A re-examination of the fate of glyceride-glycerol in neutral lipid absorption and transport

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Abstract Conventional ideas concerning the unidirectional movement of triacylglycerol from intestinal lumen to lymph with *sn*-2-monoacylglycerol being the major glyceride-glycerol precursor were challenged by our finding that steady state specific activities of radiolabeled triacylglycerol (glyceryl moiety) in the intestinal mucosa and lumen were greatly reduced as compared to the specific activity of intraduodenally infused triacylglycerol. Investigation of the point at which the radiolabel was diluted was performed in mesenteric lymph duct-cannulated rats with a duodenal cannula through which trioleoyl[^{3}H]glycerol was constantly infused. Both within the bowel lumen and in the intestinal mucosa, monoacylglycerol, diacylglycerol, and triacylglycerol specific activities were 31% or less of the specific activity of the infusate; chylomicron triacylglycerol specific activity was 75%. Efflux of neutral lipid from the mucosa into the bowel lumen was directly demonstrated by finding that when ^{3}H glucose was injected intraperitoneally during triolein infusion, luminal triacylglycerol had a higher specific activity than was present in the mucosa. We conclude that there are two pools of mucosal triacylglycerol. One is rapidly transported and derives most of its glyceride-glycerol from luminal monoacylglycerol. The second is slowly transported; it derives its glyceride-glycerol mainly from endogenous sources and may efflux back into the bowel lumen.—**Mansbach, C. M., II, and S. Parthasarathy.** A re-examination of the fate of glyceride-glycerol in neutral lipid absorption and transport. *J. Lipid Res.* 1982. **23:** 1009–1019.

Supplementary key words intestinal lipid absorption • lymphatic transport

Intestinal lipid absorption is thought to be a unidirectional process. As a first step, the products of triacylglycerol (TG) hydrolysis are absorbed from the lumen of the intestinal tract by a mechanism not requiring energy (1). Secondly, within the mucosal cells, these hydrolytic products undergo re-esterification to TG (2), which is postulated to utilize predominantly *sn*-2-monoacylglycerol (MG) as the glyceride-glycerol precursor (3). Finally, the reformed TG is transported from the intestine in chylomicrons (2). The total process is efficient in that 90% of lipid loads of up to 500 g per day in humans are absorbed (4).

We attempted to document the efficiency with which the intestinal mucosa takes up infused neutral lipid and re-esterifies the absorbed MG and fatty acid (FFA) to TG. We sought to do this in rats by observing the rapidity with which the specific activity of the intestinal mucosal neutral lipids approached that of the infused probe lipid, trioleoyl[^{14}C]glycerol. TG was constantly infused into the duodenum to the isotopic steady state in the intestinal mucosa. However, the specific activity attained in the mucosa was only 20% of the specific activity of the infused TG, not the 85% as expected from previous data (3). The studies to be described were designed to answer relevant questions raised by this experiment, i.e., what is the importance of MG as a TG precursor and at what step during the absorption of lipid did the dilution of the glycerol radiolabel occur?

MATERIAL AND METHODS

Preliminary experiment to determine adequacy of mucosal cleansing

It was important for the planned experiments that we demonstrate adequate cleansing of the mucosa of all nonabsorbed lipids. This presented a problem since it is well known that FFA and MG are absorbed by the mucosa even in the cold (1). Therefore, TG was used as the probe lipid since there is no evidence that TG is absorbed intact in the cold. Using 200-g nonfasting rats, the upper intestine was excised and a 30-cm section of proximal jejunum was removed. PE 60 cannulae were tied into the proximal and distal ends. The intestine was placed in ice and infused with a sonified TG emulsion containing trioleoyl[^{3}H]glycerol (15,000 dpm/μmol Amersham Corp., Arlington Heights, IL); triolein, 30 mM; taurocholate, 20 mM; gum acacia, 10 g/dl; Tris

Abbreviations: TG, triacylglycerol; MG, monoacylglycerol; DG, diacylglycerol; TLC, thin-layer chromatography; FFA, free fatty acid; tri[^{14}C]oleoylglycerol, triolein labeled in the fatty acid moiety; trioleoyl[^{3}H]glycerol, triolein labeled in the glycerol moiety.

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buffer 0.01 M, pH 7.4; and 0.15 M NaCl. The intestine was removed from the ice bath and rinsed with 0.15 M NaCl. Six-cm sections were cut and the sections were rinsed with one of the following: 0.15 M NaCl, 2% albumin, 20 mM taurocholate, or 2% Triton X 100, using a squirt bottle with a fine stream. It was consistently found that the Triton X 100 wash removed the greatest number of counts from the mucosa (5) compared to unwashed samples, with 97 ± 0.5% dpm being removed. After Triton washing, histologic observation showed minimal sloughing of some cells at the very tip of villi.

Subsequent experiments

Animal preparation. Male Sprague Dawley rats (250–400 g) were given a duodenal cannula (PE 50, Clay Adams, Parsippany, NJ) the afternoon prior to an experiment. They were placed in restraining cages and infused overnight with 0.15 M NaCl at a rate of 2.4 ml $hr.^{-1}$. Where indicated, in addition to the duodenal cannula, the main mesenteric lymph duct was cannulated using a PE 50 cannula (6, 7). The next day the rats were infused for 3 hr (4 hr for lymph fistula rats) with a sonified emulsion containing 20–30 mM triolein (Sigma Chemical Co., St. Louis, MO), 20 mM taurocholate (Calbiochem, San Diego, CA), and 10 mM Tris-HCl (pH 7.0) in 0.15 M NaCl at a rate of 4.5 ml hr^{-1}. Where indicated in the text, trioleoyl[^{3}H]glycerol or trioleoyl[^{14}C]glycerol was added to give a specific activity of 90–127 × 10^3 cpm μmol^{-1} triolein. The cpm/ml were the same at the beginning and end of the infusion. In other experiments, tri[^{14}C]oleoylglycerol (Amersham Corp.) was infused intraduodenally (sp act 6,400 cpm μmol^{-1}) in addition to the trioleoyl[^{3}H]glycerol. Where specified, [2-^{3}H]glucose (New England Nuclear, Boston, MA) instead of the radiolabeled triolein was injected intraperitoneally 1 hr prior to killing. Some animals were infused with only 0.15 M NaCl. Finally, to determine the location of radioactivity not present in lymph, intestinal lumen, or mucosa, lymph-fistulated rats were infused for 4 hr with trioleoyl[^{3}H]glycerol as described. Samples of lymph, intestinal fluid, mucosa, intestinal wall, colon, stomach, gastric contents, liver, and other organs were obtained. The remaining parts of the rat were homogenized with 2 l of 0.15 M NaCl in a Waring blender, model CB-5, and multiple samples were obtained.

