Use of ^{3}H-labeled triether, a nonabsorbable oil-phase marker, to estimate fat absorption in rats with cholestyramine-induced steatorrhea

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ABSTRACT A tritium-labeled glycerol triether was tested as a nonabsorbable oil-phase marker in studies of fat absorption in normal rats and in rats with steatorrhea induced by various doses of cholestyramine. Animals were fed a test meal containing ^{3}H-labeled triether and ^{14}C-labeled trilinolein. Fat absorption was estimated in the following three ways: (*a*) by isotope ratios (the change in ^{3}H/^{14}C in the test meal and in feces); (*b*) by isotope recovery (the total fecal excretion of ^{14}C radioactivity); and (*c*) by chemical recovery (the total fecal fat excretion). Absorption calculated from isotope ratios agreed well with that calculated from isotope recovery over a range of fat absorption of 50–100%, thus validating the use of this lipid marker under these conditions of fat malabsorption. Absorption calculated from chemical recovery was consistently poorer than that calculated from isotope ratios or isotope recovery, thus suggesting that cholestyramine increased the excretion of nondietary (endogenous) fat. Triether may be of value for studying the absorption of compounds present predominantly in the oil phase during digestion and may have significant advantages over other proposed lipid markers.

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

CHEMICAL AND ISOTOPIC balance techniques are generally considered to be the most accurate methods for measuring fat absorption (1, 2). In the chemical balance technique, the total intake and output of fat are determined chemically. However, since the output may include a considerable nondietary (endogenous) component, absorption calculated by this technique is not necessarily valid (3–5). Furthermore, fecal collections must be continued for several days, because of irregularity of colonic emptying, and must be complete during the collection period.

In the isotopic balance technique, a radioactive fat is fed and the fraction of radioactivity, which is excreted in the feces, is determined (3–5). If the radioactive fat is similar in composition to the dietary fat and is fed together with a normal diet, its absorption should closely approximate the absorption of dietary fat. However, as in the chemical balance technique, a collection period of sufficient duration is required to obtain all of the label which is excreted, and, in addition, the fecal collection must be complete.

The necessity for prolonged and complete fecal collection could be overcome if a nonabsorbable lipid marker were fed together with the radioactive fat. In a previous paper (6), it was shown that an ether analogue of a triglyceride possessed the properties of an ideal lipid marker. This material has, therefore, been tested in this role in rats with varying degrees of steatorrhea induced by feeding cholestyramine. Fat absorption calculated from the marker content of daily fecal collections has been compared with fat absorption measured by chemical and isotopic balances.

MATERIALS AND METHODS

The experimental design was based on the studies by Harkins, Hagerman, and Sarett (7), and fat content and

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cholestyramine levels in the diet were chosen on the basis of their data. Absorption was studied in 16 rats of the Mayo Clinic strain (Sprague-Dawley derived) each weighing about 300 g. Animals were housed in individual metabolism cages with wire-mesh floors designed to permit the quantitative collection of feces free of urine. They were fed a powdered diet containing nominally 15% corn oil plus varying amounts of cholestyramine (Mead Johnson & Co., Evansville, Ind.). The diet was prepared by blending 7290 g of powdered fat-free diet (Nutritional Biochemicals Corp., Cleveland, Ohio)[1] with 1078 g of corn oil in a commercial rotary beater at low speed for 30 min with frequent manual stirring. Three 2000-g portions of this were then blended with 40, 80, and 120 g of powdered cholestyramine, making four diets containing nominally 0, 2, 4, and 6% cholestyramine. Each diet was fed to four animals.

Food was made available to the rats in containers attached to the end of short tunnels so as to minimize the transfer of food to the cage where it would contaminate the feces. The daily food intake was measured by weighing the container each morning before and after filling. Daily fecal collections were made and analyzed to allow an estimate of daily fat excretion.

Test Meal

After the unlabeled diet had been fed for 14 days, it was replaced by a test meal containing ^{14}C-labeled trilinolein and ^{3}H-labeled triether.[2] This test meal was prepared by sprinkling tracer doses of the labeled lipids dissolved in ether over portions of the powdered 15% corn oil diet spread thinly on a tray. The food was warmed to evaporate off the ether and was then thoroughly blended. After 24 hr, this labeled test meal was replaced with the unlabeled diet.

