Synthesis and metabolism of glycerol-^{3}H triether, a nonabsorbable oil-phase marker for lipid absorption studies

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ABSTRACT A saturated mixed-chain glycerol triether, 1-hexadecyl-2,3-didodecyl glycerol (1-hexadecoxy-2,3-didodecoxypropane), was synthesized with ^{3}H at positions 9 and 10 or ^{14}C at position 1 of the hexadecyl moiety. In acute feeding experiments in rats, less than 0.2% of the triether was absorbed, based on lymph and fecal recoveries. Radioactivity was present exclusively as triether in feces, indicating that it was not degraded by digestive or bacterial enzymes. Chronic feeding experiments in rats confirmed the nonabsorbability of the triether and further indicated that it was nontoxic, did not influence the absorption of dietary fat, and mixed intimately with the fat present in colonic contents and feces. The triether that was absorbed was deposited as triether in adipose tissue, liver, and spleen. When administered intraperitoneally to mice, the triether was stored in the tissues and was not metabolized. When the triether was partitioned between an oil phase of triolein or fatty acid and monoglyceride, and an aqueous micellar phase, the triether remained exclusively in the oil phase. The triether appears to be an ideal nonabsorbable oil-phase marker for use in lipid absorption studies.

SUPPLEMENTARY KEY WORDS ^{14}C-labeled glycerol triether · 1-hexadecyl-2,3-didodecyl glycerol · 1-hexadecoxy-2,3-didodecoxypropane · glycerol triether synthesis · glycerol triether metabolism · ether lipid metabolism · nonmetabolizable lipid

In the intestine, ingested lipid is partitioned between an oil phase and an aqueous micellar phase of bile acids and lipolytic products (1). Nonabsorbable water-soluble markers have been widely used to estimate absorption from or secretion into the aqueous phase (2–4), but such markers cannot be used with certainty for substances present in the oil phase because the oil phase and aqueous phase may move through the gastrointestinal tract at different rates (5).

An oil-phase marker should be of considerable value for studies of lipid movement, absorption, and metabolism. The recent observation by Spener, Paltauf, and Holasek (6) that the ether analogue of triolein, glycerol trioleyl ether, is not absorbed by the rat suggested to us that a glycerol triether might serve as an ideal lipid marker having the required properties of nonabsorbability, nontoxicity, and resistance to digestive and bacterial enzymes. We therefore synthesized a labeled glycerol triether and have shown that such a compound appears to possess these properties. Tests of the validity of using this triether as a marker for quantitation of fat malabsorption in rats with steatorrhea induced by cholestyramine feeding are reported in the following paper.

METHODS

Synthesis of Triether

Unlabeled 1-hexadecyl-2,3-didodecyl glycerol[1] was synthesized by the method of Baumann and Mangold (7, 8). Dodecanol and hexadecanol (Lachat Chemicals Inc., Chicago, Ill.; purity >99% as determined by GLC

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

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[1] The trivial name for the triether is used in this paper to emphasize its relationship to glycerol and glycerides. The systematic name is 1-hexadecoxy-2,3-didodecoxypropane.

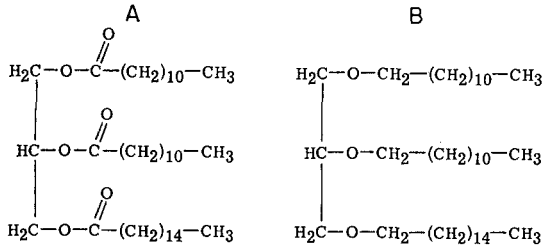

FIG. 1. 1-Hexadecyl-2,3-didodecyl triglyceride (*A*) and triether (*B*). ^3H label was on the 9 and 10 carbons of the hexadecyl side chain.

and TLC) were converted to their mesylates with methanesulfonyl chloride (Eastman Organic Chemicals, Rochester, N.Y.). Glycerol hexadecyl-1 ether was prepared using isopropylidene glycerol (Eastman Organic Chemicals) and hexadecyl mesylate; it was then reacted with 2 moles of dodecyl mesylate to give the desired product[2] (Fig. 1).