Tissue preparation. At the conclusion of the experiment, the animals were given an overdose of pentobarbitol. While asleep, but still alive, the animals were removed from their cage and the intestine was removed between clamps placed at the pylorus and at the ileocecal junction. The intestinal contents were collected in a beaker on ice with the aid of a 15-ml flush of 0.15 M NaCl. The intestine was placed on an iced glass plate and opened longitudinally. The mucosa was carefully cleaned using 2% Triton X 100 delivered as a fine stream from a squirt bottle followed by similar treatment with 0.15 M NaCl. Finally, the tissue was blotted with tissue paper. The mucosa was then scraped down to the level of the crypts (8) and collected in a beaker containing 19 ml of 0.15 M NaCl. (Cryptal cells were excluded since they are unlikely participants in lipid absorption (9) and would therefore artificially lower mucosal specific activity.) An additional 5 ml of 0.15 M NaCl was added and the tissue was homogenized in a glass-Teflon homogenizer. One ml was extracted by the method of Folch, Lees, and Sloane Stanley (10). The luminal fluid was also homogenized using a glass-Teflon homogenizer and 5 ml was extracted (10) in a 250-ml separatory funnel, with excess methanol used to break the phases. The organic extracts were evaporated under N_2 and taken up in 0.5 or 1.0 ml of chloroform–methanol 2:1 (v:v). In certain experiments 0.5 ml of the mucosal homogenate was made 5% with respect to trichloroacetic acid. A protein-free supernatant was obtained by centrifugation. Triton washing of the mucosa did not cause loss of mucosal lipids as shown by longitudinally dividing in half the intestine of a rat that had been infused with TG for 3 hr and had been given 250 μCi of [2-^{3}H]glucose (New England Nuclear, Boston, MA) intraperitoneally 1 hr prior to being killed. One half of the intestine was washed with 0.15 M NaCl. The other half was washed in the routine manner with Triton X 100. The mucosa from each half was scraped, extracted, and the lipids were separated by TLC. The half washed with Triton had 102, 101, and 94% of the radioactivity of the saline-washed half in MG, DG, and TG fractions, respectively (based on cpm per g wet weight of mucosa).

In order to determine if the neutral lipid specific activity was the same in all subcellular compartments, homogenates of intestinal mucosa infused with trioleoyl[^{3}H]glycerol were obtained (11). A sample of the total homogenate was obtained for lipid extraction and the remainder was subjected to differential centrifugation. After the homogenate had been centrifuged for 350,000 g · min in a Sorvall RC-2B (duPont Instruments, Newtown, CT), the lipid layer, which floated to the top of the centrifuge tube, was harvested. The remaining supernatant was separated into smooth and rough microsomal fractions (11) using a magnesium-containing discontinuous sucrose gradient. The pellet from the original low speed centrifugation was also collected. Lipid was extracted from all fractions (10).

Chemical methods

Neutral lipids were separated on silica gel G layers using the solvent system: hexane–diethyl ether–acetic

acid–methanol 80:20:2:6 (v:v). The lipids were visualized by using I_2 vapors and the identification of the lipid bands was aided by the co-chromatography of authentic standards. The bands were scraped from the plate; DG and TG were eluted with three portions of chloroform (5 ml) and MG was extracted at 40°C using chloroform–methanol 4:1 (v:v) (12). The ester bonds were quantitated by the method of Snyder and Stevens (13). Recovery of TG and DG from the silica was 100%, and MG was 95% as compared to standards assayed directly. Radioactivity in lipids separated in a similar manner was determined using a Packard Tricarb Model 3390 liquid scintillation spectrometer (AMBAC Industries, Downers Grove, IL) as previously described (8). Quenching of samples not put on TLC plates was determined by addition of an internal standard and, therefore, results are reported in dpm.

To determine the location of ^3H when [^3H]glucose was utilized, MG isolated from the intestinal mucosa as described was placed in a sealed test tube with 2 ml of 2 N HCl. Hydrolysis of the ester bonds was accomplished by heating the tube at 100–105°C for 96 hr. The contents of the tube were then extracted (10). The upper methanol–water phase was dried in a scintillation vial and its radioactivity was determined. The organic phase was evaporated to dryness and was taken up in 0.5 ml of chloroform–methanol 2:1 (v:v). The fatty acids were isolated by TLC. Ninety-nine percent of the radioactivity was present in the aqueous-methanolic phase indicating that the radioactivity from glucose was specifically distributed in the glycerol moiety of neutral lipids.

To determine if the ^3H from trioleoyl[^3H]glycerol infused into the lumen exchanged with hydrogen from H_2O, a 0.5-ml aliquot of intestinal fluid was placed on one side of a Rittenberg tube (14). The other side of the tube was placed in alcohol/dry ice and the tube was placed under vacuum. When the water had completely transferred to the cooled side of the tube, the vacuum was released and the radioactivity in the lyophilizand (water) was determined.

As an additional test of the lability of the ^3H, 100 μmol of triolein including 2.8×10^6 cpm of trioleoyl [^3H]glycerol was digested with 2 ml of rat intestinal juice in 7.5 ml of Tris–HCl (2 mM, pH 8.0) and 10 mM taurocholate. The reaction was carried out at 40°C and maintained at pH 8.0 utilizing a pH stat (Radiometer, Copenhagen, Denmark) with 0.1 N NaOH as titrant. The reaction was allowed to continue for 4 hr, at which point 108 μmol of NaOH was added. The reaction mixture was extracted (10) and samples were obtained from the aqueous-methanolic phase and the organic phase for determination of radioactivity. To determine if, during trioleoyl[^3H]glycerol perfusion, radioactive water-soluble metabolites were generated in the intestinal lumen either through bacterial metabolism or due to complete hydrolysis of the triolein, a rat was infused for 3 hr with the trioleoyl[^3H]glycerol emulsion as described. The rat was killed and the intestinal contents were collected. Five ml of the intestinal fluid was partitioned into organic and aqueous phases (10). A 0.5-ml sample of each phase was obtained, evaporated to dryness in a convection oven, and its radioactivity was determined.

The radiopurity of the trioleoyl[^3H]glycerol was established by finding that of $50.6 \pm 3.8 \times 10^6$ cpm infused, $48.2 \pm 0.35 \times 10^6$ cpm was in TG, $0.17 \pm 0.08 \times 10^6$ cpm was in MG, and $0.97 \pm 0.35 \times 10^6$ was in DG. Of the remaining radioactivity, 0.95×10^6 cpm was in unidentified lipid fractions, and 0.35×10^6 cpm was found in the aqueous phase after lipid extraction. (10).

DNA was measured by the method of Burton (15), RNA by the method of Cohn (16), and protein by the procedure of Lowry et al. (17). To determine if there was a leak of cytosolic proteins out into the intestinal lumen, mucosal postmicrosomal supernatant (8) was electrophoresed on a 10% cross-linked polyacrylamide gel. The protein bands, stained with Coomassie Blue, were compared with the bands found on electrophoresis of luminal fluid. Polyacrylamide gel electrophoresis and apoA-I measurements were performed through the courtesy of Dr. Steven Quarfordt, Duke University Medical Center, Durham, NC.

