Absorption of chlorophyll phytol in normal man and in patients with Refsum's disease

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ABSTRACT This study was made to determine the extent of absorption of chlorophyll phytol from the intestine of man, and the importance of chlorophyll as a source of the phytanic acid that accumulates in Refsum's disease. Uniformly ^{14}C-labeled pheophytin *a* (the Mg-free derivative of chlorophyll *a*) was fed to normal human subjects and to patients with Refsum's disease. Feces were collected and analyzed.

In all subjects, 90–95% of the administered radioactivity was recovered in the feces, still largely in the form of pheophytin *a*. The phytol radioactivity recovered in the feces averaged about 95% of that in the administered material, which indicates that there had been little absorption of the phytol moiety. Similarly, after 250 g of cooked spinach had been fed to a normal subject, almost the entire phytol content was found in the feces. Less than 5% of the ingested spinach phytol was accounted for in the thoracic duct lymph of another subject.

It was concluded that not more than about 5% of the ingested chlorophyll phytol is absorbed by man, whether normal or afflicted with Refsum's disease. On this basis we conclude that the major portion of the phytanic acid that accumulates in Refsum's disease could not be derived from dietary chlorophyll.

KEY WORDS chlorophyll · ^{14}C-pheophytin *a* · spinach · availability of phytol · intestinal absorption · thoracic duct lymph · phytanic acid · normal man · Refsum's disease

A CHARACTERISTIC feature of Refsum's disease is the accumulation in serum and tissue lipids of large amounts of the saturated isoprenoid C_{20} fatty acid *phytanic acid* (3,7,11,15-tetramethylhexadecan-1-oic acid) (1). The accumulation is due to a specific metabolic defect: the inability to oxidize this acid (2, 3). Whether the neurologic manifestations of the disease are caused by the accumulation of this acid or by other metabolic consequences of the underlying deficiency in the α-oxidation enzyme activity (4) has not been determined.

Phytanic acid is not produced endogenously (3, 5) and must therefore be derived from dietary sources. Free phytol (the corresponding α,β-unsaturated alcohol), when administered orally, is readily absorbed and converted to phytanic acid in rats (6–8), in normal human subjects, and in patients with Refsum's disease (2, 9). Phytol in esterified form, as a side chain of the chlorophyll molecule, is ubiquitous in green vegetables. Chlorophyll phytol has been suspected of being the chief source of phytanic acid in Refsum's disease (5, 10), but there is little information on the extent to which it is actually available for absorption in man.

We recently reported studies on the absorption of chlorophyll phytol in the rat, in which thoracic duct lymph was analyzed to provide precise information on the degree of such absorption (11). Only about 2% of the phytol was absorbed. In the present report, studies are presented on the absorption of chlorophyll phytol in normal human subjects and in patients with Refsum's disease, based largely on analysis of feces, but also including studies on the thoracic duct lymph of one subject. Pheophytin *a*, uniformly ^{14}C-labeled, rather than chlorophyll itself was employed in this study. Pheophytin, like chlorophyll, contains esterified phytol, and differs from chlorophyll only in the absence of the tetrapyrrole-bound Mg^{++}, which is normally removed from ingested chlorophyll by action of the acid in the stomach. Chlorophyll was also administered in the form of crude cooked spinach. Little of the chlorophyll phytol was absorbed.

METHODS

Preparation of Pheophytin-^{14}C

In this study, the pheophytin-^{14}C was prepared as previously described in detail (11), with slight modifica-

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

tions. Briefly, mature tobacco leaves that had been grown in $^{14}CO_2$ were extracted with 80% acetone, and after the acetone had been evaporated and the aqueous phase had been decanted, the waxy residue was extracted with ether. The ether was evaporated, which left the crude lipids.[1]

The crude tobacco-leaf lipids were dissolved in benzene, extracted three times with 5% HCl to insure elimination of alkaloids, and washed twice with water. The lipids were then chromatographed on a thin layer of silica gel in benzene–acetone 95:5. The foremost dark band (dense, almost black, fluorescing red, R_f 0.45), which contained 6–8% of the applied radioactivity, was promptly eluted with ether, and was identified (see below) as pheophytin *a*. The pheophytin *a* was rechromatographed on ether-washed silica gel with *n*-hexane–acetone–diethyl ether 60:20:20, which eliminated additional small amounts of impurities and substituted solvents that were easier to evaporate from the pheophytin.

