The uptake of oleic acid by rat small intestine: a comparison of methodologies

SUSANNE BENNETT CLARK*

Gastrointestinal Division, Department of Medicine,
St. Luke's Hospital Center, New York 10025

ABSTRACT The interaction between long-chain and medium-chain lipids during intestinal absorption was examined using several model systems. A decrease in steady-state triolein (LCT) output in thoracic duct lymph after addition of trioctanoin (MCT) to the duodenal infusion confirmed previous studies in unanesthetized rats which demonstrated inhibition of steady-state LCT uptake from the small intestinal lumen by MCT. In slices of everted rat jejunum octanoic acid reduced incorporation into triglyceride and initial uptake of ^{14}C-labeled oleic acid from micellar solutions. Inhibition of uptake did not occur at 0°C, when triglyceride synthesis was blocked. Incubation of slices at low pH (5.8) or in the presence of dimethyl sulfoxide also reduced uptake of oleic acid and its incorporation into triglyceride. However, when everted sacs of jejunum were similarly incubated, octanoate, dimethyl sulfoxide, or low pH caused no inhibition of oleic acid uptake or esterification. The results indicate that the significance of kinetic data describing intestinal fatty acid absorption which were obtained from experiments conducted in vitro is highly questionable, and that suitable models for in vivo uptake kinetics have yet to be developed. However, analysis of the in vitro kinetic data suggests that the intestinal mucosal membrane does not function as a simple lipid interface with respect to fatty acid absorption.

SUPPLEMENTARY KEY WORDS intestinal absorption · lymphatic transport · long-chain fatty acids · medium-chain fatty acids · membrane function · mucosal membrane transport · lipid absorption

MANY OF THE mechanisms by which ingested substances traverse the small intestinal mucosal barrier during absorption have now been described. These mechanisms include passive diffusion, facilitated diffusion, and varieties of active transport. Most studies have been concerned with water-soluble substances, since initially it had been felt that lipids would cross the supposedly lipoidal cell membrane by passive molecular diffusion. In recent years considerable controversy regarding the composition (3) and function (4) of cell membranes has arisen. The adequacy of simple molecular diffusion to account for the kinetics of intestinal absorption of lipids such as fatty acids has been questioned (5), and several studies have suggested that fatty acids may be "actively" absorbed by the small intestine (6–8). An intestinal rate-limiting step in the in vivo absorption of several fats with fatty acids of different chain length has been demonstrated (9). Competition between a long-chain fat (triolein, LCT) and a medium-chain fat (trioctanoin, MCT) has been shown to occur during steady-state intestinal absorption in unanesthetized rats (5), but interpretation of the study was complicated by the markedly increased acidity of the luminal contents found during high rates of MCT infusion. It was felt that low luminal pH might have reduced diffusion of long-chain fatty acids into mucosal cells by promoting their partition out of the luminal aqueous phase and into the oil phase, if it was assumed that the pKa of long-chain micellar fatty acid was unaltered by the presence of other lipids in the mixed micelle.

In the present series of experiments possible uptake mechanisms for a representative long-chain fatty acid, oleic acid, have been further investigated using several different techniques to study different stages of fatty acid absorption. In vivo, an experimental model (10) was used which allowed fat transport to proceed from intestinal lumen to lymph at a steady rate, while the luminal

Preliminary reports of some of these experiments have been published (1, 2).

Abbreviations: TLC, thin-layer chromatography; LCT, long-chain triglycerides; MCT, medium-chain triglycerides; DMSO, dimethyl sulfoxide.

* The author is the recipient of a Senior Investigatorship from the New York Heart Association.

lipid concentration was kept low to avoid changes in the pH of the luminal contents. In vitro, incubations of everted sacs and slices of rat jejunum were carried out in buffered solutions to control the extent of ionization of the fatty acid in the micellar bathing media. The pKa's of a series of mixed micellar solutions were also determined to clarify the relation between the present studies and earlier experimental models.

