Absorption of phytol from dietary chlorophyll in the rat

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ABSTRACT The fate of ingested chlorophyll—particularly of the phytol portion of the molecule—was studied. Uniformly ^{14}C-labeled pheophytin *a* (the Mg-free derivative of chlorophyll *a*) was prepared from an extract of tobacco leaves grown in $^{14}CO_2$, and was administered by stomach tube to rats in which the thoracic duct had been cannulated.

Only about 2% of the administered radioactivity was absorbed in 24 hr, largely into the thoracic duct lymph. Moreover, only a fraction of this lymph radioactivity was derived from phytol (i.e., was found in phytol, phytenic acid, or phytanic acid). The results indicated that not more than 1–2% of chlorophyll phytol is available for absorption by the rat. Similarly, after the administration of whole spinach or spinach extract (not labeled) to rats, only about 1% of the total phytol content was absorbed into the intestinal lymph. Nearly all of the administered phytol was found in the feces and the contents of the colon, and was still largely in the form of pheophytin. The study also indicated that little of the nonphytol portion of the chlorophyll molecule is absorbed.

KEY WORDS chlorophyll · U-^{14}C-pheophytin *a* · spinach · availability of phytol · intestinal absorption · thoracic duct lymph · phytanic acid · rat

CHLOROPHYLL IS PRESENT in leaves of green plants to the extent of about 0.2% of wet weight (1). Despite the importance of chlorophyll and its abundance in the diet of animals and man, not a great deal is known of its fate after ingestion. The principal chlorophylls of higher plants contain the isoprenoid alcohol phytol (3,7,11,15-tetramethyl hexadec-2-en-1-ol) attached by ester linkage as a side chain. The alcohol contains 20 of the 55 carbon atoms of the chlorophyll molecule. Free phytol is readily absorbed and converted to phytanic acid in rats (2–4), in normal human subjects, and in patients with Refsum's disease (5, 6). In such patients, the accumulation of phytanic acid in blood and tissues is a characteristic pathologic feature (7) which results from the patients' relative inability to oxidize this branched-chain fatty acid (5, 8). As phytanic acid is not produced endogenously (9, 10), it must be derived from dietary sources. Chlorophyll is an obvious potential source, although the availability of chlorophyll phytol for absorption has never been studied directly.

Phytanic acid has been found in tissue and milk fats of ruminants, presumably formed from chlorophyll phytol by bacteria in the rumen (11). Many of the studies on gastrointestinal degradation and absorption of chlorophyll have been made in ruminants, and are not necessarily transferable to nonruminants. Fischer and Hendschel (12), however, identified a number of phorbides and also the chlorophyll-derived porphyrin phylloerythrine in human feces, which indicated that some release of the phytol had occurred. Other workers have presented evidence for the presence of chlorophyll porphyrins in human feces, bile, and urine (see 13). Brugsch and Sheard (14), after administering chlorophyll to humans, found that pheophytin (produced by the removal of only Mg from the chlorophyll, and known to be formed by action of stomach acid on chlorophyll) was the predominant product in the feces, although varying quantities of phorbides and small quantities of substances extractable with 10% HCl (presumably porphyrins) were also present. As much as half of the administered chlorophyll was unaccounted for by products found in the feces.

Pheophytin *a* (uniformly ^{14}C-labeled) rather than chlorophyll itself was employed in the present study. After the pheophytin had been administered by stomach tube to rats, studies were made of the quantity and characteristics of the absorbed radioactive materials. Previous

Abbreviation: GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

studies had shown that free phytol is absorbed largely into the intestinal lymph, where it is found (not having been exposed to the liver and other tissues) as phytol itself or as certain distinctive derivatives of phytol (4). Hence, in order to determine how much of the absorbed radioactivity in the present study was actually derived from phytol (rather than from other parts of the pheophytin molecule or from labeled contaminants), thoracic duct lymph was collected and analyzed. Additional studies were made on the absorption of phytol from cooked spinach.