Substrate preparation

Triolein (Sigma Chemical Co., St. Louis, MO) was of practical grade and was washed repeatedly with ethanol. It was found to be 96% pure after ethanol washing.

Trioleoyl[^{14}C]glycerol was prepared by synthesizing oleoyl chloride from sodium oleate and oxalyl chloride. Excess oxalyl chloride was removed in vacuo with gentle heating. The oleoyl chloride was then dissolved in hexane, washed three times with ice, and dried with Na_2SO_4. Dried [U^{14}C]glycerol (New England Nuclear) was dissolved in redistilled pyridine and a 10-fold molar excess of oleoyl chloride was added. p-Toluene sulfonic acid was added and the mixture was refluxed for 48 hr. The resultant lipids were taken up in chloroform, washed with water, and the chloroform was evaporated in vacuo under N_2. The lipids were loaded on a silicic acid column after being dissolved in a small amount of petroleum ether. The elution of TG from the column was accomplished by using petroleum ether as the eluant. DG was eluted by adding 8% ethanol to the petroleum ether. Recovered DG could be recycled through the acylation step so that the final yield (two

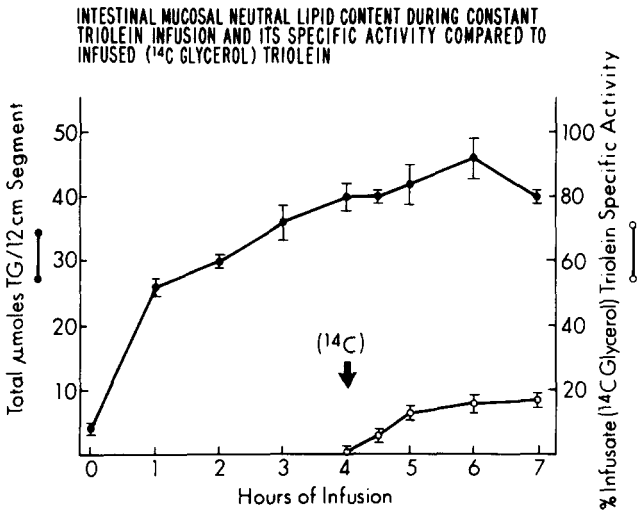

Fig. 1. Intestinal mucosal TG content and its percentage of infusate specific activity in the intestinal segment 24–36 cm from the pylorus. At 0 time a constant infusion of triolein (135 μmol hr^{-1}) was started through an intraduodenal cannula for the number of hours indicated on the abscissa. After 4 hr of infusion, trioleoyl[^{14}C]glycerol was added to the triolein infusion whose mass remained constant. Five rats were killed at each time point. TG mass and radioactivity were determined. The data represent the means ± 1 SEM.

cycles) was 80% conversion of [^{14}C]glycerol to TG. The final TG migrated as a single spot on TLC and had a radiopurity of 97%.

RESULTS

When no lipid was infused, and the MG, DG, and TG contents in the proximal three-fourths of the gut lumen were 0.54 ± 0.16, 0.14 ± 0.02, and 0.88 ± 0.47 μmol, respectively. The mucosal MG, DG, and TG contents in the proximal three-fourths of the intestine were 5.28 ± 2.8, 4.02 ± 0.54, and 23.8 ± 3.4 μmol, respectively.

Experiment utilizing trioleoyl[^{14}C]glycerol as the probe lipid

On infusion of TG, the TG content in the mucosa began to rise within the first hour (**Fig. 1**) as expected and reached an approximate steady state after 4 hr. The thesis that a steady state was reached at 4 hr is supported by statistical evidence. The mucosal TG contents at 4, 4.5, 5, 6, and 7 hr of infusion were not statistically different ($P > 0.10$ for 5 hr vs. 6 hr, the pair most closely approaching a significant difference; each point was tested against 6 hr). Although the data presented are from the 12-cm segment beginning 24 cm from the pylorus, each 12-cm segment from the proximal 48 cm of intestine also reached a steady state after 4 hr of infusion (i.e., approximately the proximal one-half of the intestine). The data are shown for the third segment because of its high steady state TG content compared to the two more proximal 12-cm segments (16.6 ± 0.92 and 30 ± 2.5 μmol for the first and second segments, respectively). The mucosal MG and DG content did not rise significantly on TG infusion (data not shown).

Upon reaching the mass steady state as regards mucosal TG content at 4 hr of infusion, trioleoyl-[^{14}C]glycerol was included in the infusion whose triolein concentration was kept constant. The relative specific activity of the mucosal TG rose, as would be expected, as the ^{14}C-labeled TG infusion was continued and reached an apparent steady state 2 hr later. However, the TG specific activity reached at the steady state was only 18% of the infusate specific activity (Fig. 1), indicating either a considerable dilution of the radiolabel by endogenous glyceride-glycerol or a major dilution of the radiolabel in a large, slowly turning over pool of TG. The other three 12-cm segments tested had similar low TG specific activities at the radioactive steady state (4 hr non-radioactive triolein infusion followed by 3 hr ^{14}C-labeled triolein infusion) as compared to the TG specific activity of the infusate (8.8 ± 0.5, 11 ± 1, and 17 ± 1.5% for the first, second, and fourth segments, respectively).

Experiments utilizing trioleoyl[^{3}H]glycerol as the probe lipid

In order to avoid the possibility of a residual large cold TG pool as a potential dilutional factor, further experiments were performed in which the radiolabeled triolein was administered at the outset. Trioleoyl-[^{3}H]glycerol was utilized after it was shown that the ^{3}H label did not significantly exchange with an aqueous hydrogen. This was established by three findings. *1*) Only 5% of the radioactivity in the intestinal lumen appeared in the lyophilizand in a Rittenberg tube. *2*) After 4 hr digestion with rat lipase in which 54% of the ester groups available for attack had been hydrolyzed, 96.5% of the total cpm remained in the organic phase after partitioning with H_2O (10). *3*) After 3 hr of trioleoyl[^{3}H]glycerol infusion, 94% of the cpm in the intestinal fluid partitioned into the organic phase (10). This would also indicate minimal biotransformation of the infused trioleoyl[^{3}H]glycerol to water-soluble products.