METHODS

Lymph Fistula Preparations

Male rats of the Wistar strain, 210–280 g in weight, were deprived of food overnight. Under ether anesthesia cannulas were inserted into the duodenum, the right external jugular vein, and the abdominal thoracic duct. The animals were then placed in modified Bollman restraint cages (11). Isotonic saline containing 0.03% KCl was continuously infused via the duodenal cannula at a rate of 1.56 ml/hr from the time of surgery until the experimental period, which began 48 hr later. No food or water were allowed by mouth during this period. It was noted in early experiments that lymph fistula animals which received MCT intraduodenally at rates readily tolerated by animals with no lymphatic drainage frequently died during, or soon after, the MCT infusion. Death was due to respiratory failure associated with a general CNS depression similar to that which is consistently produced in rats when MCT is maximally absorbed.[1] It was found that death could be prevented by intravenous administration of albumin. All animals were therefore continuously infused intravenously from the time of thoracic duct cannulation with human serum albumin (2% in 0.9% NaCl) at a rate of 1.10 ml/hr.

Lipid Stock Emulsions and Infusion Mixtures. Emulsion A contained triolein (K and K Laboratories, Inc., Plainview, N.Y.; purity 95% by TLC after ethanol extraction of unesterified fatty acid), 22%; Pluronic F-68 (Upjohn Co., Kalamazoo, Mich.), 0.6%; vegetable lecithin, 2.4%; and glucose, 4.5%, (all w/v) in water. The final lipid concentration was 672 μeq of fatty acid/ml. Emulsion B contained trioctanoin (Eastman Organic Chemicals, Rochester, N.Y.), 25%, instead of triolein; all other components (except triolein) were the same as in Emulsion A. The lipid concentration of Emulsion B was 1073 μeq of fatty acid/ml.

Both mixtures were sonicated to give stable stock emulsions which were stored in a refrigerator. A was labeled with isotopically homogeneous triolein-1-^{14}C (Nuclear-Chicago Corp., Des Plaines, Ill.). On the morning of the experiment, aliquots of A and B were mixed and diluted to give the appropriate infusion mixtures. LCT infusion: 4 ml of A diluted with 11 ml of normal saline was infused at a rate of 1.56 ml/hr. LCT and MCT infusion: 4 ml of A, 9 ml of B, and 2 ml of normal saline were infused at a rate of 1.56 ml/hr. The rates of LCT infusion were therefore identical during control and experimental time periods. Both mixtures contained comparable amounts of isotonic diluent, a condition necessary for the maintenance of a steady basal lymph flow (10).

Experimental Procedure. On the second and third postoperative days (48 and 72 hr after surgery), after a 1-hr control period on saline alone, intraduodenal infusions of the emulsified lipids containing ^{14}C-labeled triolein were given for a total of 8 hr at the same hourly volume as the previous saline infusion (1.56 ml/hr). Hourly lymph volume, lipid-^{14}C concentration, and lipid-^{14}C output in lymph were monitored during lipid infusions. Lymph secreted overnight was collected in two batches, for approximately equal time periods.

To study the effect of simultaneous absorption of MCT, in some animals the infusion mixture was changed after 4 hr from emulsified triolein-^{14}C alone (LCT infusion) to the mixture of triolein-^{14}C and nonradioactive trioctanoin (LCT and MCT infusion; lipid concentration ratio 1:5 expressed in μeq of fatty acid). In other animals this order was reversed.

Aliquots (100 μl) of thoroughly mixed lymph from each hourly collection period were counted directly without extraction. In selected experiments the distribution of lipid-^{14}C among the different lymph lipid classes was determined by TLC of the chloroform phase after extraction of aliquots of lymph with 5 volumes of chloroform–methanol 2:1.

In Vitro Incubations of Everted Sacs and Slices

Male rats of the Wistar strain were deprived food for 24 hr, stunned by a blow to the head, and the abdomen was opened. The small intestine was rinsed *in situ* with ice cold isotonic NaCl. Half of the small intestine immediately distal to the duodenal loop was removed and everted within 1 min. Slices (i.e., rings) weighing about 50 mg were cut and dropped into a beaker chilled in an ice bath and containing the oxygenated, glucose-containing buffer used in the subsequent experiment. To prepare everted sacs, the entire everted jejunum was placed immediately on an ice-cooled glass plate moistened with the buffer to be used in the subsequent experiment, and filled with 5 ml of ice-cold buffered medium containing 1% delipidated bovine albumin. As many as 10 small sacs with tissue weights of 230–300 mg were then prepared by subdivision of the whole

[1] Bennett Clark, S. Unpublished observations.

jejunum. This method substantially reduced the time of preparation of the sacs and ensured a more constant degree of distension relative to tissue weight.