METHODS AND MATERIALS

Preparation of Tobacco Leaf Extract

An extract of mature tobacco leaves that had been grown in $^{14}CO_2$ and preserved in the frozen state, was prepared[1] as follows. After they had been thawed, the leaves were covered with acetone in a volume calculated to yield an 80% solution with water in the leaves. Three additional extractions were made with 80% acetone. The extracts were combined and concentrated under reduced pressure, which left a waxy deposit on the walls of the flask. The small volume of aqueous fluid that remained was poured off. The waxy layer was extracted repeatedly with peroxide-free anhydrous ether, which was then evaporated under nitrogen to yield the lipid-soluble material.

Chromatography of Tobacco Leaf Extract

The tobacco leaf extract was chromatographed in benzene–acetone 95:5 under subdued light on glass plates (20 × 20 cm) coated with Silica Gel G (0.25 mm), 10–15 mg of extract (containing about 0.5 μc of ^{14}C-radioactivity per mg) having been applied to each plate. Various zones were promptly eluted with ether. In preliminary studies, Rhodamine 6G was included in the silica gel, so that nonpigmented lipids (which were also labeled) and added free phytol could be located under ultraviolet light. Many pigment bands were resolved. Midway between the origin and solvent front (R_f 0.45), there was a prominent, dense, almost black band, referred to below as band A. This was the uppermost pigmented band on the plate, except for yellow carotene bands and faint greenish or grayish bands. It exhibited a deep red fluorescence under ultraviolet light, appeared to be cleanly separated from adjacent bands (both colored and noncolored), and contained 6% of the radioactivity of the applied lipid extract.

[1] The tobacco-leaf extract was prepared and generously provided by Dr. W. Stepka, Medical College of Virginia, Richmond, Va.

Characterization of Band A as U-^{14}C-Pheophytin a

Band A material was directly compared with authentic pheophytin *a*. The latter was prepared from fresh spinach by sucrose column chromatography of an extract (15) and conversion of the chlorophyll *a* to pheophytin *a* by shaking with dilute acid. In TLC on mannitol (16) and on silica gel, and in column chromatography on sucrose, the two preparations moved in identical fashion. They also gave similar positive (yellow) Molisch phase tests, with intermediate products characteristic of pheophytin *a* (17).

Band A material was dissolved in ether and its absorption spectrum (visible and ultraviolet) determined with a Cary spectrophotometer. The spectrum was almost identical with that found for pheophytin *a* by Smith and Benitez (18). The absorbancy of band A material at the 667 mμ absorption peak was almost the same as the value reported for pheophytin *a* (18) and indicated a high degree of purity of the preparation. The specific radioactivity of the material was about 0.5 μc/mg.

Rechromatography of band A material on silica gel with benzene–acetone 95:5 showed that about 95% of the radioactivity again migrated with R_f 0.45. Usually 1–2% of the radioactivity migrated with free phytol, but less than half of this proved to be actually in free phytol.

A portion of the band A material containing 14,000 cpm was dissolved in ether and the ether was shaken with 22% aqueous HCl. Less than 1% of the radioactivity was extracted, which showed the virtual absence of alkaloids, phorbides, and porphyrins. Extraction of another portion with alkaline 50% ethanol (19) yielded an insignificant amount of radioactivity.

After saponification of band A material (four experiments), 33–38% of the radioactivity was found in the nonsaponifiable fraction. Of this, 85–91% migrated with free phytol in TLC (with benzene–ethyl acetate 4:1). After acetylation of the free phytol zone with acetic anhydride in pyridine, nearly all of the radioactivity migrated with phytyl acetate. Thus, about 30% of the radioactivity of band A appeared to be in (bound) phytol. Since the recovery of added phytol was only about 90%, the phytol radioactivity of band A approached the theoretical value for pheophytin (C_{20}/C_{55} = 36%), assuming uniform labeling of the chlorophyll.

On the basis of the characteristics listed above, the material from chromatographic band A was judged to be reasonably pure U-^{14}C-pheophytin *a*, and was employed in the animal studies to be described.

Studies on Absorption of Pheophytin-^{14}C

Male Sprague-Dawley rats weighing about 280 g were used. The thoracic lymph ducts were cannulated, and the animals were held in restraining cages and fed a liquid

diet for 24 hr, as previously described in detail (20). Doses of pheophytin-U-^{14}C (containing 0.6–1.2 μc of ^{14}C) were dissolved in 0.5 ml portions of corn oil and administered by stomach tube to six of the rats. Tracer doses of palmitic acid-9,10-^{3}H (containing about 4 μc of ^{3}H) were administered with the pheophytin-U-^{14}C.