The final product was purified by chromatography on columns of silicic acid (Merck) (Brinkmann Instruments Inc., Westbury, N.Y.). A solution of the impure triether in hexane (5% w/v) was applied to the column which was then developed in a stepwise manner with hexane containing 0, 10, 20, 30, 50, and 70% benzene (v/v) and finally with 100% benzene. Nonpolar impurities (probably dialkyl ethers [8]) were eluted with 10% benzene, and pure triether was eluted with 50% benzene; column fractions were monitored by TLC. Solvent mixtures of ether in hexane did not adequately separate nonpolar impurities from the triether. The final product, usually obtained after two chromatographic procedures, showed no detectable impurities by TLC (200 μg of sample, detection with concentrated sulfuric acid–water 1:1, v/v). Because these were preliminary studies with an unknown compound, and especially because of our concern regarding toxicity, we considered it necessary to use high-purity triether as added carrier. However, in routine use of the triether as a fecal marker, 95% purity would probably be adequate, provided that the impurities were nontoxic and inert.

The triether labeled with ^{14}C was prepared by using hexadecanol-1-^{14}C (made by reduction of palmitic-1-^{14}C acid with lithium aluminum hydride [9]) in the initial synthesis of the glycerol hexadecyl-1 ether. The product was purified by preparative TLC (radiopurity >99%).

The triether labeled with ^3H was prepared by synthesizing an unsaturated triether; hexadecanol was replaced by hexadec-9-enol (8)[2], and 50 mg of the resultant 1-(9-*cis*-hexadecenyl)-2,3-didodecyl glycerol was catalytically reduced with 3 Ci of tritium (performed by New England Nuclear Corp., Boston, Mass., with prereduced platinum as catalyst). The crude product contained 2.2 Ci of tritium, 93% of which was in the triether. The labeled product was diluted with unlabeled triether to a specific activity of 0.2 mCi/mg and, immediately before use, it was purified to better than 99% radiopurity by preparative TLC.

TLC was performed on 0.5-mm layers of Silica Gel H (Merck) (Brinkmann Instruments Inc.). Chloroform was used to separate mesylates from their precursors (long-chain alcohols), and ether–hexane 1:1 (v/v) was used to separate glycerol monoethers from their mesylate precursors. Ether–hexane 15:85 (v/v) was used for analytical and preparative TLC of the triether.

Triolein-1-^{14}C

This material was obtained commercially (Applied Science Laboratories Inc., State College, Pa.) and was 99% radiopure as determined by zonal scanning (10).

Absorption of Triether

Rats were prepared with cannulas in an intestinal lymphatic and in the stomach (11) and were placed in restraining cages. Food and water were withheld, but a steady infusion of 0.9% NaCl, 2.5 ml/hr, was given via the gastric cannula beginning at the time of operation. At 24 hr postoperatively, 0.5 ml of corn oil containing labeled triether was injected through the gastric cannula and rinsed in with 2 ml of saline. The syringe was weighed before and after injection to determine the dose given. Rats were studied in pairs, with a new test meal prepared for each pair. Lymph was collected as four 12-hr samples over the 48 hr following feeding. Total fecal collections were attempted with 10 of the 12 rats tested, and satisfactory collections were made from seven (three animals had diarrhea). At 48 hr after feeding, the animals were killed, and the gastrointestinal tract was excised and analyzed for residual radioactivity.

Metabolism of Triether

12 10-day-old mice were given 0.5 ml of an emulsion containing 0.5 mg of ^3H-labeled triether, by intraperitoneal injection. The emulsion was made by sonifying for 3 min a mixture of 350 mg of corn oil, 35 mg of monoolein, 7 mg of Pluronic F68 (Wyandotte Chemicals Corp., Wyandotte, Mich.), and 7 mg of triether in 7 ml of 0.9% NaCl. Four mice were killed immediately after the injection to determine the total radioactivity given. Two mice each were killed at 7, 9, and 16 days after injection, and total carcass radioactivity was measured. The remaining two mice were killed 23 days

[2] Unlabeled glycerol triether glycerol) is now available from Analabs, Inc., North Haven, Conn. 06473, and limited supplies of the ^3H-labeled compound are available from Dr. Alan F. Hofmann.

after injection and were studied as described below for chronic toxicity.

