ON METABOLIC PATHWAYS OF CAROTENES TO VITAMIN A IN ANIMALS

I. POSSIBLE INTERMEDIATES IN THE METABOLIC CONVERSION OF β -CAROTENE TO VITAMIN A

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In a previous paper (1), a procedure using paper partition chromatography for microanalysis of fat-soluble vitamins has been described, in which freshly prepared filter paper soaked with inorganic complex compounds, *e.g.*, $\text{Ca}(\text{H}_2\text{PO}_4)_2\text{-NH}_4\text{OH}$, $\text{Al}_2(\text{SO}_4)_3\text{-NH}_4\text{OH}$ or $\text{MgSO}_4\text{-NH}_4\text{OH}$, is used. Using this method, vitamin A in animal tissue has been found to be formed from β -carotene by tissue homogenate of various animals (2), and the researches in our laboratory have been extended to the enzymic study on the conversion of β -carotene to vitamin A in animals, whereby some intermediates were found to be produced (3).

It is well known that many hypothetical pathways for the conversion of β -carotene to vitamin A have been proposed but no satisfactory experimental evidence appears to have hitherto been presented. Recently, the authors have attempted to elucidate the metabolic pathways in animals. At first, β -carotene was given orally to rats, rabbits as well as frogs, and the distribution of the metabolic products in various tissues were tested by inorganic complex salt-impregnated paper chromatography, using either $\text{Ca}(\text{H}_2\text{PO}_4)_2\text{-NH}_4\text{OH}$ or $\text{Al}_2(\text{SO}_4)_3\text{-NH}_4\text{OH}$. Three organic compounds were proved to be present after spraying with Carr-Prince reagent. Vitamin A alcohol was first found as a spot, moving slowly in benzene, and two kinds (I, II) of chloroform-soluble compounds were isolated from the start of the paper chromatograms. Absorption maxima of the latter substances were consistent with β -ionone, 280 $\text{m}\mu$ in petroleum ether, and vitamin A aldehyde-protein-complexes, 365 $\text{m}\mu$ in petroleum ether, respectively. Comparing with pure synthetic β -ionone (I) freshly prepared, the one substance was identified spectrophotometrically as β -ionone, absorption maximum being 296 $\text{m}\mu$ in ethanol. β -Ionone was further found to be obtainable from vitamin A alcohol by treatment with MnO_2 in boiling petroleum ether. The second compound (II) was proved to be a kind of vitamin A aldehyde-protein-complex by paper chromatography. It was proved to be convertible to vitamin A alcohol by tissue homogenate. Standard vitamin A aldehyde, having an absorption peak at 369.5 $\text{m}\mu$ in petroleum ether, was again freshly prepared from vitamin A alcohol by oxidation with MnO_2 at 20° in

petroleum ether, and the synthetic aldehyde thus obtained was also confirmed to be convertible to vitamin A alcohol by tissue homogenate.

These findings lead to a conclusion that the plausible metabolic pathways of β -carotene to vitamin A in tissue may be the following.

EXPERIMENTAL AND RESULTS

1. Oral Administration of β -Carotene

Rats weighing 100–200 g, rabbits weighing 3–4 kg, and frogs weighing 30–50 g, were first kept fasting overnight, and the animals received orally 5–20 ml of β -carotene in Tween 80-aqueous dispersion (3 mg of β -carotene and

TABLE I
Paper Chromatographic Observation of Metabolic Intermediates after Oral Administration of β -Carotene.

R_F	Compounds present in paper chromatogram	Using the intestine homogenate of		
		Rat	Rabbit	Frog
0.0	Compound I and II	+	+	+
0.6	Vitamin A ester	+	+	+
0.8	Carotene	+	+	+

+ indicates the presence of the compound in paper chromatograms. Also the same in the following Table.