One sac or three slices were incubated in stoppered flasks with stirring or shaking in 3 ml of isotonic buffer containing sodium taurocholate (usually 10 mM) (Maybridge Research Chemicals, Cornwall, England; purity by TLC >99%), and fatty acids, in an atmosphere of 5% CO_2 in O_2. Temperature, duration of incubation, lipid concentrations, and pH were as described in the Results section.

The buffer solution used for the tissue incubations was Ca^{2+}-free, and contained 0.9% NaCl (100 parts by volume), 1.15% KCl (4 parts), 3.82% $MgSO_4 \cdot 7H_2O$ (1 part), 0.1 M phosphate buffer (20 parts), and 62.5 mg of glucose; the solution was gassed with 5% CO_2 in O_2 for 1 hr before use.

Lipids used were >99% pure as determined by gas–liquid chromatography; oleic acid-1-^{14}C was homogeneous with respect to lipid class as judged by TLC.

Analytical Procedures. Lipid uptake in everted sacs and slices was stopped when the tissues were removed from the mucosal media, rinsed once in saline, blotted gently, and placed in acid saline (pH<2). In many experiments the contents of the flasks were first acidified with 0.1 ml of 11 N HCl and the flasks placed in ice; tissues were nevertheless removed within 1 min. After weighing, slices were homogenized in 1 ml of acid saline (pH 2.5) in tubes containing carrier triolein and oleic acid (50 μg and 25 μg, respectively). Sacs were placed into individual ice-cooled homogenizing tubes containing 0.5 ml of acid saline and were opened. The serosal sides were rinsed with 1 ml of saline, the tissues were blotted, reweighed, and then homogenized together with the serosal medium. In selected experiments serosal media were analyzed separately. Initially, homogenates were extracted with 5 volumes of chloroform–methanol 2:1, and the first aqueous methanol fraction was reextracted with 1 ml of chloroform. The chloroform phase contained >99% of LCT and long-chain fatty acid. Later, a method developed by Cohen, Morgan, and Hofmann (12), in which medium-chain-length fatty acids were more completely extracted, was used. The homogenates were acidified to pH<1, shaken with 3 volumes of toluene–ethanol 2:1, centrifuged for 3 min, and the aqueous ethanol phase was reextracted with 1 ml of toluene. Total extraction of LCT, MCT, and fatty acids into the combined toluene phases was greater than 97%. Aliquots of chloroform containing long-chain labeled lipids were dried slowly under N_2 at room temperature and the radioactivity in the residue was determined; toluene extracts were counted directly without removal of solvent. Other lipid aliquots were separated further by a two-stage, one-dimensional TLC procedure.

TLC of Lipid Extracts. Aliquots of organic solvents containing approximately 100 μg of lipid were spotted on 0.25-mm-thick, precoated Silica Gel G plates (20 × 20 cm, Analtech, Inc., Wilmington, Del.). 100 μg of a mixture of nonradioactive lipids containing unsaturated monoglycerides, diglycerides, and triglyceride (courtesy of Dr. F. H. Mattson, Procter and Gamble Co., Cincinnati, Ohio) were added to each spot. The plate was developed to within 2 cm of the top with acetone–chloroform 4:96. It was dried for 10 min, placed with the origin-edge down in acetone–ethyl acetate–glacial acetic acid–heptane 5:11:3:81, and the second solvent was allowed to run to a level 5 cm below the first solvent front (i.e., to the approximate level reached by diglyceride in the first solvent system). The plate was again dried and then exposed to iodine vapor; spots corresponding to phospholipid, monoglyceride, fatty acid, 1,2 diglyceride, 1,3 diglyceride, and triglyceride were marked. The iodine was allowed to evaporate over a 2-hr period in a fume hood and the marked areas were scraped into vials and counted directly without extraction of the lipid from the silica gel.