The labeled diet contained 1.0 μCi of ^{3}H-labeled triether per g in every case. However, the dose of ^{14}C-labeled trilinolein varied depending upon the amount of cholestyramine being fed: none, 0.1 μCi/g of test meal; 2% cholestyramine, 0.05 μCi/g; 4 or 6% cholestyramine, 0.025 μCi/g. This variation in ^{14}C dosage was designed to produce approximately equal ^{3}H/^{14}C ratios in all fecal collections, on the basis of the decrease in fat absorption anticipated in animals fed cholestyramine. Fecal collection was continued for a further 48 hr (days 16 and 17) after removal of the labeled test meal.

[1] Composition stated by manufacturer: casein, 21.10%; Alphacel "cellulose," 16.45%; sucrose, 58.45%; salt mixture (USP XIV), 4.00%; vitamin supplements.

[2] Triether = 1-hexadecyl-2,3-didodecyl glycerol triether (trivial name) or 1-hexadecoxy-2,3-didodecoxypropane (systematic name). The ^{3}H label was in the 9 and 10 positions of the hexadecyl moiety.

Analytical Methods

Daily fecal collections were analyzed for saponifiable fat content by a modification of the method of Jover and Gordon (8). Each daily collection was refluxed for 90 min in a mixture of 22.5 ml of 95% ethanol and 2.5 ml of 66% KOH. The mixture was then cooled and acidified with 13.0 ml of 2.5 N HCl. Fatty acid was extracted into 30 ml of toluene, and 3 ml of the organic phase was taken for titration against 0.08 N tetrabutylammonium hydroxide in absolute methanol (9), with 3 drops of 0.1% bromthymol blue in absolute ethanol as the indicator. For radioactivity determinations, a 1.0 ml sample of the toluene phase was pipetted into 15 ml of a toluene-based liquid scintillation counting solution and counted in a Picker Liquimat liquid scintillation counter. Weighed samples of the four test diets taken at 2-day intervals were analyzed in quadruplicate by the same technique. Values agreed within 5% in all cases, indicating that the diets were homogeneous. The mean value for saponifiable fat for each test diet was used in calculating the daily fat intake for each group. Weighed samples of the labeled test meals were also analyzed for fat and radioactivity.

Calculations

The daily fat intake was calculated from the weight of food eaten and the measured fat content of the diet. Over the last 3 days of the experiment (the 24 hr during which the labeled test meal was available and the following 48 hr), fat absorption was calculated by the following three methods.

Chemical Recovery. Fat balance was estimated by the formula:

$$\% \text{ fat absorbed} = (1 - [\text{daily fecal excretion per daily fat intake}]) \times 100.$$

For the 3 day period a mean daily fat balance was calculated.

Isotope Recovery. Absorption of ^{14}C-labeled trilinolein was estimated by the formula:

$$\% \text{ trilinolein absorbed} = (1 - [\text{total } ^{14}\text{C excreted over 3 days per total } ^{14}\text{C fed}]) \times 100.$$

Isotope Ratio. Absorption of ^{14}C-labeled triglyceride was also calculated from the ratio of ^{3}H-marker to ^{14}C in the daily fecal collection by the formula:

$$\% \text{ trilinolein absorbed} = (1 - [^{3}\text{H}/^{14}\text{C in test meal per } ^{3}\text{H}/^{14}\text{C in daily fecal collection}]) \times 100 \text{ (10).}$$

RESULTS

Cholestyramine decreased the fat absorption with all dose levels used with the decrease being proportional to the dose of cholestyramine fed (Tables 1 and 2). Animals