FIG. 1 *Paper Chromatograms of Metabolic Intermediates after Oral Administration of β -Carotene.*

A, standard vitamin A and β -carotene; B, rat; C, rabbit; D, frog; E, negative control (using intestine alone)

30 mg of Tween 80 in 100 ml of water) by catheterization. After 5–12 hours the animals were killed and the digestive tracts were rapidly homogenized. To the homogenate was added a few ml of water, and the mixture thus obtained was extracted with 10–20 ml of petroleum ether or chloroform. The solvent layer was completely washed with water and dried over anhydrous sodium sulfate. The solvent was evaporated under diminished pressure and the syrupy concentrate thus obtained was tested by paper chromatography with $\text{Ca}(\text{H}_2\text{PO}_4)_2\text{-NH}_4\text{OH}$ paper, using petroleum benzene as a mobile phase. The results are shown in Table I and Fig. 1.

The spots, R_F 's 0.60 and 0.8, were identified as vitamin A ester and β -carotene respectively. The spot, R_F 0.0, was cut out of the paper, 2 cm in length, and the metabolic products present at the start were eluted with

FIG. 3 *Paper Chromatograms of Metabolic Intermediates of β -Carotene by Tissue Homogenate in Vitro.*

A, rats; B, rabbit; C, frog; D, negative control.

FIG. 2 *Absorption Spectrum of the Compound Appearing at the Start of Paper Chromatograms.*

FIG. 4 *Absorption Spectrum of the Compound Present at the Start of Paper Chromatogram.*

petroleum ether or chloroform. The intermediate substance isolated here was shown to have an absorption maximum at $365\text{ m}\mu$ in pure ethanol (Fig. 2).

2. Experiments with Tissue Homogenates from Various Animals

The intestine homogenates were prepared by grinding in a mortar with Ringer-Locke solution or 0.25 M sucrose solution as usual. To $0.5\text{--}1.0\text{ g}$ of each tissue homogenate was added 5 ml of an aqueous dispersion of β -carotene freshly prepared from 3 mg of β -carotene crystal and 30 mg of Tween 80 in 100 ml . After $1\text{--}3$ hours, the reaction mixture was thoroughly extracted with petroleum ether or chloroform. The solvent was evaporated *in vacuo*, and the concentrate thus obtained was tested as described above. The results are shown in Table II, and Figs. 3 and 4.

TABLE II
Paper Chromatograms of Metabolic Intermediates by Intestine Homogenate from Various Animals *In Vitro*.

R_F	Compounds present in paper chromatogram	Using the intestine homogenate of		
		Rat	Rabbit	Frog
0.0	Compound I and II	$\pm$	$\pm$	+
0.6	Vitamin A ester	+	+	+
0.8	β -Carotene	+	+	+

3. Isolation and Identification of Metabolic Products

Isolation was performed by one-dimensional ascending paper chromatography, using $\text{Ca}(\text{H}_2\text{PO}_4)_2\text{-NH}_4\text{OH}$ or $\text{Al}_2(\text{SO}_4)_3\text{-NH}_4\text{OH}$ paper and petroleum benzine

FIG. 5 Absorption Spectra of the Degradation Products Appearing on Paper Chromatograms when Vitamin A Alcohol (a) or β -Carotene (b) was Treated with MnO_2 .

Vitamin A alcohol or β -carotene (10 mg)
 ↓ dissolved in 50 ml of petroleum ether and
 boiled with 200 g of MnO_2 for 5 hr.
 Precipitate
 ↓ extracted with petroleum ether or
 chloroform
 Degradation products (solution)
 ↓ concentrated under diminished pressure
 to a syrupy mass
 Reddish-brown substance
 ↓
 Paper chromatography

FIG. 6 Degradation of Vitamin A Alcohol
 and β -Carotene with MnO_2

Parallel experiments were performed for the preparation of β -ionone from 10 mg of natural vitamin A alcohol and β -carotene by treatment with 200 mg of MnO_2 (4) in boiling petroleum ether for 5 hours (Fig. 6). The precipitate in the reaction mixture was filtered, and extracted with petroleum ether or chloroform. Petroleum ether-sparingly-soluble material thus obtained gave an absorption maximum at 280 $\text{m}\mu$ in petroleum ether, at 295 $\text{m}\mu$ in chloroform and at 296 $\text{m}\mu$ in ethanol. The Compound I was identified as β -ionone by paper chromatography, comparing with a synthetic sample (Fig. 7).

β -Ionone was prepared from lemon grass oil. After repeated rectifications it gave an absorption maximum at 296.5 $\text{m}\mu$ in ethanol, ϵ being 10030, whereas the absorption maximum of β -ionone has been reported to lie at 296 $\text{m}\mu$ in ethanol, ϵ being 10700.