Chronic Toxicity of Triether

Four male rats (mean weight, 292 g) and four female rats (mean weight, 213 g) received a powdered diet (Fat-Free Test Diet; Nutritional Biochemicals Corp., Cleveland, Ohio) containing 5% corn oil plus 0.5% triether for 29 days. During the test period, food intake and weight gain were measured in these animals and in a control group of eight animals which simultaneously received the same diet containing corn oil but without the triether. Then, the animals were killed, and blood was taken for determinations of hematocrit value, total and differential leukocyte counts, blood cell morphology, urea nitrogen, and sugar. Tissue was also taken from bone marrow, liver, spleen, kidney, myocardium, adrenal gland, intestine, pancreas, lung, adipose tissue, and brain for histologic examination. Weighed tissue samples were also taken from liver, spleen, kidney, brain, and adipose tissue and homogenized in chloroform–methanol 2:1 (v/v); aliquots were taken for measurement of tissue radioactivity.

Effect of Triether on Lipolysis

The hydrolysis of a corn oil emulsion by rat pancreatic juice was studied in the presence and absence of triether. An emulsion of 50 μmoles of triolein plus 20 μmoles of synthetic taurocholate per ml of phosphate buffer (pH 6.0, 0.15 M in Na^+) was mixed in varying proportions with a similar emulsion containing, in addition, 50 μmoles of unlabeled triether per ml. Samples (1 ml) of the mixed emulsions were added to 0.9 ml of buffer plus 0.1 ml of a solution of rat pancreatic juice (10 mg of lyophilized rat pancreatic juice per ml, equivalent to approximately 25 μl of fresh juice per 0.1 ml) and incubated at 37°C for 15 min. Lipolysis was stopped by adding 0.2 ml of 5 N H_2SO_4, and the liberated fatty acids were extracted and titrated (12).

Partition of Triether

Sealed 13.5-ml cellulose nitrate ultracentrifuge tubes containing an oil and a bile salt phase, together with ^3H-labeled triether and ^{14}C-labeled triolein, were shaken (132 strokes/min; amplitude, 39 mm) at 37°C. The oil phase consisted of either 2 ml of corn oil containing 40 mg of triether-^3H (approximately 6 μCi) and a tracer dose of triolein-^{14}C (approximately 0.2 μCi), or 2 ml of a mixture of oleic acid and monoolein (2:1 molar ratio) together with 40 mg each of triether-^3H and triolein-^{14}C. The bile salt phase was 6 ml of a mixture of 7 mM sodium taurocholate and 3 mM sodium taurodeoxycholate in sodium phosphate buffer (pH 6.3, 0.14 mM in Na^+).

After 24, 48, or 72 hr the tubes were centrifuged for 90 min at 20,000 rpm at approximately 37°C in a Beckman Model L-2 ultracentrifuge (no. 50 rotor). Aliquots of the clear lower phase were removed by piercing the tube, and the lipids were extracted with ether–heptane–ethanol 1:1:1 (v/v/v) (13). The radioactivity of the lipid extract and of a weighed aliquot of the original oil phase was determined by liquid scintillation counting.

Analytical Methods

Lymph. Lymph volume was measured; the lymph was then centrifuged (10 min, 2000 rpm) and a 0.5 or 1.0 ml sample of the supernate was taken. To this was added 20 volumes of chloroform–methanol 2:1 (v/v) and, after standing overnight, the solution was filtered through glass wool. To the filtrate was added 0.3 volume of 0.1 M NaH_2PO_4, forming two phases. The lower phase was removed and adjusted to 25.0 ml with chloroform–methanol 2:1. An aliquot of the chloroform–methanol phase was transferred to a counting vial, evaporated under a nitrogen stream, and counted in a toluene-based scintillation solvent system. In most studies, aliquots of lymph were also counted directly in Bray's dioxane-based scintillation mixture (14). Counts by both methods closely agreed.

To study the metabolism of triether during its passage through the intestinal mucosa, the percentage of lymph radioactivity present as triether was determined. In early studies in which lymph radioactivity was very low, this was done by determining the percentages present as saponifiable (nontriether) and nonsaponifiable (triether) fractions. In later studies, extracts of lymph lipids were also subjected to direct zonal scanning and to zonal scanning after saponification, acidification, and extraction of total lipid. The results of all three methods agreed within 10%.

