Absorption, transport, and storage of retinyl-15-^{14}C palmitate-9,10-^{3}H in the rat

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ABSTRACT Retinyl-15-^{14}C palmitate-9,10-^{3}H was fed to rats in order to study hydrolysis and reesterification of this ester during digestion, absorption, transportation, and storage. After administration there was a progressive increase in the ^{14}C/^{3}H ratio of the retinyl esters as they moved from intestinal contents to intestinal mucosa, lymph, and liver, which indicates that repeated hydrolysis and reesterification occur during the digestion and assimilation of this ester.

KEY WORDS retinyl ester · palmitate · hydrolysis · reesterification · absorption · lymph transport · liver · intestinal mucosa · microsomes · retinol · vitamin A · rat

THE MECHANISM for the intestinal absorption of retinyl esters has been the subject of investigation for many years (1–7). At present the consensus seems to be that prior to absorption the esters are hydrolyzed by a digestive esterase(s) (8, 9) and that the major portion of absorbed retinol is reesterified in the mucosal cell (10). This esterification apparently takes place primarily in the microsomal fraction of the cell (8, 9). Palmitic acid seems to be the fatty acid most commonly involved in this esterification process. During absorption retinol is found largely in the particulate fraction, whereas retinyl ester is concentrated mainly in the soluble fraction (10) of the mucosal cell. Thus it appears that the intestinal absorption of retinyl esters involves some degree of both hydrolysis and reesterification, but the magnitude of these processes is at present not clear.

The aim of this investigation was to follow the extent of hydrolysis and reesterification of retinyl ester during digestion, absorption, transport, and storage. This was accomplished by isolating retinyl esters from intestinal contents, mucosa, lymph, and liver of rats fed doubly-labeled retinyl-15-^{14}C palmitate-9,10-^{3}H and comparing the ^{14}C/^{3}H ratios of the retinyl ester isolated from these sites with that of the respective starting material.

MATERIALS AND METHODS

Preparation of Retinyl Palmitate

The retinyl-15-^{14}C palmitate-9,10-^{3}H (hereafter referred to as labeled retinyl palmitate) was synthesized according to the method of Baxter and Robeson (11) and purified by chromatography over deactivated alumina (12). All samples used were more than 90% pure.

Preparation of Corn Oil Solution of Retinyl Palmitate

Approximately 10 mg of labeled retinyl palmitate was dissolved in 25 ml of petroleum ether. A 1 ml aliquot was taken to determine the radioactivity of the sample and the remainder was evaporated to approximately 2 ml under a stream of nitrogen. Approximately 2 g of corn oil was added and the remaining petroleum ether was evaporated under nitrogen.

Administration of Reitnyl Ester

Albino, female rats weighing 175–200 g and fasted for 18 hr were anesthesized with ether and dosed by stomach tube with labeled retinyl palmitate dissolved in corn oil. Approximately 3 mg of this ester (1,300,00 cpm of ^{14}C and 700,000 cpm of ^{3}H) was administered to each rat. The rats were again anesthesized with ether $^{1}/_{2}$, 3, and 24 hr after feeding, and the small intestines were ligated at a point $^{3}/_{4}$ inch distal to the ligament of Treitz and at a point 18 inches beyond the first ligature. These intestinal segments and livers were quickly removed and immediately prepared for lipid extraction or cell fractionation.

Collection of Lymph from Thoracic Duct

The thoracic duct was cannulated by the method of Boll-

man and coworkers (13, 14). The retinyl ester was administered as previously described. Lymph was collected hourly for the first 6 hr and then for 6–12 and 12–24 hr periods after dosing.

Preparation of Intestinal Segment for Cellular Fractionation

The intestinal segments were washed first with 3–10 ml portions of cold 0.25 M sucrose, then once with 2 ml of corn oil and finally again with 10 ml of the sucrose solution. The washings, excluding the one with corn oil, were pooled as the intestinal contents. After washing, the mucosa was scraped with a glass slide, weighed, and diluted with 0.25 M sucrose (10 volumes) containing 0.003 M calcium chloride. The mucosae of three rats were pooled and homogenized, and cellular fractions were prepared essentially as reported by Shelton, Krause, and Gross (15) with a major modification in the preparation of the nuclear fraction (Fig. 1). Supernatant fractions I and II