Characterization of Pheophytin-^{14}C

The material prepared as described above was found to be identical with authentic pheophytin *a* in TLC on sucrose and on mannitol as well as on silica gel. It gave a positive Mollisch phase test (12). Its absorption spectrum was characteristic of pheophytin *a*, and its absorbancy (1 cm light path, concentration in g/liter) at the 667 mμ absorption peak was approximately the same as the reported value for pheophytin *a* (13). The specific radioactivity of the material was about 0.5 μc/mg. Rechromatography of the material showed that about 95% of the radioactivity again migrated in a band with the characteristics of pheophytin *a*. Insignificant amounts of radioactivity were extracted from the preparation (dissolved in ether) by 22% aqueous HCl or by alkaline 50% aqueous ethanol. After saponification of the preparation, 35% of the radioactivity was found in the nonsaponifiable fraction, and 33% migrated with phytol in TLC. Thus, considering that the recovery of added phytol was incomplete (about 95%), the phytol radioactivity of the preparation approached the theoretical value for uniformly ^{14}C-labeled pheophytin (C_{20}/C_{55} = 36%).

Studies with Pheophytin-^{14}C

The normal subjects studied were J.B., a 53 yr old man, and R.I., an 18 yr old college student. The patients with Refsum's disease were siblings J.S., a 34 yr old man, and K.S., a 29 yr old woman, who have been described in detail elsewhere (14). The patients had been subsisting for several months on a diet low in phytol; the same type of diet was fed to the normal subjects for a week prior to administration of the pheophytin-^{14}C.

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

In the test procedure, each of the subjects was given a single dose of the uniformly labeled pheophytin *a* containing about 1–1.5 μc of ^{14}C (which was dissolved in corn oil and spread on a slice of toast). All of the feces were then collected (in 3-day collections) for 9 days. Urine was collected for 24 hr and a blood sample was taken about 6 hr after the pheophytin had been administered.

Feces suspensions were made essentially as described by Jover and Gordon (15), but with quantities of diluents calculated to bring the total volume to 1000 ml instead of to 2000 ml when possible. Duplicate 5 ml aliquots of each suspension (which contained 50% alcohol) were subsequently extracted by homogenization with 50 ml of chloroform–methanol 2:1 in a Waring Blendor. After the addition of 10 ml of 0.02–0.05 N H_2SO_4 to each extract, the chloroform and aqueous phases were separated.

Fecal lipids (from 10 ml of the chloroform phases) and portions of the original pheophytin preparation were saponified by heating at 80°C for at least 1 hr with 10 ml of 2% alcoholic KOH. After dilution of the alcoholic KOH with an equal volume of water, the nonsaponifiable fraction was extracted four times with hexane.

Phytol-^{14}C was separated from the other nonsaponifiable lipids by TLC on Silica Gel G containing Rhodamine 6G, with the solvent benzene–ethyl acetate 4:1 (after carrier phytol had been added). Recoveries of applied radioactivity averaged 95%. Identification of the phytol zone was made under ultraviolet light. Verification of phytol radioactivity was made by rechromatography of the phytol after acetylation, followed by radioassay of the phytyl acetate zone.

The nature of the ^{14}C-labeled substances in the native feces was investigated by TLC of the feces extracts (without preliminary saponification), followed by elution and further study of the various TLC zones (pheophytin, free phytol, origin, rest of plate). Recoveries of applied fecal radioactivity in these studies averaged 88%. In order to study the radioactivity present in phorbides or porphyrins, we shook chloroform phases of the feces extracts with 0.01 N NaOH and with 20% HCl. The aqueous phases of the original feces extracts were also assayed for radioactivity.

In order to concentrate the lipid-soluble substances in the urines for radioassay, we brought aliquots (200 ml) of the urines to pH 4 with HCl and extracted with chloroform (20 ml). Blood sera were extracted by injection into chloroform–methanol 2:1 (25 ml/ml).

For quantification of radioactivity in the lipid extracts and their fractions, the lipid residues were dissolved in 15-ml amounts of 0.5% diphenyloxazole in toluene and assayed in a Packard liquid scintillation counter. The aqueous phases were assayed on anthracene crystals (16).