Saponification of lymph lipids was performed on 10-ml samples of the chloroform–methanol extract. The solvent was removed by a stream of nitrogen, and 5 ml of ethanolic KOH (4 g of KOH in 4 ml of H_2O plus 96 ml of absolute ethanol) and petroleum ether (1:1, v/v) (13) was added. The tube was shaken and then allowed to stand at room temperature for 72 hr; 2.5 ml of water and 2.5 ml of diethyl ether were then added, and the tube was vigorously shaken. After separation of the two phases, the upper phase was removed to obtain nonsaponifiable radioactivity, and the lower phase was acidified and extracted twice with 5 ml of ether–hexane 1:1 (v/v). The upper phases were combined and evaporated to dryness in a counting vial. Toluene-based scintillation mixture was added, and the sample was counted for determination of saponifiable lipid radioactivity.

Test Meal. Samples of the corn oil–triether mixtures fed to the rats were weighed into counting vials, dissolved in toluene scintillant mixture, and counted.

Feces. Fecal collections were refluxed for 8 hr with 70 ml of chloroform–methanol 2:1 (v/v). The extract was then filtered into 100-ml volumetric flasks and made up to volume with chloroform–methanol. Samples (0.1 ml) of this were pipetted directly into 20 ml of toluene scintillant for counting. Saponification, acidification, and extraction showed negligible radioactivity remaining in the fecal debris.

Gastrointestinal Tract. The stomach plus small bowel, the cecum, and the large bowel were individually saponified, together with their contents, in 125-ml vessels in 2.5 ml of 66% KOH plus 22.5 ml of absolute ethanol. The vessels were refluxed for 90 min and allowed to cool, and the contents were then acidified with 13.5 ml of 3 N HCl. Lipid was extracted into 50 ml of toluene, and 1 ml of this extract was taken for liquid scintillation counting.

Total Carcass Radioactivity. The carcass was digested with 200 ml of 25% KOH for 72 hr at room temperature and then for 24 hr on a boiling water bath. The vessel was then cooled in an ice bath, and the contents were acidified with 100 ml of concentrated HCl. Lipid was extracted into 500 ml of petroleum ether, and 0.5-ml samples of this solution were taken for liquid scintillation counting.

Zonal Scanning. Purity of radiochemicals and the identification of the radioactive lipids were determined by zonal scanning of thin-layer chromatograms, using a zonal scraper based on the design of Snyder and Kimble (10). Thin-layer chromatograms of lipids were developed for 12 cm in appropriate solvents and then scanned (4 mm resolution); appropriate standards were run with each separation.

RESULTS AND DISCUSSION

Synthesis of Triether

The over-all yield of triether synthesized by the method of Baumann and Mangold was about 30%, based on the hexadecanol used. The 1-hexadecyl-2,3-didodecyl glycerol was a white solid melting at 23°C. GLC on a 1% OV-17 column at 280°C showed a single broad peak with a retention time of 1.74 relative to methyl 3,7,12-triketo-5β-cholanoate.[3] TLC in a number of solvent systems showed a single component with an R_f slightly greater than that of triglyceride (Table 1). The infrared spectrum (Perkin–Elmer, Model 337) of the triether was identical with that of a sample of 1,2-dioctadecyl-3-dodecyl glycerol generously supplied by Dr. W. J. Baumann of the Hormel Institute (Austin, Minn.).

[3] Methyl 3,7,12-triketo-5β-cholanoate (methyl dehydrocholate) was chosen as a reference compound because it has an appropriate retention time, is a stable solid, and is available commercially in high purity and at low cost.

TABLE 1 MOBILITY OF TRIETHER ON THIN-LAYER CHROMATOGRAMS

	Solvent System*			
	A	B	C	D
Cholesterol oleate	1.90	1.18	1.42	—
Triether	1.63	1.18	1.34	1.11
Methyl oleate	1.41	1.01	1.12	0.95
Triolein	1.00	1.00	1.00	1.00
Cholesterol	0.11	0.20	0.21	0.45
Monoolein	0.00	0.04	0.00	0.08

Mobilities relative to triolein in four solvent systems. Migration distance 10 cm on Silica Gel H (Merck), 0.5 mm thickness; lined tank; values are means of three determinations.

* Solvent systems (v/v): A, hexane–diethyl ether, 85:15; B, hexane–diethyl ether–methanol–glacial acetic acid, 85:20:3:3 (15); C, hexane–diethyl ether, 75:25; D, benzene–acetone–glacial acetic acid, 90:10:1.