TABLE 1 INTAKE AND EXCRETION OF FATTY ACID, DAYS 1–17

Cholestyramine	Rat No.	Food Ingested	Fatty Acid Ingested	Fatty Acid Excreted
%		g	mEq	
0	1	17.45 ± 2.90	7.56 ± 1.26	0.41 ± 0.12
	5	17.42 ± 2.80	7.55 ± 1.21	0.42 ± 0.12
	9	16.16 ± 2.38	7.01 ± 1.03	0.47 ± 0.89
	13	19.12 ± 1.95	8.29 ± 0.85	0.59 ± 0.15
	Mean	17.54 ± 2.51	7.60 ± 1.17	0.47 ± 0.14
2	2	15.98 ± 3.78	6.82 ± 1.61	0.98 ± 0.45
	6	17.12 ± 2.76	7.32 ± 1.17	1.44 ± 0.35
	10	15.59 ± 1.45	6.62 ± 0.62	1.28 ± 0.37
	14	16.49 ± 1.97	7.06 ± 0.84	1.12 ± 0.26
	Mean	16.29 ± 2.64	6.95 ± 1.06	1.20 ± 0.36
4	3	17.57 ± 3.60	7.32 ± 1.53	2.33 ± 1.35
	7	17.52 ± 3.23	7.32 ± 1.38	1.95 ± 0.93
	11	17.40 ± 3.69	7.25 ± 1.55	2.62 ± 1.07
	15	14.30 ± 3.51	5.95 ± 1.46	1.33 ± 0.71
	Mean	16.70 ± 3.71	6.95 ± 1.56	2.06 ± 1.14
6	4	21.16 ± 4.11	8.64 ± 1.69	5.21 ± 1.55
	8	20.75 ± 2.43	8.52 ± 0.99	3.45 ± 0.81
	12	21.10 ± 2.16	8.67 ± 0.88	5.45 ± 0.47
	16	17.74 ± 2.55	7.28 ± 1.05	3.70 ± 1.09
	Mean	20.19 ± 3.18	8.29 ± 1.30	4.45 ± 1.36

The results are means ± SD; 17 day experimental period.

receiving no cholestyramine absorbed a mean of 94.1 ± 1.8% (± SD) of the dietary fat, based on the daily chemical fat balances performed over the 17 day experimental period. When 2% cholestyramine was present in the diet, calculated absorption decreased to 82.9 ± 5.0%, and 4% cholestyramine reduced calculated absorption to 68.5 ± 13.1%. As discussed below, the steatorrhea reflects not only malabsorption of dietary fat but also increased excretion of endogenous fat. Despite this degree of steatorrhea, diarrhea was not a problem, and a complete daily fecal collection was possible with all groups.

Moderate contamination of feces with food occurred frequently, but no correction for this contamination was attempted (see Discussion). When the radioactive test meal was fed, two animals (one fed no cholestyramine and one fed 4% cholestyramine) showed gross contamination of feces with the test meal, and data from these animals were discarded. The remaining 14 rats excreted a mean of 79.2 ± 7.5% of the ingested triether, about 20% on the day of feeding, 50% on the following day, and 10% on the third day. Fecal collection was not continued beyond this period.

Absorption calculated from the recovery of ^{14}C in the feces for the 3 days of the test period agreed closely with absorption calculated from the isotope ratios (Table 2). A comparison of the two methods showed correlation coefficients of 0.93 on the first day (day 15), 0.99 on the second dry (shown in Fig. 1), and 0.92 on the third day.

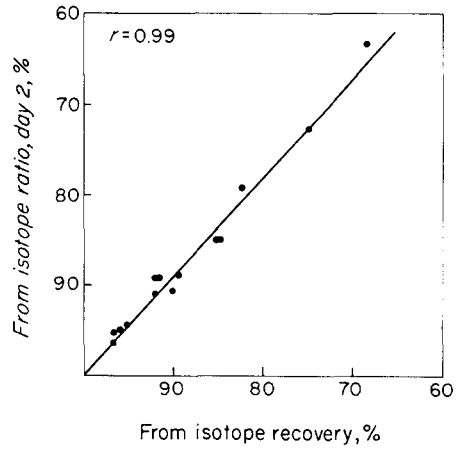

FIG. 1. Correlation of estimates of fat absorption by isotope recovery (during 3 days after administration of test meal) and by isotope ratio on feces obtained during second day after the radioactive test meal. No significance can be assigned to slightly greater absorption calculated from isotope recovery (slope = 1.1).

