogen atoms attached to the 2 central carbon atoms of β-carotene are not removed during these reactions. Since molecular oxygen is required for the conversion in *vivo* of β-carotene to retinal (1), it seems highly likely that the mechanism of this reaction involves a direct reaction between molecular oxygen and the 2 central carbon atoms of β-carotene, followed by the cleavage of the central double bond of β-carotene, to yield 2 molecules of retinol. The biosynthesis of vitamin A from β-carotene is hence most likely a dioxygenase reaction, and can be formulated as follows.

\[
\begin{align*}
\text{H} & \rightarrow \text{O} + \text{O} \rightarrow \left[ \begin{array}{c} \text{H} \\ \text{C} \end{array} \right] & \rightarrow & \left[ \begin{array}{c} \text{H} \\ \text{C} \end{array} \right] & \rightarrow & \left[ \begin{array}{c} \text{H} \\ \text{C} \end{array} \right] \\
\text{14C} & \rightarrow & \text{O} & \rightarrow & \text{O} & \rightarrow & \text{H}
\end{align*}
\]

The fine details of this dioxygenase reaction, i.e., whether the initial step consists of a hydroperoxylation or of a concerted reaction involving both carbon atoms and molecular oxygen, whether there is a cyclic peroxide intermediate as pictured above, etc., cannot as yet be defined. The outline of the over-all reaction seems, however, clearly established. Complete proof for this formulation will require the demonstration that the oxygen atom in vitamin A is directly derived from molecular oxygen.

**Acknowledgments**—We are grateful to Mrs. Joan Vormbaum for expert assistance during this work. We wish to thank Dr. Rolf Blomstrand of Stockholm, Sweden, for the gift of β-carotene-15,15'-3H2.

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**Table II**

<table>
<thead>
<tr>
<th>Sample analyzed</th>
<th>Yield of retinal 3H</th>
<th>Yield of retinal 14C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate β-carotene-14C, 3H</td>
<td>46%</td>
<td>4.01</td>
</tr>
<tr>
<td>β-Carotene from control flask</td>
<td>3.33</td>
<td></td>
</tr>
<tr>
<td>Reaction product: retinal*</td>
<td>3.28</td>
<td></td>
</tr>
<tr>
<td>Experimental Flask 1</td>
<td>3.27</td>
<td></td>
</tr>
<tr>
<td>Experimental Flask 2</td>
<td>3.21</td>
<td></td>
</tr>
</tbody>
</table>

* Assayed as Fraction 3 after alumina column chromatography.

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Note Added in Press—While the above manuscript was awaiting publication, Olsen and Hayashi reported (13) the conversion of β-carotene to retinal in *vivo*, in yields generally in the range of 5%, by soluble protein fractions of rat liver and intestinal homogenates. These workers postulated that the mechanism of this conversion involved a dioxygenase reaction between the 2 central carbon atoms of β-carotene and molecular oxygen, followed by the central cleavage of β-carotene into 2 molecules of retinal. We are in agreement with this postulated mechanism which is identical with the one suggested in the present manuscript and is supported by the evidence presented here.
REFERENCES

Mechanism of the Biosynthesis of Vitamin A from $\beta$-Carotene
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