The hexadecyl didodecyl product was used because it offered significant advantages, in its preparation and properties, over the glycerol trioleyl ether used by Spener et al., (6). Thus, the preparation of saturated mesylates and monoethers is simple because precautions against oxidation and *cis-trans* isomerization are not needed. Furthermore, the intermediate products crystallize more readily and at higher temperatures. However, the major advantage of the synthetic scheme described is that the unsaturated alkyl residue can be reduced with tritium to give a highly labeled product containing non-exchangeable tritium. The choice of chain length in the triether was dictated by our desire that the final product be liquid at body temperature.

Absorption of Triether

Recovery from Lymph. Two rats were fed 10 mg of ^{14}C-labeled triether, two were fed 10 mg of triether labeled with both ^{3}H and ^{14}C, four were fed 10 mg of ^{3}H-labeled triether, and four were fed 100 mg of ^{3}H-labeled triether (Table 2). A mean of 0.14% (0.07–0.29%) of the dose fed was recovered; the percentage recovery of the dose was lower after feeding 100 mg of triether than after 10 mg. However, the output after a dose of 100 mg of triether was equivalent to a recovery of 122.4 nmoles of triether while the output after a 10 mg dose equaled only 24.4 nmoles. This difference is statistically significant ($P < 0.001$, "t" test).

The mean value for lymphatic recovery, 0.14%, closely agrees with the lymphatic recovery of oleyl triether reported by Spener et al. (6) (less than 0.14%). However, since the intestinal lymph fistula preparations used in our experiments gave only about a 60% recovery of fed radioactive triglycerides and fatty acids, the total lymphatic transport of triether may have been as high as 0.25% of the dose fed. This value presumably would represent all of the absorbed triether.

TABLE 2 Recovery of Radioactive Triether from Intestinal Lymph

Triether Labeled With:	Dose		Recovery in Lymph	
			% of Dose Fed	nmoles
	mg	*μmoles*		
^{14}C	10	15.3	0.12	18.4
			0.09	13.6
			0.29*	44.4
			0.08†	12.2
		Mean	0.14	22.2
^{3}H	10	15.3	0.12	18.4
			0.08	12.2
			0.28*	42.8
			0.08†	12.2
			0.28	42.8
			0.19	29.1
		Mean	0.17	26.3
^{3}H	100	153	0.11	168.3
			0.07	107.1
			0.07	107.1
			0.07	107.1
		Mean	0.08	122.4

*† These animals were fed a mixture of ^{14}C-labeled and ^{3}H-labeled triether and are included in both groups.

The amount of triether absorbed was dose related in that a 10-fold increase in dose caused a five-fold increase in absorption. If the partition coefficient of triether between an oil and a micellar phase is independent of concentration, then a larger dose of triether would cause an increased level of triether in the lipid phase. An increased concentration of triether in the micellar phase with correspondingly increased transport to the mucosa and increased absorption would follow. The possibility that nonsolubilized triether is absorbed, with or without associated oil phase, by pinocytosis cannot be excluded.

The lymphatic recovery of ^{3}H-labeled triether was similar to that of ^{14}C-labeled triether, indicating that the ^{3}H label was stable during passage of the triether through the upper gastrointestinal tract. In the two animals fed both labels, the ratio of ^{3}H to ^{14}C in the lymph, cecal and colonic contents, and feces was essentially the same as that in the test meal (Table 3).

Form of Radioactivity in Lymph. The percentage of lymph radioactivity present as triether varied considerably from group to group (Table 4). However, the percentages were similar in rats fed the same test meal. This suggested the possibility that the test meals contained varying trace amounts of a readily absorbed impurity. To test this, a sample of triether was purified to more than 99.7% purity by repeated preparative TLC and was fed at two doses (10 and 100 mg) to two rats each. Lymph lipids were subjected to zonal scanning directly and after saponification, acidification, and extraction. A mean of 80% of the lymph activity was present as triether (Fig. 2).

TABLE 3 Ratio of ^{3}H to ^{14}C After Feeding Triether Labeled with ^{3}H and ^{14}C

Sample	^{3}H:^{14}C Ratio	
	Rat 1	Rat 2
Test meal	1.00	1.00
Lymph	0.96	0.96
Cecum	0.85	*
Colon	0.92	*
Feces	0.94	0.96

* Insufficient counts.