4. Production of a Metabolic Product II.

The Compound II was separated from the chloroform-soluble portion. It shows a purplish-red coloration by Carr-Price reagent, and has an absorption maximum at 365 $\text{m}\mu$ in ethanol. Standard vitamin A aldehyde, having an absorption maximum at 369.5 $\text{m}\mu$ in ethanol, was prepared from vitamin A

FIG. 7 Absorption Spectrum of Pure Synthetic β -Ionone.

— Petroleum ether - - - Ethanol
 Chloroform

as a developing solvent. At the end of experiment, the spots at R_F 0.0 were cut out of the paper, 2 cm in length, and the metabolic products were extracted with petroleum ether or chloroform. The Compound I, which was isolated, was sparingly soluble in petroleum ether and gave purplish-red coloration by Carr-Price reagent and showed an absorption maximum at 280 $\text{m}\mu$ in petroleum ether and 290–298 $\text{m}\mu$ in ethanol (Fig. 5).

Vitamin A acetate (50 mg)
 ↓ 10 ml of N KOH alcoholic solution was
 added and saponified for 30 min.
 Reaction mixture
 ↓ 300 ml of water was added and extracted
 with 100 ml of petroleum ether
 Vitamin A alcohol freshly prepared
 in petroleum ether
 ↓ treated with 1–2 g of freshly prepared
 MnO_2 at 20° for 10 days
 Filtrate
 ↓ concentrated by evaporation *in vacuo*
 Orange-red syrupy substance
 ↓
 Paper partition chromatography

FIG. 8 Preparation of Standard Vitamin A Aldehyde Freshly Liberated from Vitamin A Acetate.

alcohol by treating with freshly prepared MnO_2 at 20° for 10 days in petroleum ether by Katsui (5) as shown in Figs. 8–9. The metabolic intermediate II was tested for vitamin A aldehyde, but the result of repeated paper chromatography was negative.

5. Determination of Vitamin A Aldehyde-Protein-Complex.

Intestine homogenate was prepared by grinding in a mortar with Ringer-Locke solution or 0.25 *M* sucrose solution. To 0.5–1.0 g of each tissue homogenate was added 5 ml of an aqueous dispersion of vitamin A aldehyde freshly prepared from 3 mg of vitamin A aldehyde crystal and 30 mg of Tween 80 in 100 ml. After 1–3 hours, the reaction mixture was thoroughly extracted with petroleum ether or chloroform. The solvent was removed under diminished pressure, and the resultant residue thus obtained was tested for identifying the metabolic product II.

In further experiments the enzymic investigation of the metabolic product II was attempted by treating the substance extracted from the spot with rat intestine homogenate and it was found to be convertible to vitamin A ester.

Parallel experiments were carried out for the metabolic conversion of synthetic vitamin A aldehyde to vitamin A alcohol, with a positive result as shown in Figs. 10 and 11.

FIG. 10 Paper Chromatograms Showing the Conversion of Synthetic Vitamin A Aldehyde to Vitamin A by Intestive Homogenate in Vitro.

A, standard vitamin A and vitamin A aldehyde; B, before incubation; C, after incubation; D, negative control.

FIG. 9 Absorption Spectrum of Vitamin A Aldehyde Prepared from Vitamin A Alcohol with MnO_2 .

FIG. 11 Absorption Spectrum of the Compound Appearing at R_F 0.6 in Paper Chromatogram.

SUMMARY

1. Metabolic conversion of β -carotene to vitamin A was studied by oral administration to rats, rabbits as well as frogs, and several unknown compounds were found on paper chromatograms.

2. Further studies on metabolic conversion of β -carotene to vitamin A *in vitro* by rat tissue homogenate, revealed two metabolic intermediates in paper chromatograms.

Compound I with an absorption maximum at 296 m μ in ethanol was identified as β -ionone, and the Compound II with an absorption maximum at 365 m μ in petroleum ether was identified as a kind of vitamin A aldehyde-protein-complex.

These findings lead to the plausible conclusion that vitamin A aldehyde-protein-complex may be an intermediate in the metabolic conversion of β -carotene to vitamin A alcohol.

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