Counting Methods

Samples were counted in Bray's solution (13) or in Triton–toluene (14); there was no significant quenching in any of the samples analyzed.

Estimation of pKa of Mixed Fatty Acid Micellar Solutions

A series of solutions containing sodium taurocholate, oleic acid, and octanoic acid, (Applied Science Laboratories Inc., State College, Pa.), monoolein (Eastman Organic Chemicals; technical grade, class-purified by TLC), and a mixture of monooctanoin and monodecanoin (4:1), in proportions chosen to include combinations similar to those present in the intestinal lumen during maximal lipid absorption, was prepared. The solutions were acidified to pH<2 with 11 N HCl and were titrated with 0.05 N NaOH to pH>12. The pH of the solution was recorded after each fractional addition of alkali. A constant Na^+ concentration of 150 mM was maintained throughout each titration. Most solutions were clear initially; of those with the highest lipid concentrations which were initially cloudy, most cleared before the final end point was reached. However, sharp endpoints were obtained even in solutions where opalescence persisted.

RESULTS AND DISCUSSION

Lymph Fistula Animals

When ^{14}C-labeled triolein was infused (LCT infusion), lipid first appeared in lymph between 25 and 35 min

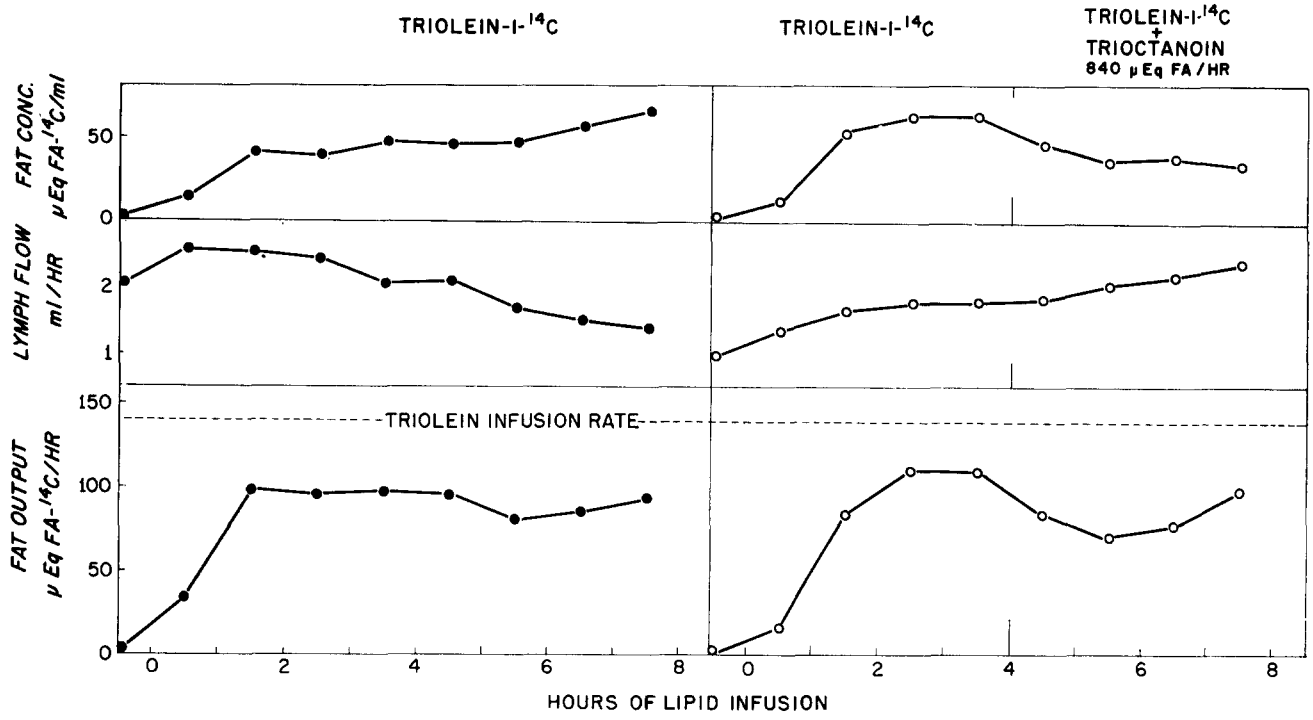

FIG. 1. Effect of trioctanoin on steady state lymphatic transport of triolein. Rate of intraduodenal infusion of triolein-1-^{14}C was 140 μeq FA/hr.