In each experiment, thoracic duct lymph was collected for 24 hr and analyzed. In three of the experiments, studies were made not only on the lymph, but also on expired CO_2, urine, feces, tissues, and intestinal contents. Expired CO_2 was collected after the rat (held in restraining cage) had been placed in a large desiccator fitted with gas inlet and outlet. CO_2-free air was circulated through the jar and then through a 20%-NaOH trap, which was changed periodically. Urine and feces were collected from the jar, and tissues and intestinal contents were obtained at the end of the experiment.

Analytical Methods

Lymph and urine were extracted by injection into chloroform–methanol 2:1 (25 ml/ml). Tissues, intestinal contents, and feces were extracted by homogenization with chloroform–methanol (25 ml/g) or, in some cases, with slightly acidified ethanol–acetone 1:1 (25 ml/g). After 0.2 volume of 0.02 N H_2SO_4 had been added, chloroform and aqueous phases of the chloroform–methanol extracts were separated.

Tissue and fecal lipids and pheophytin preparations were saponified by heating a few milligrams at 80°C for 1–2 hr with 5 ml of 2% alcoholic KOH. After an equal volume of water had been added, the nonsaponifiable fraction was extracted with hexane (10 ml, 3×), and after acidification of the residual aqueous mixture, the saponifiable fraction was extracted with hexane followed by ether. The residual aqueous solution was also retained for analysis. Methylation of the saponifiable fraction was accomplished by heating overnight at 60°C in methanol–5% H_2SO_4.

Phytol-^{14}C was separated from other nonsaponifiable lipids (after addition of carrier phytol) by TLC on Silica Gel G containing Rhodamine 6G with the solvent benzene–ethyl acetate 4:1. Lipid zones were identified under UV light.

Radioactivity of the lipid extracts and their fractions was determined after dissolving them (in counting vials) in 15 ml of toluene containing 0.5% diphenyloxazole. ^{14}C- and ^{3}H-radioactivity were assayed simultaneously at efficiencies of 46 and 14%, respectively, and corrected for quenching as determined from internal standards. ^{14}C-radioactivity of the CO_2 solutions and of the aqueous phases of the extracts was assayed on anthracene crystals (21) at an efficiency of 11%, and corrected to 46% efficiency.

Determination of Free and Bound Phytol in Spinach

Since vegetable foods might contain significant quantities of free phytol in addition to the phytol present as part of the chlorophyll molecule, the free phytol content of uncooked and cooked spinach was investigated.

After blotting them to remove excess water (from cooked preparations), we homogenized 2-g portions of spinach with chloroform–methanol 2:1 (50 ml). The chloroform phases (separated after the addition of 0.2 volume of water) were dried with anhydrous Na_2SO_4, and were evaporated under N_2. The residues were taken into hexane and placed on a sucrose column. The column was washed with hexane until a drip appeared at the outlet, then development was continued with 2% *n*-propanol in hexane. Free phytol (together with carotenes and other nonpolar lipids) was thereby eluted from the column before pheophytin *a* reached the outlet (i.e., before any of the chlorophyll-related pigments had been eluted), as verified after adding ^{14}C-phytol to the spinach extract. The free phytol was then separated from the carotenes and other lipids in the front fraction by TLC on silica gel with benzene–ethyl acetate 4:1. After elution, the phytol was quantified—either as free phytol or, better, after acetylation or silylation—by GLC on a 12 ft column of ethylene glycol succinate polyester, 17% on Gas-Chrom P, at 150–180°C. After the spinach extract had been saponified and the phytol had been separated from the other nonsaponifiable lipids by TLC, total phytol of the spinach was determined by GLC.

Studies on Absorption of Spinach Phytol

A homogenate of whole cooked spinach (1 part spinach and 2 parts water) in three doses of 5 ml at 3-hr intervals (total of 5 g) was administered by stomach tube to a rat that had been fed a phytol-free diet. Another such rat was given the total lipid material (including the chlorophyll) extracted from 15 g of frozen spinach (by homogenization with chloroform–methanol), after the material had been dissolved in 1 ml of corn oil, together with a tracer dose of ^{3}H-palmitic acid. The quantity of phytol absorbed into the thoracic duct lymph in each case and the quantity excreted in the feces were determined by methods described in the sections above.