TABLE 4 Percentage of Lymph Radioactivity as Triether after Test Meals

Test Meal	Triether Fed		Purity of Triether	% of Lymph Radioactivity as Triether	
	Amount	Label		Rat 1	Rat 2
	mg		%		
1	100	^{3}H	99.1	28	28
2	10	^{14}C	99.1	89	84
3	10	^{3}H and ^{14}C	^{3}H, 99.1; ^{14}C, 100.6	67	63
4	10	^{3}H	99.1	90	83
5	10	^{3}H	>99.7*	77	85
6	100	^{3}H	>99.7*	91	69

Each test meal was fed to two animals.
* The triether was purified by repeated (three times) preparative TLC.

Our finding that more of the lymph radioactivity was present as triether with increasing radiopurity of administered compound suggests that the radioactivity present in lymph in compounds other than triether originates from radioactive impurities in the administered triether. Spener et al. (6) also observed that there was considerable hydrolysis of the oleyl triether during its

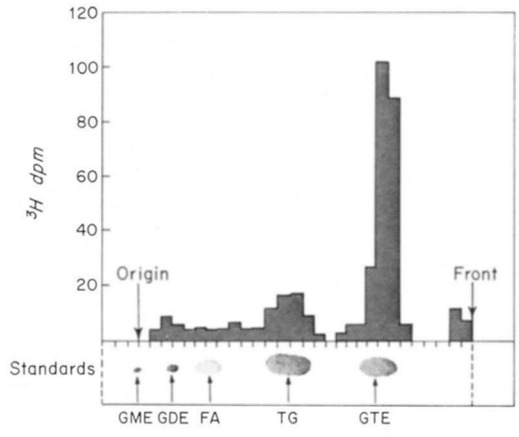

Fig. 2. Zonal scan of lipid extract of intestinal lymph obtained after feeding highly purified (>99.7%) ^{3}H-labeled triether. Standards: GME, glycerol monoether; GDE, glycerol diether; FA, fatty acid; TG, triglycerides; and GTE, glycerol triether.

absorption, but their results could be explained by diether impurities present in their sample. Paltauf (16) has now reported that diethers are well absorbed in the rat.

Recovery from Feces and Gastrointestinal Tract. In the seven rats from which satisfactory collection was possible, a mean of 93.3% of the triether (individual values, 80, 85, 90, 97, 98, 100, and 103%) was recovered in the feces plus cecal and colonic contents. In five animals, radioactivity was present only in the feces, but in two rats 15% of the triether was retained in the cecum.

Zonal scans of fecal extracts showed fecal radioactivity present solely as triether, indicating that the triether was not degraded by digestive and bacterial enzymes in the normal rat. In seven rats, a mean of 99.1% (range, 98.2–100.4%) of the radioactivity was present in the triether peak. Radioactivity present not as triether was spread throughout the chromatogram, and no other identifiable peaks were detected (Fig. 3). Both the lymph and fecal recoveries show that very little of the triether is absorbed by the normal rat.

Metabolism of Triether

At 7 and 9 days after intraperitoneal injection of an emulsion containing ^{3}H-labeled triether into 10-day-old mice, virtually all the injected radioactivity could be recovered from the carcass. At 16 days after injection, all the radioactivity was recovered from one mouse but only 60% from a second mouse (Table 5). In all animals studied, zonal scans showed that carcass radioactivity was confined to triether fractions.

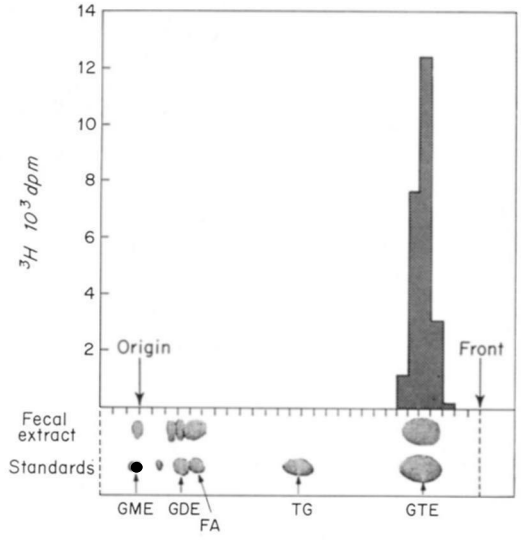

FIG. 3. Zonal scan of lipid extract of feces obtained after feeding ^{3}H-labeled triether. Abbreviations as for Fig. 2. A zonal scan of the lipid extract of omental fat from a mouse given triether intraperitoneally was identical to that shown for fecal extract. Both chromatograms indicate that the triether was not metabolized.