Studies with Spinach

One normal subject (E.B., a 49 yr old female), after eating a chlorophyll-free diet for a week, ingested 250 g of whole cooked spinach (weighed after being blotted to remove excess water) in the course of a day. The feces were collected (in 3-day collections) for 9 days for analysis.

Another subject, whose thoracic duct had previously been cannulated through a supraclavicular incision, was fed 180 g of cooked spinach over a period of 2–3 hr. The total lymph flow was then collected (in four successive collections) for a total period of about 24 hr. Volumes of the collections were measured, and representative aliquots of each were taken for analysis.[2] It had already been shown in rats that the absorption of phytol (and of phytanic acid) occurs largely into the intestinal lymph (8).

For determinations of total phytol (in the cooked spinach, in the feces, and in the lymph), the lipids were extracted, nonsaponifiable fractions were prepared, and the phytol (no carrier added) was separated from other nonsaponifiable lipids by TLC, using the methods already described. The trimethylsilyl ether derivative of the phytol was prepared by treatment with bis-(trimethylsilyl)-acetamide, and was quantified by GLC on a 12 ft column of ethylene glycol succinate on Gas-Chrom P at 150°C. In many cases, phytol determinations were also made by GLC, without prior isolation of the phytol by TLC. Silylation was necessary to prevent serious loss of phytol during column transit.

Since some phytol is converted to phytanic and phytenic acids during absorption into the intestinal lymph of the rat (8), determinations of these acids (in addition to the determinations of phytol) were made on the human lymph specimens. For this purpose, the saponifiable fractions of the lymph lipids (extracted with hexane, after the residual saponification mixtures had been acidified) were methylated by heating overnight at 65°C with fresh methanol–5% H_2SO_4, and were analyzed by GLC on the ethylene glycol succinate column at 168°C. Phytanic and phytenic acids were also quantified together, after the saponifiable fractions had been hydrogenated and the phytenic acid (if present) thereby converted to phytanic acid.

RESULTS

Absorption of Pheophytin-^{14}C

Recovery of Radioactivity in Feces. After uniformly ^{14}C-labeled pheophytin *a* (containing about 1–1.5 μc of ^{14}C radioactivity) had been ingested by each of two normal subjects and two patients with Refsum's disease, 90–95% of the ingested radioactivity was recovered in the total lipid extracts of the feces (Table 1). Most of the radioactivity was found in the first (3-day) collections, although up to about 10% was present in the second collections. Virtually none was present in the third collections.

Phytol Radioactivity in Feces. After the fecal lipids had been saponified, radioactivity equal to 31–35% of the total administered radioactivity was found in the nonsaponifiable fractions, and almost all of this migrated with phytol (Table 1). Thus, nearly 32% (on average) of the administered radioactivity was recovered in fecal phytol, compared with a recovery of 33% of the radioactivity in phytol on direct analysis of the administered pheophytin-^{14}C. This means that about 95% of the administered phytol passed into the feces without being absorbed. The results were similar in normal and diseased subjects.

Distribution of Radioactivity in Feces. After direct TLC of the fecal lipids (without saponification), 72–74% of the administered radioactivity recovered was still in the form of pheophytin *a* (Table 1). These values are probably too low, since the recovery of applied radioactivity was incomplete. About 1–2% migrated approximately with free phytol, and most of the remainder appeared to be in altered pigments, which migrated more slowly than pheophytin *a*.

The aqueous phases of the fecal extracts contained little radioactivity, and only about 1–2% of the chloroform phase radioactivity was extracted by 0.01 N NaOH or by 20% HCl. Therefore, it was concluded that the feces contained only small quantities of chlorophyll-derived phorbides or porphyrins.

Radioactivity in Serum and Urine. Determinations of serum radioactivity in each subject at about 6 hr after

TABLE 1 RADIOACTIVITY FOUND IN PHEOPHYTIN-^{14}C AND IN FECES COLLECTED AFTER ADMINISTRATION OF PHEOPHYTIN-^{14}C

Substance Analyzed	Fraction: Total Lipids	Non-saponifiable Fraction	Phytol	Pheophytin *a*
		% of radioactivity		
Pheophytin-^{14}C	(100)	35	33	95
		% of administered radioactivity		
Feces of subject:				
Normal				
J. B.	95	35	33	73
R. I.	94	33	32	72
Refsum's disease				
K. S.	94	31	30	72
J. S.	90	33	32	74

Each subject received about 1–1.5 μc of ^{14}C radioactivity.