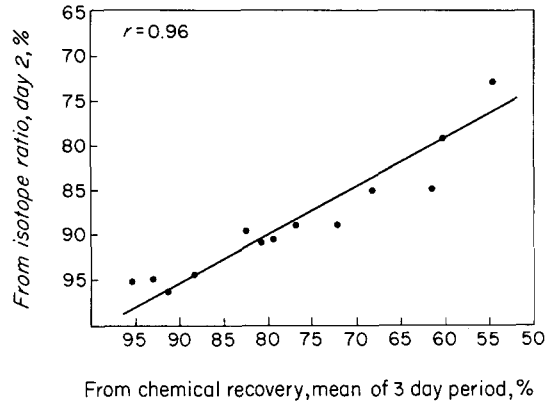

FIG. 2. Correlation of estimates of fat absorption by chemical balance (during 3 days after administration of test meal) and by isotope ratio in feces obtained during second day. The slope (0.54) is considered to indicate increased excretion of endogenous fat which is determined chemically. The high correlation coefficient suggests that cholestyramine reduced the absorptions of dietary and endogenous fat similarly.

The mean chemical balance over the 3 day period after the test meal agreed closely with the fat balance determined over the entire 17 day period for 11 of the 14 rats (Table 2). When absorption calculated from chemical recovery on days 15–17 for each rat was compared with absorption calculated from isotope ratios, a correlation coefficient of 0.89 was found for day 15, 0.96 for day 16, and 0.87 for day 17. However, the chemical balance technique consistently indicated poorer absorption than did the isotope ratio method. In Fig. 2, absorption calculated from the 3 day mean chemical balance is plotted against absorption calculated from the isotope ratio in the feces on the second day after feeding. The slope of this line is 0.54 (calculated by the method of least squares).

TABLE 2 FAT ABSORPTION CALCULATED FROM CHEMICAL FAT BALANCE, ISOTOPE RECOVERY (^{14}C), AND ISOTOPE RATIOS ($^{3}H/^{14}C$) AFTER A TEST MEAL CONTAINING ^{14}C-LABELED TRILINOLEIN AND ^{3}H-LABELED TRIETHER FED ON DAY 14

Cholestyramine	Rat No.	Fat Absorption (%) Calculated by Different Methods						
		Chemical Balance		Isotope Recovery	Isotope Ratio			
		Days 1–17	Days 14–17	Days 14–17	Days 14–15	Days 15–16	Days 16–17	Days 14–17*
%								
0†	1	94.6	95.4	96.5	95.5	95.2	91.0	95.2
	5	94.4	93.2	96.0	93.7	94.9	89.0	94.3
	9	93.3	91.4	96.7	93.4	96.3	96.1	95.4
Mean ± SD		94.1 ± 1.8	93.3	96.4	94.2	95.5	92.0	95.0
2	2	85.6	88.4	95.1	91.1	94.4	93.9	93.6
	6	81.1	82.6	92.2	86.9	89.2	88.8	88.5
	10	80.6	76.9	91.6	93.3	89.0	83.6	89.2
	14	84.2	80.9	92.2	89.0	90.8	90.2	90.4
Mean ± SD		82.9 ± 5.0	82.2	92.8	90.1	90.8	89.1	90.4
4†	3	68.2	79.6	90.1	86.3	90.6	85.6	89.0
	7	73.4	72.1	89.7	87.0	88.8	84.0	87.9
	11	63.9	68.1	85.3	79.7	85.0	76.0	83.2
Mean ± SD		68.5 ± 13.1	73.3	88.4	84.3	88.1	81.9	86.7
6	4	40.1	54.9	74.8	59.9	72.8	51.4	68.9
	8	59.4	60.4	82.4	63.8	79.2	76.0	75.1
	12	37.1	36.3	68.3	63.4	63.4	−6.9‡	62.2
	16	49.3	61.5	84.8	81.2	84.9	74.3	83.6
Mean ± SD		46.5 ± 13.1	53.3	77.6	67.1	75.1	48.7	72.4

* These ratios were calculated from the total radioactivity present in feces during days 14–17.
† One rat from this group excluded because of gross contamination of feces by test meal.
‡ This sample contained insufficient ^{3}H counts for estimation of a valid $^{3}H/^{14}C$ ratio.