RESULTS

Absorption of Pheophytin-U-^{14}C

After pheophytin-U-^{14}C (1.2–2.4 mg, containing 0.6–1.2 μc of radioactivity) had been administered by stomach tube to each of six rats, the thoracic duct lymph collected during the subsequent 24 hr contained only 2% (1.0–2.4) of the administered radioactivity.

It had previously been established that over 70% of free phytol absorption from the intestine occurs through

the lymphatic route (4). However, because of the possibility that pheophytin phytol and free phytol might not be absorbed in identical fashion, complete studies were made on the disposition of the administered pheophytin-U-^{14}C radioactivity in three rats. 92% was recovered (Table 1). About 1.5% had been absorbed into the thoracic duct lymph, and 2.7% had been absorbed through all available routes, including the lymphatics (i.e., was recovered in lymph, CO_2, tissues, and urine). Thus, of the total absorbed ^{14}C-radioactivity, over half was absorbed into the thoracic duct lymph.

Although most of the pheophytin-U-^{14}C radioactivity remained unabsorbed (in the intestinal contents and feces), over 75% of the simultaneously-administered palmitic acid-^{3}H radioactivity was absorbed (Table 1), so there was no general defect in absorption.

Nature of Absorbed ^{14}C-Labeled Material

After free phytol-U-^{14}C had been administered in previous studies, about $^{2}/_{3}$ of the radioactivity found in the lymph was present in the form of phytol itself, and most of the remainder was in the form of phytenic and phytanic acids (4). Therefore, to determine whether the lymph ^{14}C-radioactivity found in the present experiments was principally derived from the phytol portion of the pheophytin molecule, we saponified the lymph lipids and looked for radioactivity in phytol and in the branched-chain fatty acids.

After saponification of the lymph lipids, 30% of the ^{14}C-radioactivity (average of five experiments) was found in the nonsaponifiable fraction, and 70% in the saponifiable fraction. Less than 20% of the nonsaponifiable fraction radioactivity migrated in TLC with phytol, and less than 20% of the saponifiable fraction radioactivity migrated in GLC with phytanic and phytenic acids.

TABLE 1 ABSORPTION OF ^{14}C-PHEOPHYTIN AND ^{3}H-PALMITIC ACID

	Distribution of Radioactivity	
	Pheophytin	Palmitic Acid
	% of administered radioactivity	
Recovered, total	91.8	92.6
lymph(thoracic duct)	1.5	73.8
expired CO_2	0.3	—
carcass	0.8	1.4
urine	0.3	0.2
gut contents	18.6	9.0
feces	70.6	8.3
Absorbed, total*	2.7	75.3

Doses of the two labeled compounds (containing, respectively, 0.6–1.2 μc of ^{14}C and about 4 μc of ^{3}H) were dissolved together in corn oil and administered to each of three rats by gastric tube. The rats were killed after 24 hr. Mean values are presented.

* Recovered in lymph, expired CO_2, carcass (including organs), and urine.

Studies on the ^{14}C-labeled substances in the liver and carcass, although less satisfactory than the lymph studies, likewise indicated that only a small part of the radioactivity was in the form of phytol or phytanic acid. Thus, the absorbed radioactivity actually derived from phytol was equivalent to not more than about 0.5% (0.2 × 2.7%) of the administered pheophytin radioactivity, or about 1.5% of the administered pheophytin phytol radioactivity.

Nature of ^{14}C-Labeled Substances in Feces

Ethanol–acetone extracts of the feces were diluted with 1 or 2 volumes of 0.02 N H_2SO_4 and extracted with hexane and with benzene or ether. These pooled extracts contained most of the fecal ^{14}C-radioactivity. TLC of the extracted lipid with benzene–acetone 95:5 showed that 65% of the recovered ^{14}C-radioactivity migrated in a band with the characteristics of pheophytin *a* (R_f 0.45, charcoal color, red fluorescence). 6–12% of the radioactivity migrated approximately with free phytol, but further studies (TLC with other solvent systems, and rechromatography after saponification) indicated that less than half of this was actually present in phytol.[2] Some phytol-containing pigments other than pheophytin appeared to be present. In TLC of the fecal lipids with hexane–ether–acetic acid 80:20:2, almost no ^{14}C-radioactivity migrated with phytyl palmitate, which indicates that little phytol had been reesterified with fatty acid in the intestinal lumen. Approximately $^{1}/_{3}$ of the total fecal-lipid ^{14}C-radioactivity was found in the nonsaponifiable fraction after saponification, and most of this migrated with phytol, which further indicates that little phytol had been released from its ester linkage and absorbed.