No macroscopic abnormalities could be detected in two mice killed 23 days after injection. Histologic examination of organs also showed no abnormalities apart from an inflammatory response on the serosal surface of the liver and the perirenal fat, which was attributed to the emulsifying agent.

Extraction and liquid scintillation counting of weighed aliquots from various organs showed radioactivity to be confined to the adipose tissue, liver, and spleen. Calculation of the probable total radioactivity in each organ, using published values for average organ weights (17), showed that about 80% was present in adipose tissue with the remainder equally distributed between liver and spleen. Zonal scanning of the lipid extract from omental and epididymal fat showed that activity was confined to triether.

Using calculated total organ radioactivity it was estimated that each mouse still retained most of the injected dose 23 days after intraperitoneal injection (Table 5). An emulsion injected intraperitoneally should be rapidly absorbed by the subdiaphragmatic lymphatics (18) and from there enter the bloodstream and pass to the liver. Some of the triether was taken up by the liver and spleen, presumably by macrophages, but the majority was present in the adipose tissue. Further mobilization of the triether from that site would probably be very slow indeed because young growing animals were used, and the turnover of adipose tissue itself in these animals would probably be slow (19).

Some of the triether thought to be in omental fat may have been lipid taken up by macrophages from the serosal surface, in view of the inflammatory response seen on microscopy. This may explain the smaller percentage of radioactivity found in adipose tissue after triether was fed to rats for 28 days; in these animals most of the radioactivity was present in the liver. The sequestration of a nonmetabolizable lipid by the reticuloendothelial cells in the liver and spleen might be expected in view of their role in removing foreign emulsions from the bloodstream (20).

TABLE 5 METABOLISM OF TRIETHER AFTER INTRAPERITONEAL INJECTION INTO 10-DAY-OLD MICE

Days after Injection	No. of Mice	Recovery	
		% of Dose	% as Triether
0	4	100	98, 98, 99, 99
7	2	103, 88	98, 98
9	2	98, 95	98, 98
16	2	100, 60	Not estimated
23	2	60–70*	99
		70–80	

* Estimated from average organ weights (see text). Adipose tissue, 80%; liver, 10%; spleen, 10%.

Toxicity of Triether

No animals receiving triether died, but three control rats died of endemic pneumonia during the test period. Food intake and weight gain of animals fed the triether were similar to those of control animals. Blood tests on triether-fed animals and surviving control animals produced identical results (Table 6). Histologically, tissue from animals fed triether was normal in every case.

Radioactivity was distributed chiefly in liver (mean ± SEM, 54.7 ± 4.0%), adipose tissue (40.5 ± 4.0%, assuming it was 10% of body weight), and spleen (2.7 ± 0.6%). Negligible radioactivity was present in the kidney and brain. The radioactivity recovered in liver, adipose tissue, and spleen was equivalent to 0.14 ± 0.02% of the dose ingested over 28 days, in agreement with percentage absorption measured by lymphatic output or fecal balance.

Zonal scanning of the lipid extracts was difficult due to the extremely low specific activity of the extracts. However, in the eight test animals, the only peak of radioactivity in the liver extracts corresponded to triether. This finding, as well as the agreement between percentage absorption estimated from lymphatic output or fecal balance and that estimated from recovery of administered radioactivity, is additional evidence that absorbed triether is metabolized slowly if at all, despite the existence of an active "etherase" in intestinal mucosa and liver (21, 22).

The dose of triether used in all of the studies with rats (400 mg/kg in acute studies and 890 mg/kg in chronic studies), is considerably in excess of that which would be needed for absorption studies in humans. For clinical use, a single dose of about 1 mg/kg would suffice, and about 0.2% of this would be absorbed, so that the amount of triether entering the body would be 0.002 mg/kg. This is 1/10,000 of the amount injected into the mice.