after the infusion began. Thereafter, the hourly lymphatic output of labeled lipid increased rapidly and reached a steady rate after the second hour, at about 60% of the duodenal infusion rate. In particular, the mean outputs during the third and fourth hours were not significantly different from the means of the fifth and sixth hours during continuous LCT infusion (paired comparisons by Student's "*t*" test). A typical experiment is shown in Fig. 1. The total lipid recovered from the thoracic duct by the following morning was 75–80% of the total infused.

To evaluate the effect of MCT on LCT output in lymph, the mean hourly outputs of radioactivity during the steady-state periods (third and fourth hours vs. fifth and sixth hours) were compared for all animals on both experimental days, using a paired "*t*" test. In the presence of trioctanoin, the lymphatic output of radioactivity was significantly decreased in 10 experiments on six animals ($P<0.001$). The average output of radioactivity during mixed lipid infusion was 20% less than the control level. The lower lipid-^{14}C output was due entirely to a decrease in the concentration of lipid in the lymph; lymph flow did not decrease when MCT was added to the triolein-^{14}C infusion.[2]

It has been shown by a pulse-labeling technique[3] that the time required for the transport of reesterified oleic acid into thoracic duct lymph during steady-state duodenal infusion of triolein is between 35 and 45 min. Since in the present experiments the concentration of ^{14}C-labeled lipid decreased significantly within the first 60 min of mixed infusion, the effect of trioctanoin on triolein absorption probably occurred at an early step in the absorption process. Thus, the possibility remains that some form of competition was operating in the region of the brush border membrane.

In previous experiments in which competitive effects were demonstrated (5) the luminal pH had fallen during high rates of MCT infusion, and this might have contributed to the reduced triolein absorption rate which was then observed. After 7 hr of the mixed lipid infusion in one lymph fistula animal, however, the pH of the luminal micellar phase was still 6.58, i.e., similar to that found during absorption of triolein alone under steady-state

[2] Available information (Ref. 10) indicates that halving the duodenal fluid infusion in this experimental model reduces the steady-state fat output in the third and fourth hour by no more than 7%. In the present experiments, the isotonic fluid infusion rate during the mixed lipid infusion was only about 20% less than during the LCT infusion, and unpublished control experiments have established that duodenal infusions of isotonic glucose and isotonic saline affect lymph flow similarly. Moreover, infusion of the mixed lipids caused an *increase* in lymph flow beyond that caused by LCT alone (Fig. 1). It is conceivable that fat-stimulated lymph flow does not produce the same small "wash-out" of fat from the mucosa as seems to occur with duodenal fluid perfusion (Ref. 10), but no information on this point exists at present. However, even if only the component of the lymph flow that is dependent on duodenal fluid administration were to affect lymphatic LCT output, in the present experiments such an effect should have amounted to no more than a 2% decrease during the mixed lipid infusion, yet a 20% decrease was observed.

[3] Bennett Clark, S., and P. R. Holt. Unpublished observations.

conditions. The decrease in lymphatic output of ^{14}C-labeled lipid during simultaneous absorption of trioctanoin at the relatively low infusion rates used in the present experiments was therefore unrelated to any change in pH of the luminal contents.

Uptake and Glyceride Incorporation of Oleic Acid-1-^{14}C In Vitro

In each of the following experiments, only total ^{14}C-labeled lipid, ^{14}C-labeled fatty acid, and ^{14}C-labeled triglyceride are considered, since other products together comprised less than 5% of the total lipid radioactivity in the tissue.

Experiments Using Everted Slices

Effect of Temperature on Initial Rate of Uptake of Oleic Acid. The total lipid-^{14}C in the tissue increased linearly with time at 0°C, at room temperature, and at 37°C, and the slopes of the lines did not differ significantly (Fig. 2). This result is consistent with a simple diffusion mechanism for oleic acid uptake under these conditions.