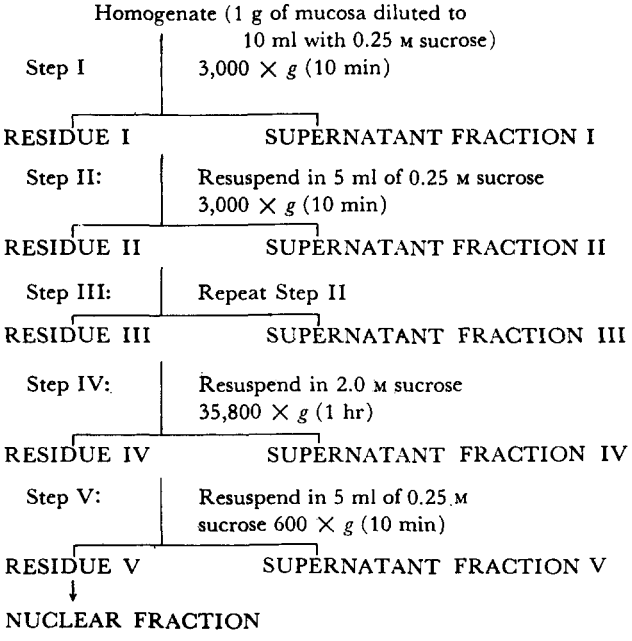

FIG. 1. Isolation of nuclear fraction from intestinal mucosa.

were combined for further fractionation of mitochondria and microsomes according to the procedure described (15). Supernatant fractions III, IV, and V were combined and designated "debris fraction." Residue V, the nuclear fraction, was stained with hematoxylin and eosin and was found to be essentially free of debris material. The mitochondrial and microsomal fractions appeared to be essentially pure as judged by techniques previously described (15).

Lipid Extraction and Isolation of Retinyl Esters

The lipids were extracted from all tissues, cellular fractions, and lymph according to the technique of Bligh and Dyer (16). The chloroform extract was filtered, dried over anhydrous sodium sulfate, and evaporated to dryness on a flash evaporator at 40–50°C. The residue was dissolved in approximately 20 ml of *n*-hexane containing 1 mg of α-tocopherol and chromatographed on 18 g of specially prepared silicic acid. The chromatography equipment and eluting solvents were the same as used by Hirsch and Ahrens (17).

The silicic acid was prepared by mixing 200 g of silicic acid ("minus 325 mesh," obtained from Bio-Rad Laboratories, Richmond, Calif.) with 1 liter of 0.1 N sodium hydroxide for 2 hr. The silicic acid was filtered by suction and dried at 110°C for 24 hr; the product was called "B-silicic acid."

The efficiency of the separation of the retinyl ester by chromatography over "B-silicic acid" was tested by placing a mixture of approximately 200 mg of intestinal mucosa lipid, 1 mg of α-tocopherol, and a known amount (0.5–1.0 mg) of ^{14}C-labeled retinyl ester in hexane on a column and eluting with 350 ml of 1% diethyl ether in hexane (v/v). In all instances more than 94% of the radioactivity was recovered in this eluate.

Similar experiments with retinol-^{14}C showed that the column did separate retinol from retinyl ester since no activity was found in the 1% diethyl ether in hexane eluate; 80% of the activity was present in the 8% diethyl ether in hexane fraction.

Preparation of Maleic Anhydride Derivative

Since it was recognized that the retinyl ester fraction separated from lipid extracts contained cholesterol ester, it was necessary to determine if the cholesterol esters contributed to the radioactivity of this fraction. This was examined by comparing the ^{14}C/^{3}H ratio of the mixed retinyl ester–cholesterol ester fraction with the ratio obtained for a maleic anhydride derivative of retinyl ester prepared from the same mixture (18). Since the ratio found for the derivative did not differ significantly from that found for the mixed fraction, the ^{14}C/^{3}H ratio of the combined retinyl ester–cholesterol ester fraction is reported in this study to represent the ratio present in the retinyl ester fraction.

Radioactivity Measurements

The radioactivity in the doubly-labeled samples was determined by liquid scintillation counting with the Packard Scintillation Spectrometer (Model 3324, with a counting efficiency of 50% and 20% for ^{14}C and ^{3}H, respectively). Quenching was corrected for by the use of internal standards.