Experiments to determine if dilution of the infused radiolabel occurred in the intestinal lumen, mucosa, or mesenteric lymph

In order to know more accurately at which point radiolabel dilution occurred, experiments were performed in lymph-cannulated rats in which the specific activity of the neutral lipids of the luminal contents,

mucosa, and chylomicrons could be known. **Fig. 2** shows the intestinal lumen and intestinal mucosal relative specific activities of acylglycerols. It is evident that the mucosal content of MG and DG is not greatly different than that observed under fasting conditions. However, the mucosal TG content increased over 6-fold. In the lumen the MG content was found to be the major neutral lipid species present and was 20-fold greater than fasting values. TG and DG content were more modestly increased. This attests to the rapidity and adequacy of lipolysis in these rats. Of particular interest are the relative specific activity data for each of the neutral lipids in both lumen and mucosa. As shown in the figure, none of these was greater than 31% of the infusate specific activity. This was true even in the intestinal lumen, the site of radiolabel infusion. These data would indicate that at the steady state for TG, for example, 108 μmol of glyceride-glycerol was generated by the intestinal mucosa (150 μmol $\times$ (1–0.28)) and 2.7 μmol of TG was effluxed from the mucosa into the intestinal lumen vide infra (3.7 μmol $\times$ (1–0.27)). These calculations with reference to mucosal efflux assume that the TG effluxed is of low specific activity as compared to that present in the whole mucosa (vide infra).

To rule out the possibility that mucosal sloughing contributed to the decreased luminal specific activity, the DNA contents of the mucosa and lumen were determined. The mucosal DNA content was found to be 26.2 ± 2.1 mg and the luminal DNA content was 0.041 ± 0.056 mg. Since the total neutral lipid present in the intestinal mucosa was 158 μmol, it can be calculated on the basis of the DNA data that only 0.25 μmol of neutral lipid in the bowel lumen was due to sloughed mucosal cells as compared to the 14.7 μmol actually present. The possiblity of a generalized leak of protein from the mucosa due to the infusate was ruled out by the inability to detect apoA-I in the intestinal fluid (<5 μ/ml intestinal fluid) and by the absence of identifiable cytosolic proteins in the luminal fluid. To assure that the small molecular weight proteins seen in the luminal fluid were not the result of degraded cytosolic proteins, postmicrosomal supernatant was incubated with luminal fluid for up to 2.5 hr at 37°C. No small molecular weight bands were produced.

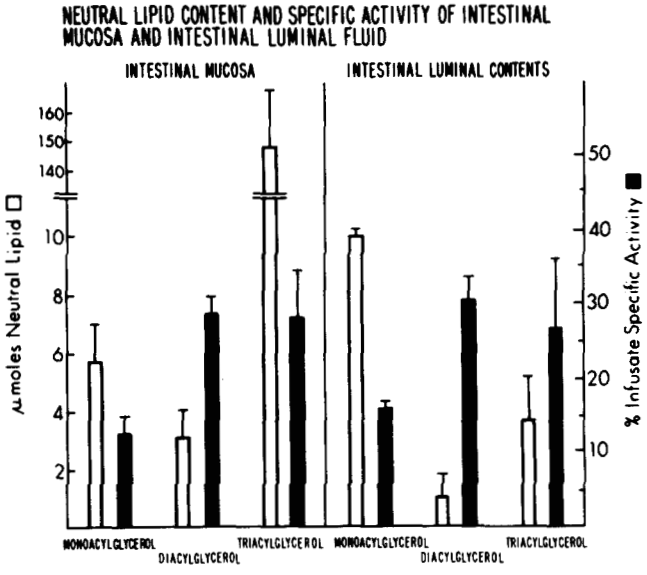

Fig. 2. Neutral lipid content and its percentage of infusate specific activity in the intestinal mucosa and lumen of the proximal three-fourths of the intestine. Four rats with intraduodenal and mesenteric lymph cannulae were constantly infused with TG (90.5 μmol hr^{-1}) containing trioleoyl[^{3}H]glycerol for 4 hr. The intestinal luminal contents were collected and the mucosa was scraped. Both were analyzed for mass and radioactivity of MG, DG, and TG. The infusate TG specific activity was 128,000 ± 4,400 cpm μmol^{-1}. The data are the means ± 1 SEM.

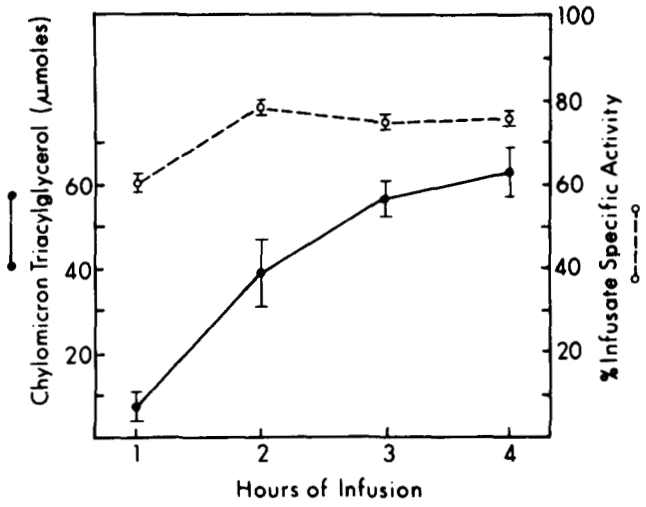

Fig. 3. Chylomicron TG content and relative specific activity from the rats presented in Fig. 2. Lymph was separately collected for each hour of the 4-hr triolein infusion and the chylomicrons were isolated. The TG mass is shown by the solid circles. The open circles show the percent of infusate specific activity (128,000 ± 4,400 cpm μmol^{-1}) found in the chylomicron TG. The data and the means ± 1 SEM.

The TG content and specific activity of chylomicrons from the rats whose data were shown in Fig. 2 are presented in **Fig. 3.** The TG output in lymph chylomicrons increased each hour to approach a steady state at 4 hr of infusion corresponding to the steady state attained in the mucosa at this time. In contrast to the large increment in TG output into the lymph on TG infusion, the TG relative specific activity remained nearly constant throughout the 4 hr of infusion. The relatively low value at the first hour of infusion is most likely due to the dilution of the radiolabeled TG by the 23 μmol of TG already present in the mucosa in the fasting state. In sum, these data would suggest that the glyceride-glycerol in the chylomicrons was derived from a constant proportion of the absorbed radiolabeled glyceride-

TABLE 1. Specific activity of intestinal and luminal fluid and chylomicrons as the percentage of infusate specific activity

	MG	DG	TG
Lumen[a]	16 ± 1.0%	31 ± 3.2%	27 ± 9.6%
Mucosal[b]	12 ± 2.4%	29 ± 2.2%	28 ± 6.4%
Chylomicrons[c]			73 ± 8%

[a] Data from Fig. 2, mean ± 1 SEM.
[b] Data from Fig. 2, mean ± 1 SEM.
[c] Data from Fig. 3, mean ± 1 SD of each hour's mean percentage of infusate specific activity.

glycerol pool in the mucosa. Of particular interest is that fact that the specific activity of the chylomicron TG was much closer to the specific activity of the infusate (avg. for all 4 hr was 73% of infusate specific activity) than was its presumed precursor, mucosal TG (27% of the specific activity of the infusate). The specific activity of the neutral lipids in the intestinal lumen, mucosa, and chylomicrons relative to that of the infusate is summarized in **Table I.** In two experiments, the TG specific activity was determined in the lymph remaining after the chylomicrons had been removed (3rd and 4th hr of infusion). The specific activity of the TG associated with the heavier lipoproteins was much reduced by comparison with the chylomicron TG (avg. for 3rd and 4th hr was 1% of infusate TG specific activity) indicating an origin from a TG pool that is different than the TG pool which provides chylomicron TG.