[2] This study was made possible by the generous cooperation of Dr. Harry E. Sarles, Department of Internal Medicine, University of Texas Medical Branch, Galveston, Tex., who made the necessary arrangements and provided the appropriate lymph specimens.

the pheophytin-^{14}C had been ingested (at the time of peak levels as shown by previous studies) showed that less than 0.5% of the ingested radioactivity was present in the plasma. The significance of this radioactivity is uncertain, since the radioactive substances were not identified. They might have been derived from labeled contaminants, which were present in small amounts in the pheophytin-^{14}C preparation. Much less than 1% of the ingested radioactivity was found in the urine collected during the first 24 hr. These results are consistent with the recovery of most of the administered radioactivity in the feces.

Absorption of Spinach Phytol

During the period of the low-phytol diet, the feces of normal subject E.B. contained only a fraction of a milligram of phytol per day. After 250 g of spinach (containing 155 mg of phytol, with about 2 mg in the free form) had been eaten by the subject, the feces (collected for 9 days) contained 152 mg of phytol (Table 2). This experiment indicates that little of the phytol was absorbed. However, the extremely close agreement between the amounts of administered and recovered phytol must be considered fortuitous. As found in studies with pheophytin-^{14}C, phytol of the feces obtained after the spinach feeding was predominantly present in combined form, largely in the form of pheophytin *a* and *b*.

To avoid the imprecision inherent in determining small quantities of absorbed phytol (particularly unlabeled phytol) by difference between dietary intake and fecal excretion, spinach (180 g) was fed to one patient with a thoracic duct fistula, and the lymph was collected and analyzed for phytol. The lymph contained a mere trace of greenish color, demonstrated by extracting and concentrating the lipids. Only about 2% of the spinach phytol was found in the lymph in 24 hr (Table 3). A slightly smaller quantity of phytanic acid was also present, but no phytenic acid was demonstrated. Unlike the phytol, which clearly arose from the spinach, the phytanic acid was distributed fairly uniformly throughout all of the lymph collections (including the control collection), and probably arose in large part from preformed phytanic acid contained in dairy products (17) of the patient's diet. This view is supported by the failure to find phytenic acid, which appears to be an intermediate in the conversion of phytol to phytanic acid (8). Even assuming that the phytanic acid arose from spinach phytol, and that some phytol was also absorbed directly into the portal capillaries rather than into the lymphatics, the results indicate (in agreement with the other experiments) that the absorption of chlorophyll phytol did not exceed 5%.

TABLE 2 PHYTOL FOUND IN FECES OF NORMAL SUBJECT AFTER COOKED SPINACH HAD BEEN INGESTED

Feces Collection	Phytol Content
	mg
Control (3 days)	<1
No. 1 (3 days)	134
No. 2 "	15
No. 3 "	3
Total	152
Spinach, ingested (250 g)*	155

* Ingested during first 12 hr of collection period No. 1.

TABLE 3 PHYTOL FOUND IN THORACIC DUCT LYMPH OF A NORMAL SUBJECT AFTER COOKED SPINACH HAD BEEN INGESTED

Lymph Collection	Volume	Phytol Content
	ml	*mg*
Control (1:15 a.m.–6:00 a.m.)	780	<0.1
No. 1 (6:00 a.m.– 2:50 p.m.)	900	0.1
No. 2 (2:50 p.m.– 7:00 p.m.)	900	1.3
No. 3 (7:00 p.m.–12:45 a.m.)	1020	0.6
No. 4 (12:45 a.m.– 8:30 a.m.)	1080	0.1
Total		2.1
Spinach, ingested (180 g)*		108

* Ingested 6:00–8:20 a.m. during collection period No. 1; the phytol content is based on analyses of other lots.

DISCUSSION

The present results indicate that little phytol is absorbed from chlorophyll in the diet. Since free phytol is readily absorbed (2), one must conclude that the phytyl–ester linkage in the chlorophyll molecule resists the action of the intestinal enzymes. Even after being exposed to the bacterial action in the colon, a large part of the administered material remained intact.