RESULTS

The results of a typical 3 hr postprandial experiment are

TABLE 1 DISTRIBUTION OF ^{14}C AND ^{3}H IN TOTAL LIPID AND RETINYL ESTERS FROM INTESTINAL CONTENTS, INTESTINAL MUCOSA, AND LIVER 3 HR AFTER FEEDING RETINYL-^{14}C PALMITATE-^{3}H*

	^{14}C	^{3}H	$^{14}C/^{3}H$
	cpm		
Total retinyl palmitate fed (10.59 mg)	3.68×10^6	1.24×10^6	2.97
Intestinal Contents			
Total Lipid	5.38×10^5	1.69×10^5	3.18
Retinyl ester	2.64×10^5	6.03×10^4	4.38
Intestinal Mucosa			
Total lipid	8.07×10^4	4.86×10^4	1.66
Retinyl ester	3.77×10^4	3.77×10^3	10.00
Liver			
Total lipid	1.03×10^5	1.38×10^4	7.46
Retinyl ester	6.12×10^4	3.06×10^2	200.00

Extracts from three female rats were pooled to yield the results.
*$E_{1\,cm}^{1\%}$ at 328 mμ in *n*-hexane = 925.

TABLE 2 DISTRIBUTION OF ^{14}C AND ^{3}H IN TOTAL LIPID AND RETINYL ESTERS FROM SUBCELLULAR FRACTIONS OF INTESTINAL MUCOSA 3 HR AFTER FEEDING RETINYL-^{14}C PALMITATE-^{3}H*

	^{14}C	^{3}H	$^{14}C/^{3}H$
	cpm		
Total retinyl palmitate fed (10.59 mg)	3.87×10^6	1.70×10^6	2.28
Intestinal Mucosa Homogenate			
Total lipid	1.19×10^5	7.87×10^4	1.51
Retinyl ester	6.43×10^4	7.38×10^3	8.71
Nuclei			
Total lipid	750	350	2.14
Retinyl ester	78	19	4.11
Mitochondria			
Total lipid	1.56×10^4	1.05×10^4	1.49
Retinyl ester	8.49×10^3	1.47×10^3	5.78
Microsomes			
Total lipid	9.48×10^3	6.65×10^3	1.43
Retinyl ester	4.20×10^3	4.56×10^2	9.21
Soluble fraction			
Total lipid	6.95×10^3	2.80×10^3	2.48
Retinyl ester	2.66×10^3	5.44×10^2	4.89
Debris			
Total lipid	8.58×10^4	5.84×10^4	1.47
Retinyl ester	4.89×10^4	4.89×10^3	10.00

Extracts from three female rats were pooled to yield the results.
*$E_{1\,cm}^{1\%}$ at 328 mμ in *n*-hexane = 897.

recorded in Tables 1 and 2. In Table 1 it may be noted that there was a considerable increase in the $^{14}C/^{3}H$ ratio of the retinyl ester from intestinal contents during digestion and absorption. The $^{14}C/^{3}H$ ratio for the total lipid extract of intestinal contents was not significantly different from that of the administered retinyl ester. At the end of 3 hr the $^{14}C/^{3}H$ ratio in the total lipid extract from the intestinal mucosa was considerably lower than that in the original retinyl ester, whereas the ratio was significantly increased in the retinyl ester fraction. The uncharacterized debris fraction of intestinal homogenate contained the highest $^{14}C/^{3}H$ in the retinyl ester with the microsomes, mitochondria, and soluble fraction ranking next in decreasing order (Table 2). The ratios found in the liver for total lipid and retinyl ester were both increased over the starting ratio.

The results of the half-hour postprandial experiments are not recorded because they gave essentially the same ratios as are reported for the 3 hr animals. The only significant difference was a reduction in the radioactivity found in the various fractions.

The results of injecting radioactive retinyl palmitate into segments of small intestine and immediately washing out the contents as previously described revealed that 1% of the total activity administered could be accounted for as material adhering to the luminal surface of the mucosa. The $^{14}C/^{3}H$ ratio of the adhered material was the same as that of the retinyl ester administered.