Recoveries of infused TG mass and radioactivity

During the 4 hr of infusion, 166 μmol of TG was transported in the lymph as chylomicrons and 149 μmol of TG was present in the mucosa. When combined with the other neutral lipids present in the gut lumen and in the mucosa (23.5 μmol), a total of 338.5 μmol of neutral lipid can be accounted for. This should be compared to the 362 μmol of TG that was infused.

Infused into the rats was $4.8 \pm 0.24 \times 10^7$ cpm. Present in chylomicrons, was $1.7 \pm 0.19 \times 10^7$ cpm. Minor amounts remained in the gut lumen ($3.9 \pm 1.1 \times 10^5$ cpm). Amounts of $4.6 \pm 0.87 \times 10^6$ cpm were in the various neutral lipid fractions in the mucosa and $2.6 \pm 0.47 \times 10^6$ cpm was present in the supernatant after the addition of trichloroacetic acid to the mucosal homogenate. Very minor amounts of radioactivity were present in the phosphatidylcholine fraction in the mucosa ($3.0 \pm 0.42 \times 10^4$ cpm) and even smaller amounts were in the other phospholipid fractions ($1.7 \pm 0.19 \times 10^4$ cpm). Thus, only 2.45×10^7 cpm was recovered in the intestine and lymph.

Because not all the infused radioactivity could be accounted for in the lymph and intestinal mucosa, it was assumed that the missing radioactivity was distributed into other parts of the rat either by being transported via the plasma (via the portal vein) or via lymph channels that did not feed into the main mesenteric lymph duct. Therefore two rats were specifically investigated to determine the location of all retrievable infused radioactivity. After 5.5×10^7 dpm was infused in the two rats so studied, 3.0×10^7 dpm was found in the bowel lumen, intestinal mucosa, and lymph. An additional 0.2×10^7 dpm was in the remaining intestinal wall after the mucosa was removed and in the mesentery; and 2.0×10^7 dpm was found in other parts of the rat, 1.1×10^7 dpm in the aqueous phase (10), and 0.9×10^7 dpm in the chloroform phase (10). Less than 0.06×10^7 dpm was found in the gastric lumen and colon. Thus, 5.2×10^7 dpm was recovered of the 5.5×10^7 dpm infused. This suggests that the reduced specific activity of the neutral lipids of the intestinal mucosa and lumen, as compared to infusate specific activity, was not produced artifactually.

Experiment to show the segment of bowel where dilution of radiolabel occurred

Because of the reduced specific activity of the neutral lipids in the bowel lumen compared to that of the infused triolein, it was of interest to see where the reduction occurred down the length of the bowel. For this experiment, trioleoyl[^{3}H]glycerol was infused for 3 hr. At the termination of the experiment, the proximal

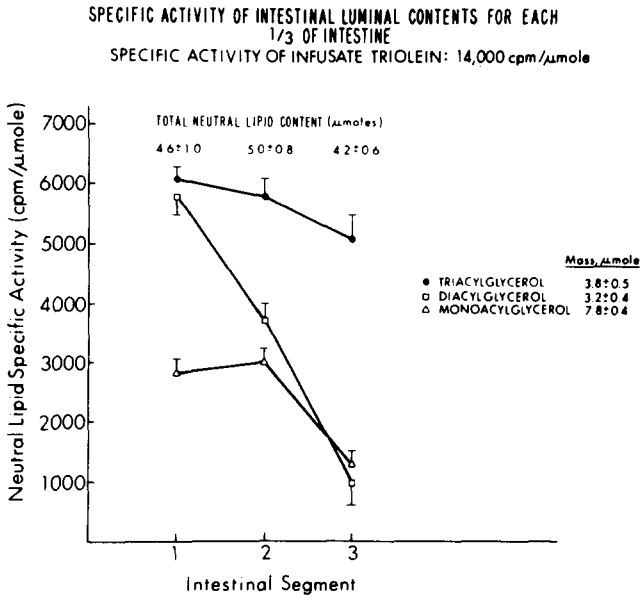

Fig. 4. The neutral lipid specific activity of the intestinal contents of each third of the proximal three-fourths of the intestine. Intestinal segments 1 to 3 are numbered beginning from the pylorus. The total neutral lipid content (MG + DG + TG) after a 3-hr intraduodenal triolein infusion (135 μmol hr^{-1}) is given for each segment at the top of the figure. The neutral lipid composition of all three segments combined is given on the right hand side of the figure. The data are the means ± 1 SEM of four rats.

three-fourths of the intestine was divided into thirds and the luminal contents were separately collected. As shown in **Fig. 4,** the quantity of neutral lipid was evenly distributed down the length of the bowel with MG being the predominant neutral lipid species present. TG specific activity was found to be significantly reduced compared to infusate specific activity even in the proximal one-third intestinal segment and decreased only modestly in the more distal bowel. DG specific activity progressively decreased down the length of the bowel. MG specific activity, which was lower than the TG and DG specific activity in the most proximal segment, did not demonstrate further reduction until the most distal segment was reached.

Experiments showing conservation of infused radiolabeled TG acyl groups and the effect of varying the infused TG load

To determine if the oleoyl moiety of trioleoylglycerol was better conserved as regards its reutilization for mucosal neutral lipid synthesis than was the glyceryl moiety, tri[^{14}C]oleoyl[^{3}H]glycerol was infused intraduodenally for 3 hr. At the conclusion of the experiment, luminal fluid and mucosal scrapings were analyzed for neutral lipid specific activity with respect to both ^{14}C and ^{3}H. The results (**Fig. 5**) show that both in the bowel lumen and in the mucosa, oleate was more avidly retained in neutral lipids than glycerol. In agreement with the observations of Karmen, Whyte, and Goodman (18), the ^{14}C specific activity in the mucosal neutral lipids was never greater than 75% of the infusate specific activity, indicating significant dilution of the oleate by endogenous acyl groups.