Recoveries of total radioactivity and of phytol radioactivity in the feces were a little lower (on average) in the two Refsum's disease patients than in the two normal subjects, after pheophytin-^{14}C had been administered. This difference, which was probably due to chance or to the diminished dexterity of the patients, was not considered significant.

The availability of chlorophyll phytol in man has previously been studied only indirectly and imprecisely. Fischer and Henschel (18) identified a number of phorbides and the chlorophyll-derived porphyrin *phylloerythrine* in human feces, indicating that some phytol had been released. Brugsch and Sheard (19) found pheophytin to be the chief degradation product of chlorophyll in human feces. However, they also noted the presence of phorbides and porphyrins, and they completely failed to account for $^1/_3$ to $^1/_2$ of the administered

chlorophyll. Kohler, Elvehjem, and Hart (20) believed that large quantities of chlorophyll-derived pigments in the feces no longer contained phytol. These various investigators made no studies of the phytol moiety itself, but their results could be interpreted to suggest that a significant part of the phytol and, indeed, of the chlorophyll itself is absorbed. Various therapeutic effects have been ascribed in the past to ingested chlorophyll, but the present results indicate that little chlorophyll is absorbed in any form.

It is not known whether phytanic acid *causes* the neurologic damage in Refsum's disease, or, if it does, how much is required. Avigan (21) has pointed out that ruminants regularly exhibit appreciable levels of phytanic acid in the serum, which they tolerate without developing neurologic damage. The objective of the present study was not to elucidate the relationship between phytanic acid and neurologic damage, but rather, to discover the source of the phytanic acid, and particularly to evaluate the importance of chlorophyll, which has been indicted as a major precursor (5, 10, 22). A definite conclusion that chlorophyll is not an important source derives from the following considerations.

Although phytanic acid analyses have usually been made only on serum of patients with Refsum's disease, the tissue analyses reported by Klenk and Kahlke (1) and by Hansen (23) indicate that the total body accumulations of the acid reach levels of at least several hundred grams, even in children. If a person should eat 100 g of spinach[3] every day for 20 yr, and absorb and retain the entire phytol content, about 440 g of phytanic acid could accumulate from this source. However, if only 5% were absorbed, as the present results indicate does occur, the maximum accumulation would be only 22 g, which is a small fraction of the quantities that have been found. The diet of the usual patient with Refsum's disease does not appear to have been abnormal, and the patients are known to degrade some phytanic acid (3). Thus, unless the tissue analyses cited above should prove to be very misleading, chlorophyll could not be a quantitatively important source of the phytanic acid that accumulates in Refsum's disease.

Despite the low absorbability of chlorophyll phytol, composition of the diet *is* important in Refsum's disease, at least insofar as the accumulation of phytanic acid is concerned. This was shown by the decreases in serum and adipose tissue phytanic acid that occurred in patients who were maintained for about a year on diets low in green vegetables and animal fats (22, 24). The simultaneous clinical improvement that occurred in some of the cases is difficult to interpret, because of the natural remissions and exacerbations of the disease. Further studies are needed to identify the important sources of phytanic acid. Possibly some vegetables contain significant quantities of phytol that have already been liberated from chlorophyll through the action of natural chlorophyllase or by other means, and some foods might be rich in nonchlorophyll-derived phytol or in other isoprenoid substances that are convertible to phytanic acid. However, it now seems likely that preformed phytanic acid itself—known to be present in dairy products and in meats from ruminants (17, 25, 26) and in fish oils (27), which in some countries are extensively used in margarine—may be the most important source. Giving some support to this view are the observations of Ackman and associates on the diastereoisomers (28) of phytanic acid in the sera of patients with Refsum's disease. They found widely different ratios of the D,D,D and L,D,D isomers in patients of different countries (29), and the patterns of the isomers seemed to correspond in some degree to the patterns found in preformed phytanic acid of the prevalent foods in the respective countries.

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[3] This quantity of spinach (as weighed without excess water) is sufficient for two small servings, and contains about 60 mg of chlorophyll phytol. Spinach ranks very high among vegetable foods in chlorophyll content.

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