In these comparisons the assumption is that labeled trilinolein and unlabeled corn oil are absorbed to the same extent. Since corn oil consists largely of unsaturated fatty acids (52% linoleic and 30% oleic) (11), digestion of it will produce a mixture of unsaturated fatty acids and monoglyceride, as will digestion of trilinolein. There is no evidence to suggest that the absorption of linoleic and oleic acids and monoglyceride differs.

DISCUSSION

Validation

This study shows that ^{3}H-labeled triether is a valid lipid marker in studies of fat absorption in normal rats and in rats with cholestyramine-induced steatorrhea. Isotope recovery for the 3 day test period and isotope ratio on the second day of the test period appear to estimate the same parameter with equal accuracy. However, the estimates of fat absorption based on isotope recovery did not correlate as well with those based on the isotope ratios of feces collected on the first and third days of the test period (r = 0.93 and 0.92, respectively). In part, this may be due to difficulty in obtaining enough ^{14}C counts in these early and late samples to allow an accurate estimate of the isotope ratio. However, it seems likely that the movement of the marker is, in fact, slightly different from that of fat and its digestion products. Nonetheless, these studies show that triether can be used as an effective lipid marker if the fecal collections contain sufficiently large amounts of radioactivity.

Fat absorption measured by chemical recovery was strikingly lower than that calculated from the isotope ratios, despite the linear correlation between the methods. The apparently lower value indicated by the chemical recovery method is probably caused by increased endogenous fat in the feces. This finding that bile-fistula animals have a significant increase in the excretion of endogenous fat, agrees with the observation by Bernhard, Ritzel, and Hug (12) and Holasek (13), but it does not agree with that by Blomstrand and Lindquist (3). Endogenous fecal fat is not measured by the isotope ratio method.

The slope of the line (0.54) in Fig. 2 suggests that about half of the fecal fat is of endogenous origin, and cholestyramine appeared to inhibit the absorptions of dietary and endogenous fat similarly. Animals receiving no cholestyramine excreted a mean of 0.40 ± 0.11 mEq of

fatty acid per day. Endogenous fat output in these animals should, therefore, be about 0.2 mEq of fatty acid, which agrees with the fatty acid output reported for rats on a fat-free diet (14, 15).

Some food spillage occurred in all cages. In most cases the food coated the collecting funnel and was washed off during the daily cleaning. This removal of food did not affect the fecal analysis, but it did cause a slight overestimate of the calulated marker intake. Such an overestimate of triether intake could explain the recovery of only 80% of the calculated dose compared to >93% fecal recovery of triether administered intragastrically (6). In further support of this argument, the lowest recoveries of triether were observed in the two animals excluded because of gross fecal contamination (52% recovery in both cases). Lastly, the 3 day collection period may have been too short for complete excretion of the administered triether.

A similar overestimate of the dose of labeled trilinolein would lead to an overestimate of the absorption based on fecal recoveries of ^{14}C radioactivity. However, since contamination of feces by diet would have the opposite effect, no significance can be assigned to the slightly lower absorption calculated from isotope ratios than from isotope recovery.

Nonabsorbable Markers

In recent years, nonabsorbable markers have found increasing use as dilution indicators in studies of intestinal absorption and secretion (16). Although polyethylene glycol (mol wt 4000) seems ideal for perfusion or intubation studies concerned with substances well dispersed in an aqueous phase (17), it does not appear to be valid as a marker for the study of lipids, such as triglyceride, which separate from it in the stomach (18, 19).

Insoluble salts have also been used as nonabsorbable markers in studies of lipid absorption. The most frequently used is chromium sesquioxide, either after attaining a steady state (20–23) or as an acute dose (24–27). As a steady-state marker, chromic sesquioxide allows a valid estimate of absorption to be made from incomplete fecal collections (20), but prolonged feeding of the marker and test diet is still required. This compound also appears to be satisfactory as an acute marker, although there is evidence that the insoluble salt and lipids move through the gastrointestinal tract at different rates (24). Furthermore, the particulate nature and high density of chromic sesquioxide could cause sampling errors in experiments in which fine tubes are used to collect intestinal content (27).