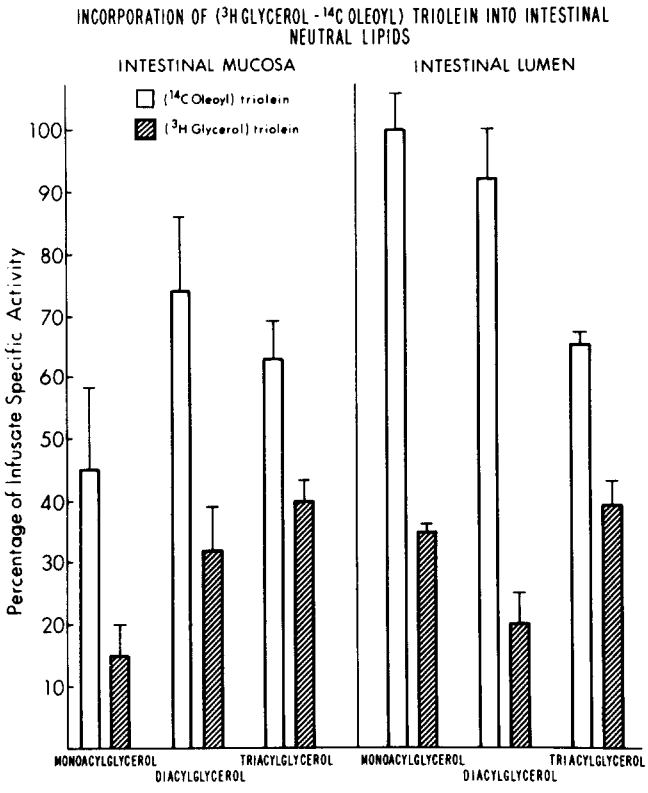

Fig. 5. Neutral lipid specific activity in the intestinal lumen and mucosa of rats infused intraduodenally with triolein (135 μmol hr^{-1}) containing (tri[^{14}C]oleoyl[^{3}H]glycerol. The data are the means ± 1 SEM of four rats.

TABLE 2. Specific activity of intestinal mucosa and luminal fluid as the percentage of infusate specific activity

TG Infusion Rate	Mucosa			Luminal Fluid		
	MG	DG	TG	MG	DG	TG
11.4 μmol hr^{-1}	2.7[a]	4.2	14	7.0	15	51
49 μmol hr^{-1}	7	49	59	25	43	33
90.5 μmol hr^{-1}	12[b]	29	28	16	31	27

[a] The data are the average of two experiments and are calculated as follows: specific activity mucosa or fluid/specific activity infusate × 100. Only the proximal half of the intestine was utilized since grossly this was the portion involved in active absorption.

[b] Data from Fig. 2 from the proximal three-fourths of the intestine.

Because of the relatively large TG loads infused (90.5 μmol hr^{-1}), observations were made at two other TG infusion rates as shown in **Table 2.** At the lowest infusion rate studied, severe depression of the specific activity of all neutral lipids in both mucosa and lumen was noted, except for luminal TG. The severity of the depression may, in part, be accounted for by the dilution of the radiolabel into pre-existing cold neutral lipid pools under conditions of a low TG load. When 49 μmol hr^{-1} TG was infused, significant reductions from infusate specific activity were still observed, although not as severe in mucosal DG and TG as when 90.5 μmol hr^{-1} was infused. Presumably this related to the reduced quantity of TG presented for export from the enterocytes, resulting in a greater percentage of mucosal TG in the quickly exported pathway than when the TG infusion rate was 90.5 μmol hr^{-1}.

Since the taurocholate concentration used (20 mM) was well above its critical micellar concentration, it is possible that this comparatively high bile acid concentration affected the enterocyte's luminal cellular membrane in such a way as to account for the results. To investigate this possibility, two rats were infused for 4 hr at a rate of 66 μmol of trioleoyl[^{3}H]glycerol (in 6 mM taurocholate) per hr. Neutral lipid specific activity in the lumen averaged 44% of the specific activity of the infusate, suggesting that the taurocholate concentrations routinely used did not produce the results obtained.

Direct demonstration of the mucosal to lumen efflux of neutral lipids

Since the data indicated that there was a large contribution of endogenous glyceride-glycerol to neutral

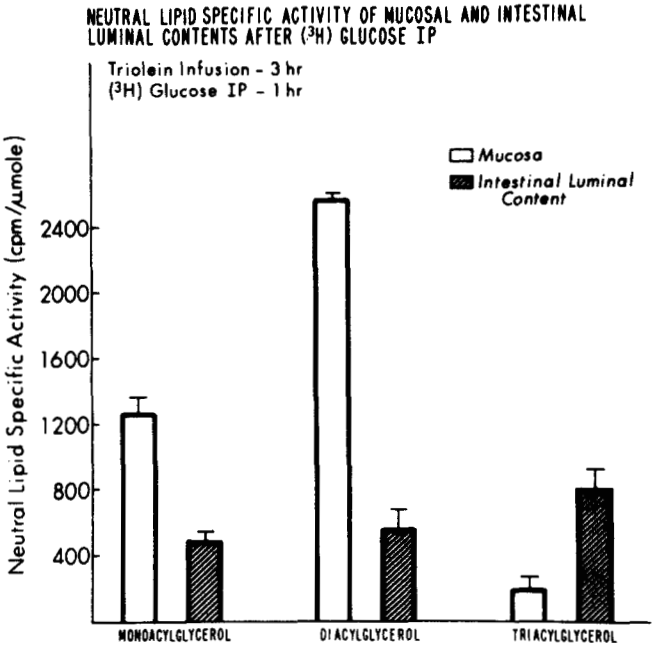

Fig. 6. Neutral lipid specific activity in rats infused for 3 hr intraduodenaly with triolein, (135 μmol hr^{-1}). One hour before killing, [2-^{3}H]glucose was given IP. The mucosa was scraped and the intestinal contents were collected from the proximal three-fourths of the intestine. The specific activities of the various neutral lipids were determined. The data are the means ± 1 SEM from four rats. Mucosal specific activity: MG < DG $P < 0.04$. MG and DG > TG $P < 0.003$. Luminal specific activity: TG > MG and DG $P < 0.05$. Mucosal specific activity compared to luminal specific activity MG $P < 0.01$, DG $P < 0.02$, TG $P < 0.001$ (Student's t test).

lipid synthesis, a major source was presumed to be *sn*-glycerol-3-phosphate. Entry into the pathway which leads to glycerol-3-phosphate production may be obtained by administering glucose. Accordingly, [2^{3}H]glucose was injected intraperitoneally 1 hr before the conclusion of a 3-hr intraduodenal triolein infusion to document the contribution of endogenous glycerol-3-phosphate to glyceride-glycerol. The results of this experiment are shown in **Fig. 6.** As can be seen in the figure, the specific activities for both MG and DG are considerably higher in the mucosal lipids than in the luminal lipids, while the reverse is true for TG. Furthermore, it is evident that the precursors of TG, MG, and DG, have specific activities in the intestinal mucosa that are much greater than TG. This would be expected since MG and DG should turn over more rapidly than TG as indicated by their small mass compared to TG. Thus during the hour after [^{3}H]glucose injection, a greater proportion of MG and DG should be labeled than TG, not only because of the precursor-product relationship between them but also because of the large non-radiolabeled TG pool which would dilute newly synthesized TG. Of particular interest is the finding that luminal TG had a greater specific activity than mucosal TG (Fig. 6). It should be noted that there is no way in which [^{3}H]glucose could be incorporated into TG in the bowel lumen (99% of all cpm were found in glyceride glycerol rather than the fatty acid group).