Absorption of Phytol from the Colon

Because there seemed to be some free phytol present in the feces and colon contents after the administration of pheophytin, the degree of absorption of free phytol-U-^{14}C introduced into the cecum (tracer doses dissolved in corn oil emulsified in rat bile) was investigated. The experiments indicated that not more than 10% of the phytol was absorbed.

Free and Total Phytol Content of Spinach

Fresh uncooked spinach was found to contain an in significant quantity of free phytol (<1 μg/g). However, several lots of cooked spinach contained 1–2% of the total phytol in free form. The total phytol content

[2] In a more recent experiment in which the feces were extracted with chloroform–methanol and the chloroform phase was chromatographed directly, a higher pheophytin value and a lower free phytol value were observed.

(determined after saponification of the lipid extract) was found to be approximately 600 μg/g.

Availability of Spinach Phytol for Absorption

After 5 g of whole cooked spinach and the lipid extract from 15 g of frozen spinach, respectively, had been administered to individual rats, the thoracic duct lymph (collected for 30 hr in each case) contained only about 1% of the phytol content of the spinach. This result included the phytol that had been converted to phytenic and phytanic acids, as well as that which was present in the lymph as phytol itself. Moreover, the lymph showed none of the intense green color of the administered material. On the other hand, the feces and large gut contents contained an amount of phytol approximately equal to that present in the spinach preparation. TLC of extracts of the feces showed the presence of much pheophytin *a* and less pheophytin *b*, which together contained a large part of the phytol originally present in the spinach.

DISCUSSION

After the administration of ^{14}C-labeled pheophytin *a* to rats, not more than 1–2% of the phytol component was absorbed in 24 hr. The greater part of the pheophytin passed the entire length of the intestinal tract intact. Any significant liberation of phytol that did occur presumably occurred principally in the large intestine, where absorption of free phytol was shown to be relatively inefficient. This minute absorption of pheophytin phytol by the rat is in contrast to the previously observed 50% absorption of orally administered tracer doses of free phytol (4).

Strain and Manning have pointed out (22, 23) that various manipulations may cause isomerization of chlorophylls and pheophytins, and Bacon (24) has emphasized the danger that chlorophyll pigments may be altered (oxidized) during chromatography on active adsorbents such as silica gel. It is not known whether isomerization or allomerization of pheophytin would affect the biological availability of its phytol component. The result of the phase test indicated that the pheophytin-U-^{14}C employed in the present study had not been altered by allomerization, and the chromatographic behavior appeared to be characteristic of the normal isomer. It is probable that the preparation contained a small amount of labeled impurities, which may have been the source of some of the absorbed radioactivity. However, the impurity was probably not greater than about 5%. The result obtained after feeding whole cooked spinach and spinach extract supported the results obtained with the purified pheophytin-U-^{14}C.

Previous studies have shown that free phytol is an excellent source of phytanic acid, being readily absorbed and metabolized both in animals and in man. The present results, however, if they can be extrapolated to man, suggest that the esterified phytol in the chlorophyll molecule is not a quantitatively important dietary source. Although these results indicate that purified chlorophyll and whole spinach are poor sources of absorbable phytol, it is possible that some crude vegetables may be better sources of phytol or other phytanic acid precursors. We have shown here that cooked spinach may contain significant amounts of free phytol, and it is known that under certain conditions phytol may be split from the chlorophyll molecule through the action of chlorophyllase which is present in the leaves. Also, vegetables contain additional possible phytanic acid precursors, such as vitamin K, vitamin E, the ubiquinones, and the polyprenols, and some foods possibly contain appreciable quantities of nonchlorophyll phytol. Dairy products (25) and beef and lamb tissues (26, 27) are known to contain phytanic acid itself, which may prove to be a more important source of phytanic acid in man than phytol.

Manuscript received 16 June 1967; accepted 4 August 1967.

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