Other insoluble salts have been suggested. Both $^{131}BaSO_4$ (10, 28) and $^{95}ZrO_2$ (29) have been combined with ^{131}I-labeled triolein to test fat absorption, with promising results. However, as with chromium sesquioxide, some separation of marker and lipid occurred, and accurate estimates of fat absorption were possible only with stool collections near the middle of the excretion period. In addition, both are gamma ray emitters with short half-lives, and the estimation of ^{14}C-labeled lipids in the presence of these markers would require additional handling. After our work had been completed, Seife and Shils (28) reported studies in which fat absorption was measured in a group of patients with various degees of malabsorption, by both conventional chemical techniques (saponification and titration) and by the marker technique (^{131}I-labeled triolein with $^{133}BaSO_4$ as the nonabsorbable marker). The results by the two methods correlated well ($r = 0.94$) even in patients with significant malabsorption. In contrast to our findings, they did not observe a consistent increase in excretion of endogenous fat in patients with malabsorption, although the number of patients studied was small. Their work may indicate that the convenience of counting marker and fat labeled with gamma ray emitters may outweigh some loss in validity caused by separation of fat and marker or by deiodination or both.

Oil-Phase Marker

A problem inherent in the use of any lipid-soluble marker in studies of triglyceride absorption arises from the change in physical properties of triglyceride during its digestion. Undigested triglyceride has properties similar to those of triether, but its digestion products, fatty acid and monoglyceride, are transferred to the aqueous micellar phase (30) as they are formed and thus separate from the oil phase containing the triether marker. Such phase separation of marker and triglyceride digestion products could cause a difference in the rate of movement through the intestinal tract. Triether thus appears to be an oil-phase marker and complements aqueous-phase markers such as polyethylene glycol or particulate phase markers such as chromium sesquioxide.

It is possible that the effectiveness of triether as a fecal marker in studies of steatorrhea in man would depend on the cause of the defect in fat absorption. Thus, if a mucosal defect were involved, as in sprue, normal hydrolysis and solubilization of the dietary fat would result in the digestion products being transferred to the aqueous phase while the marker remained in the oil phase. In this case, separation of the test lipid and the marker might result. On the other hand, if digestion were defective, as in pancreatic disease, the test lipid and the marker would remain together in the lipid phase. If solubilization were defective, as in biliary disease, the digestion products and the marker would remain together in the lipid phase. In our experiments, cholestyramine feeding probably greatly reduced the concentration of micellar lipid, and, under these conditions, most of the fat, whether tri-

glyceride or lipolytic products, remained in the oil phase. This may be the reason for the validity with which fat absorption could be estimated by the isotope ratio technique in the studies.

Silicone oil has also been suggested as an oil-phase marker for lipid absorption studies (31). This material has many of the properties of an ideal marker, but quantitative measurement of it is difficult. Nevertheless, it proved to be an excellent marker in studies in 22 normal rats, in which absorption of ^{14}C-labeled glycerol tristearate closely paralleled absorption measured by the ratio of marker to test lipid in the feces on the day of feeding. It was not tested in animals with steatorrhea. ^{3}H-labeled triether should theoretically be at least as effective as silicone oil as a marker and has the advantage of ready quantitation.

If quantitation of unlabeled triether is necessary, for example, in very young children, gas–liquid chromatographic analysis of a saponified fecal extract probably would be feasible because the triether chromatographs as a single peak on a 1% OV-17 column (6).

Sitosterol has been suggested as a nonabsorbable marker for studies of cholesterol absorption (32), but the rationale for its use in these studies is that it undergoes degradation and metabolism by bacteria to the same extent as cholesterol. It would not, therefore, be suitable for studies of absorption of other lipids.

One other obvious candidate as an oil-phase marker is ^{3}H-labeled squalene; it could be prepared by reduction of squalene with tritium.

Future Studies

As a marker for the oil phase of gastric and intestinal contents, triether should be more valid than water-soluble markers for estimating the absorption by the stomach and small intestine of substances present predominantly in the oil phase during digestion, such as lipid-soluble vitamins, sterols, and insecticides. Further studies are indicated to define the validity of triether for estimating fat absorption in clinical conditions associated with fat malabsorption in man.

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