Since luminal MG and DG, potential precursors of luminal TG, have lower specific activities than TG, and luminal TG had a 4-fold greater specific activity than mucosal TG (Fig. 6), it is proposed that luminal TG is derived from only a portion of the mucosal TG pool. If luminal TG were derived from a homogenous portion of the mucosal TG pool, its specific activity should either be equal to or less than that of the entire mucosal pool assuming dilution in the lumen by non-radioactive infusate TG.

Variation of TG specific activity in mucosal subcellular compartments

Because of the results of the [^{3}H]glucose experiment and because the discrepancy in the specific activities between mucosal TG and chylomicron TG suggested that there was more than one compartment of TG in intestinal mucosa, the following experiment was undertaken to establish this compartmentation of TG directly. As shown in **Table 3,** only about 50% of the total TG present in the mucosa was recovered from the various compartments. This was due to incomplete recovery of the prechylomicron fraction. Nevertheless, representative samples were obtained. The amount of TG in the low speed pellet was greater than expected and might represent TG trapped nonspecifically by sedimenting particles or TG in the Golgi apparatus. However, as noted in the table, TG in this compartment had the highest specific activity of all compartments tested and therefore it is unlikely that this represents nonspecifically trapped TG.

The results of the specific activity data clearly indicate that there is compartmentation of TG in intestinal mucosa. The lowest specific activity was found in the rough microsomal fraction, which is less involved than the smooth microsomal fraction in TG transport within the cell as judged by electronmicrographs (19). The low spin pellet and the smooth microsomes had the highest specific activity. In no instance was a compartment identified that had a TG specific activity that would be expected for true chylomicrons i.e., 70–75% of infusate specific activity. As shown in the table, the prechylomicron fraction of easily floatable lipid has a specific activity no different from that found for the total mucosa.

DISCUSSION

The data presented in this report raise questions concerning current thinking about neutral lipid metabolism in the intestine. It has been assumed that MG provides the major source of glyceride-glycerol in the intestine.

This assumption is based on a variety of studies in which a doubly labeled MG (glycerol and fatty acid) was utilized and its conversion to TG was followed (3, 20–22). The results of these studies have been buttressed by biochemical evidence that would lead to a similar conclusion (8, 23, 24).

In analyzing the previous data, it is evident that the dominance of the MG pathway rests in the calculation of μmol of TG synthesized based on the specific activity of the substrate fed rather than the quantitation of the total TG present in the mucosa. However, in the studies of Skipski, Morehouse, and Deuel (20) utilizing infused 1,3 dioleyl-2-deuteriostearyl [^{14}C]glycerol TG, the amount of TG synthesized from labeled stearate and glycerol was calculated to be, respectively, 18.5 and 9.4 mg of TG as compared to the 215 mg of TG actually found in the mucosa. By contrast to these studies in intestinal mucosa, Mattson and Volpenhein (22) showed that the specific activity of lymph TG closely approximated that of fed TG when the glycerol and *sn*-2-acyl groups were radiolabeled but not when the acyl labels were placed at the 1 or 3 position of glycerol (22). The present observations support these findings.

Since chylomicron TG is derived from mucosal TG, it is obvious that the expected precursor product relationship with regard to specific activity is not obeyed in the present study. That is, the specific activity of the product (chylomicron TG) is greater than that of its precursor (mucosal TG) during constant infusion of the radiolabel.

The most reasonable resolution of this paradox would be to consider that there are at least two pools of TG in the mucosa. One pool is quickly transported from the intestine as chylomicron TG. Entrance into this pool is predominantly from MG-derived TG, either because of the rapidity of TG synthesis via this pathway or because this pathway delivers TG preferentially to the intestinal TG transport mechanism. The second pool is slowly transported from the mucosa. It is this pool that loses its exogenous glyceride-glycerol either due to hydrolysis of 2-MG in the intestinal lumen after its isomerization to 1-MG (25) and subsequent lipolysis, or by the action of mucosal MG lipase. The free glycerol released under these circumstances is likely to be lost to the neutral lipid pool since glycerol kinase is not very active in the intestinal mucosa (26) and a ready source of glycerol-3-phosphate is available from glucose or amino acid metabolism. The possibility of two distinct TG pools is enhanced by the demonstration of differing specific activities of TG in various subcellular compartments. Additionally, precedent for separate neutral lipid pools has been set by the demonstration of the metabolic inequality of microsomal-bound DG in the intestine (8, 24). DG derived from MG is solely a TG precursor, whereas glycerol-3-phosphate-generated DG may be either a TG or phosphatidylcholine precursor. This is not due entirely to formation of the 2,3 DG isomer since the 1,2 isomer is preferred (27).

TABLE 3. Subcellular distribution of triacylglycerol and [^{3}H]triacylglycerol in intestinal mucosa

Subcellular Compartment	Quantitation	Specific Activity
	total μmol/compartment[a]	*cpm μmol*$^{-1}$[a]
Total mucosa	148 ± 20	38,100 ± 2,160
Prechylomicrons[b]	62 ± 10 62 ± 10	36,700 ± 3,850
Low speed pellet[c]	11 ± 1.8	49,500 ± 3,150[f]
Rough microsomes[d,e]	0.28 ± 0.04	25,100 ± 2,300[f]
Smooth microsomes[d,e]	0.29 ± 0.13	46,500 ± 3,000[f]
Infusate		94,100[g]

[a] The data are the means ± 1 SEM of five rats.
[b] The lipid that floated on the surface at 340,000 *g* min centrifugation.
[c] The pellet from the 340,000 *g* min centrifugation.
[d] Smooth and rough microsomes obtained by the method of Hubscher et al. (11).
[e] Microsomal μgm RNA mg prot.$^{-1}$ was 74 ± 7.7 and 37 ± 2.6 for the rough and smooth microsomal fractions, respectively.
[f] Means are different from total mucosa, $P < 0.05$ by Student's *t* test.
[g] All specific activities were normalized to this mean value.