TABLE 6 DIETARY INTAKE DURING, AND HEMATOLOGIC INDICES AND BLOOD CHEMISTRIES (MEANS ± SEM) AFTER A 28 DAY CHRONIC TOXICITY STUDY OF TRIETHER

	Controls	Test
Deaths	3/8	0/8
Intake (g/day)	14.1 ± 1.4	16.2 ± 0.7
Weight gain (g)*	11 ± 40	30 ± 5
Blood determinations:		
Hematocrit (%)	44 ± 4	45 ± 3
BUN (mg/100 ml)	41 ± 2	43 ± 2
Blood glucose (mg/100 ml)	104 ± 9	108 ± 5
Differential leukocyte count	Normal	Normal

Control and test groups each consisted of four male and four female rats. Total triether intake of the test group was 235.2 ± 10.1 mg which corresponds to 31.8 ± 1.7 mg/kg per day.

* Small gain and large SEM for controls were caused by endemic pneumonia in the colony; two males and one female of the control group died of pneumonia.

Effect of Triether on Lipolysis

When triether was added to emulsified triolein, moderate depression of lipolysis was seen with molar ratios of triether to triglyceride up to 0.4. However, further increases in triether content caused only slight further depression of lipolysis (Fig. 4).

Triether might be a potent competitive inhibitor of lipase because, although its structure is similar to that of triglyceride, it cannot be hydrolyzed; however, little effect was seen. In view of the small amount of triether which would be needed in lipid absorption studies, any effect of triether on lipolysis during use of the material as a fecal marker would be expected to be undetectable.

Partition of Triether

Partition values of both labeled lipids varied considerably in the 24-, 48-, and 72-hr equilibration studies, probably due to the very low aqueous solubilities of the lipids and the consequent low level of radioactivity in the aqueous samples. Because no consistent variation with time was seen, the results for the three studies have been averaged. When corn oil was present as the oil phase, ^{3}H-labeled triether showed an oil phase-micellar bile acid phase distribution coefficient of approximately 5×10^4 while that for ^{14}C-labeled triolein was 4×10^4. When fatty acid–monoolein was used as the oil phase, the distribution coefficients were higher, 7×10^5 for triether and an unmeasurable value for triolein.

These findings agree with both old (23) and recent (1, 24) reports that triglycerides are insoluble in bile acid solutions and also with the findings of Borgström (24) that the partition of triglyceride between an oil phase and a micellar bile acid solution is greatly in favor of the oil phase. Our experiments indicate that triether

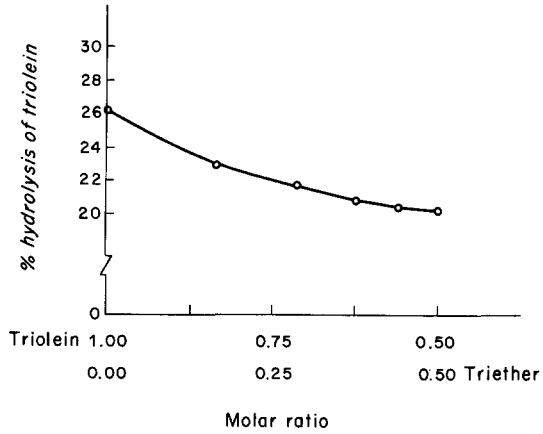

FIG. 4. Influence of added emulsified triether on hydrolysis of emulsified triolein by rat pancreatic juice: 50 μmoles of triolein or triether was emulsified with 20 μmoles of sodium taurocholate in 2.0 ml of phosphate buffer, pH 6.0 (0.15 M in Na^+). Mixture was incubated for 15 min at 37°C, and amount of lipolysis was determined by extraction and titration of the released fatty acids.

has physical properties which are similar to those of triglyceride when partitioned between an oil phase and a micellar bile acid solution. It is interesting that the triether partitions even less readily into the micellar phase from an oil phase composed predominantly of lipolytic products.

COMMENT

These studies show that 1-hexadecyl-2,3-didodecyl glycerol triether possesses a number of the properties required for a substance to be an oil-phase marker: it is very poorly absorbed, it is readily measured, and it is stable to digestive and bacterial enzymes (at least in the normal rat). There is no evidence of toxicity, and it does not significantly affect digestion. It has a very low solubility in the intestinal micellar phase. Triether is, therefore, a promising marker for the oil phase in studies of lipid absorption. Sie, Valkema, and Loomeijer (25) have shown that silicone oil also has a number of the properties of an ideal oil-phase marker, and it is possible that the only major advantage of triether over other potential oil-phase markers such as silicone oil or branched-chain hydrocarbon is the ease of measurement of the triether.

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