Table 3 summarizes the results of a typical thoracic duct cannulation experiment after administering doubly-labeled retinyl palmitate. Here it may be noted that the $^{14}C/^{3}H$ ratio found for the retinyl ester in lymph was 63. This ratio was approximately the same for all of the hourly periods up to 6 hr and for the 6–12 and 12–24 hr periods after dosing. The ratio found for the retinyl ester of the liver at the end of 24 hr was about 300.

TABLE 3 DISTRIBUTION OF ^{14}C AND ^{3}H IN TOTAL LIPID AND RETINYL ESTERS FROM THORACIC LYMPH AND LIVER 24 HR AFTER FEEDING RETINYL-^{14}C PALMITATE-^{3}H*

	^{14}C	^{3}H	$^{14}C/^{3}H$
	cpm		
Total retinyl palmitate fed (5 mg)	1.39×10^6	1.07×10^6	1.30
Lymph (19.75 ml)			
Total lipid	1.35×10^5	1.98×10^5	0.68
Retinyl ester	1.02×10^5	1.62×10^3	63.00
Liver			
Total lipid	1.00×10^5	7.60×10^3	13.16
Retinyl ester	7.87×10^4	2.53×10^2	311.00

Extracts from three female rats were pooled to yield the results.
*$E_{1\,cm}^{1\%}$ at 328 mμ in *n*-hexane = 861.

The $^{14}C/^{3}H$ ratios of the maleic anhydride derivatives from all retinyl esters were essentially the same as those reported in Tables 1 and 2.

DISCUSSION

The primary objective of this study was to gain some insight into the extent that retinyl esters are hydrolyzed and

reesterified during the process of digestion, absorption, transport, and storage in the liver. Retinyl palmitate was used since it is one of the more abundant natural esters and perhaps not subject to as extensive hydrolysis as other less common esters.

The results of this study show that extensive hydrolysis and reesterification of retinyl ester does take place in the intestinal mucosa and liver. The increase in the $^{14}C/^{3}H$ ratio of the retinyl ester from intestinal contents also points to hydrolysis and reesterification. The latter effects may be the result either of the action of intestinal enzymes or of the countertransport of the ester from the mucosa to the lumen or a combination of both.

In all experiments the $^{14}C/^{3}H$ ratio of the retinyl esters from characterized subcellular fractions was highest in the microsomes and lowest in the nuclear and soluble fractions. Different ratios for the various subcellular fractions suggest that at some site(s) in the intestinal mucosa further hydrolysis and reesterification occurs, the activity being highest in the microsomal fraction. This finding agrees with the report of Mahadevan, Sastry, and Ganguly (8, 9) that the microsomes from intestinal mucosa contain esterases capable of hydrolyzing and reesterifying retinyl ester. The findings of Futterman and Andrews (19) also place this activity in the microsomal fraction of the liver.

The $^{14}C/^{3}H$ ratio of the total lipid extract from tissues and lymph was always lower than that in the retinyl ester fraction. This finding may be explained by the postulate that the hydrolyzed palmitic acid from the administered retinyl ester has been incorporated into other lipid components of the total lipid extract while various amounts of unlabeled (endogenous) fatty acids have been used in reesterifying newly formed retinyl ester.

A valid explanation for the low $^{14}C/^{3}H$ ratio found in the lipid fraction of the intestinal mucosa (Table 1) cannot be derived from this study. Since no significant quantities of radioactivity were found in the aqueous fraction remaining after lipid extraction of the intestinal mucosa, it seems improbable that retinol is preferentially degraded to a nonlipid component and that palmitate is not so affected. A more plausible suggestion might be that palmitate is more rapidly absorbed by the intestine than retinol. This would account for the decrease in the $^{14}C/^{3}H$ ratio in the lipid fraction of the intestinal mucosa and an increase in the $^{14}C/^{3}H$ ratio of the lipid fraction of the intestinal contents.

The $^{14}C/^{3}H$ ratio progressively increases as retinyl ester is formed in the intestine, transported by lymph, and stored in the liver. Therefore, hydrolysis and reesterification of retinyl ester are common occurrences in the absorption and storage of vitamin A. The question remains as to whether the alcohol or ester form is the species preferentially transported across the cellular membrane.

In view of the considerable activity found in retinyl ester fractions from the livers of lymphatic cannulated rats, it appears that a circulation route(s) other than the thoracic lymph is(are) concerned with the transport of retinyl ester from the intestine to the liver.

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