It is not clear what controls the distribution of dietary MG into the fast or slowly transported pool. One potential factor is the intestinal site of lipid absorption and transport. Since the rate of lipid transport from the distal intestine is less than from the proximal intestine (28), it is probable that more TG would enter the slowly transported pool in the distal gut. This thesis was not tested in the present studies since the proximal half of the intestine appeared grossly to absorb most of the infused lipid. In this context, it should be noted that each 12-cm segment of the proximal half of the intestine had approximately the same TG specific activity. An additional factor that might influence TG distribution into either the fast or slowly transported pool is the infused TG load. As shown in Table 2, at very low TG infusion rates, pre-existig neutral lipid pools (see Results), which are non-radioactive, blur any effect because of their dilution of the radiolabel, in addition to any reduction of the radiolabel by mucosal metabolism. However, at TG infusion rates approximately one-half that routinely used (Table 2), mucosal TG specific activity was 2-fold greater than when the routine TG infusion rate was used. This would suggest that at this more modest infusion rate, a greater percentage of the infused glyceride-glycerol entered the rapidly transported TG pool than when higher infusion rates were given. Finally, it is possible that the acyl groups of the resynthesized TG molecule direct, in some manner, entry into the rapidly transported or slowly transported pool.

Of particular interest is the finding of significant dilution of the infused radiolabeled glyceride-glycerol in the bowel lumen. Two lines of evidence from this study suggest that this luminal dilution of radioactivity is due to the efflux of neutral lipid from the intestinal mucosa into the bowel lumen. First, in considering the source of the non-radiolabeled glyceride-glycerol, the intestinal mucosa is the most reasonable since biliary phospholipids cannot be converted to neutral lipids in the intestinal lumen nor would the mass of biliary phospholipid (2 μmol hr^{-1})[3] be enough to effectively dilute the specific activity of the infused TG (90.5 μmol hr^{-1}). Secondly, the [^{3}H]glucose study provides a direct demonstration of the passage of neutral lipid into the bowel lumen. In these studies, the only neutral lipid radiolabeled source must have originated in the intestinal mucosa for the reasons already given (see Results). Therefore, any radioactivity in neutral lipids in the lumen under these circumstances must have come from the mucosa.

No precise information can be given on the size of the mucosal neutral lipid pool that is available for efflux from the bowel. However, at the conclusion of the 4-hr infusion period, when mucosal neutral lipids have reached steady state conditions, luminal neutral lipid pools would also be expected to have reached constant size. At this time, as shown in Fig. 2, there were 14.7 μmol of neutral lipid present in the lumen. If each neutral lipid species in the lumen is multiplied by (1 − its fraction of infusate specific activity, Table 2) it can be calculated that 11.7 μmol of neutral lipid would be required to efflux from the mucosa to account for the reduced specific activity of the neutral lipids in the lumen as compared to infusate specific activity. Similar calculations applied to the lowest infusion rate (Table 2) indicate that 0.6 μmol of neutral lipid must be effluxed and, for the mid-range infusion rate (Table 2), 4.1 μmol. The amount of lipid required to efflux represents 2, 5, and 7% of the total mucosal neutral lipid pool in ascending order of TG infusion rates. Thus, there is a direct correlation between the mass of lipid infused and both the amount of lipid effluxed and its percentage of the total mucosal neutral lipid pool.

In considering which neutral lipid fraction would be the most likely to efflux from the mucosa, TG would seem the most reasonable. The most compelling evidence derives from the [^{3}H]glucose study. Since mucosal MG has a specific activity 2.5-fold and DG 4.5-fold greater than luminal MG or DG, respectively, it is unlikely that there is rapid efflux of mucosal MG or DG. By contrast, TG had a specific activity 4-fold greater in the lumen than in the mucosa, suggesting that the heavily labeled TG pool that did efflux was the source (via lipolysis) of radiolabeled luminal MG and DG, since TG had the highest specific activity of luminal neutral lipids. An additional point in favor of TG as the neutral lipid species that effluxes from the mucosa into the lumen is the study in which tri[^{14}C]oleoyl[^{3}H]glycerol was used; it was seen that the dilution of the oleate specific activity occurred at the level of TG within the intestinal lumen.

Although the large specific activity differences and the small luminal DG pool rule out the possibility of DG transfer from the mucosa to the lumen, some efflux of MG from the mucosa is not eliminated, viz. since the luminal mass of MG is large compared to mucosal MG, a moderate efflux of newly synthesized and therefore labeled MG (from [^{3}H]glucose) from the mucosa would result in a decreased specific activity in the lumen because of its large non-radiolabeled MG pool. MG efflux is also suggested by the data presented in Fig. 4 in which it is shown that MG specific activity is lower in the intestinal lumen than TG specific activity in each third of the proximal three-fourths of the intestine.

Although transmembrane movement of MG in the intestine from lumen to mucosa has been known for some time, DG and TG movement have not been described. Since MG movement is passive (1), there is no a priori reason to suppose that it is unidirectional. Nevertheless, the suggested lack of major MG efflux becomes more understandable when its chemical gradient is considered. Since the average luminal volume is 5 ml and the average mucosal weight is 6 g, it is evident that MG could be considered to be moving down its concentration gradient by passing from the lumen (MG concentration 2 mM) into the mucosa (MG concentration 1 mM). This is not true for TG whose concentration in the mucosa is 50-fold greater than in the lumen. DG would also have a higher mucosal than luminal concentration but its intracellular movement is likely to be restricted because of its membrane bound nature (8, 24).

The present data have two important implications, both of which are under current investigation. First, it is evident that neutral lipid efflux from the mucosal cell might be increased in disease states and be implicated in the development of steatorrhea. For example, in the steatorrhea associated with diabetes mellitus, only a minority of such cases can be explained on the basis of a stasis syndrome (30) or concomitant gluten sensitive enteropathy (31). The speculation that these patients might have an exaggerated efflux of neutral lipid into the bowel is enhanced by the finding in juvenile diabetes that TG is retained in the mucosa after a lipid meal (32) which would increase the opportunity for such efflux to occur.

Secondly, these and previous data raise speculations concerning intestinal neutral lipid synthesis. If the endogenously synthesized glyceride-glycerol-derived TG

[3] Bile phosphatidylcholine concentration (2 mM, ref. 29) × bile flow per hour (1 ml; Mansbach, C., and M. A. Cox, unpublished observations in TG-infused rats).

pool is separate from the MG-derived TG pool, it would be implied that the enzymes associated with these two very different pathways might also be different. For example, MG acyltransferase might acylate DG as well as MG, although this appears unlikely. Another postulate is that there are two DG acyltransferases, one which would accept the product of MG acyltransferase and the other which would accept DG synthesized from glycerol-3-phosphate. A final and more likely thesis is that the two DG synthetic pathways might be segregated on the microsomal membrane in such a way that the DG produced by both pathways is effectively separated. In this event one DG acyltransferase may be present but act on each of the two DG pools separately. With each of these hypotheses the final product, TG, would be segregated on the basis of its pathway of origin and would likely have the opportunity of entering different pools with subsequently different fates with regard to rapidity of TG transport.

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