Oleic Acid Uptake in the Presence of Dimethyl Sulfoxide (DMSO). If oleic acid uptake by the mucosal membrane could be likened to molecular diffusion of lipid from an aqueous to a lipid phase, it seemed reasonable that addition of a solvent miscible with both lipid and water might accelerate uptake of fatty acid by lowering interfacial tension. However, the initial uptake of ^{14}C-labeled oleic acid at 0°C from micellar solutions at pH 6.6 was not increased by the addition of 1.0 M DMSO (Fig. 3).

When initial rates of uptake were measured at 37°C (Fig. 4), there was no difference, in the first 2 min, between controls and slices incubated in 1.0 M DMSO, but thereafter uptake in the presence of DMSO gradually decreased. This decrease was paralleled by a reduced rate of incorporation of ^{14}C-labeled oleic acid into triglyceride. The levels of ^{14}C-labeled fatty acids in tissues remained the same as in control slices. After 20 min, the total lipid-^{14}C in everted slices was less when DMSO was present (444 ± 115 nmoles ^{14}C-labeled fatty acid/g wet wt for controls, compared with 302 ± 65 for 0.3 M DMSO and 281 ± 26 for 1.0 M DMSO, means ± SD; $P<0.10$ and $P<0.05$, respectively). The levels of labeled fatty acids in the slices were the same in all three groups (140 ± 27, 114 ± 16, 124 ± 22), but incorporation of oleic acid into triglyceride was reduced by DMSO (267 ± 77 for controls, compared with 168 ± 55 for 0.3 M DMSO and 138 ± 19 for 1.0 M DMSO; $P<0.10$ and $P<0.02$, respectively).

Effect of pH on Oleic Acid Uptake and its Incorporation into Triglyceride. Since unionized fatty acid is more lipid-soluble and less water-soluble than the ionized form, the relation between the fatty acid uptake rate and the extent of ionization was studied. The total oleic acid concentration in three micellar solutions was kept constant at 0.33 mM, but the extent of its ionization was changed by adjustment of the pH to 7.8, 6.8, and 5.8 respectively. Relative to the concentration present at pH 7.8 (arbitrarily taken as 1), the concentration of unionized oleic acid should have increased approximately five times at pH 6.8, and approximately nine times at pH 5.8. 5-min uptakes of ^{14}C-labeled oleic acid from micellar solution at 37°C were not greater at pH 5.8 than at 6.8 or 7.8.

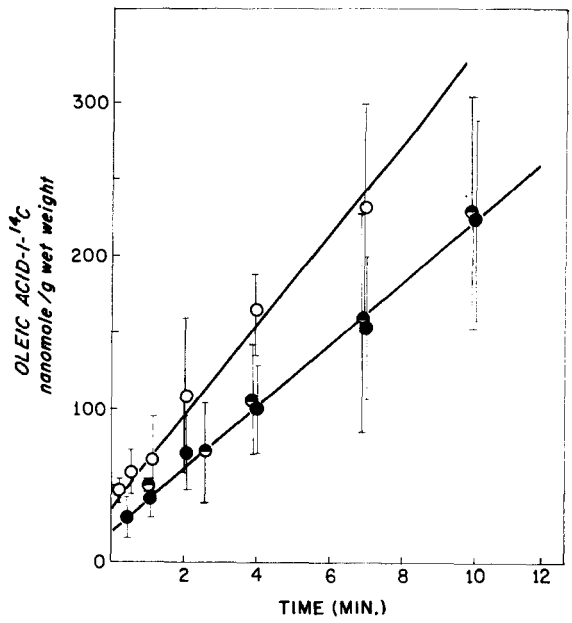

FIG. 2 Effect of temperature on oleic acid uptake by everted slices. ●, 0–6°C; ◓, 23–28°C; ○, 37°C. Means of 9–27 slices ± SD, from 3–10 animals. Incubation media contained 0.33 mM oleic acid-1-^{14}C, 10 mM sodium taurocholate, pH 6.6 buffer.

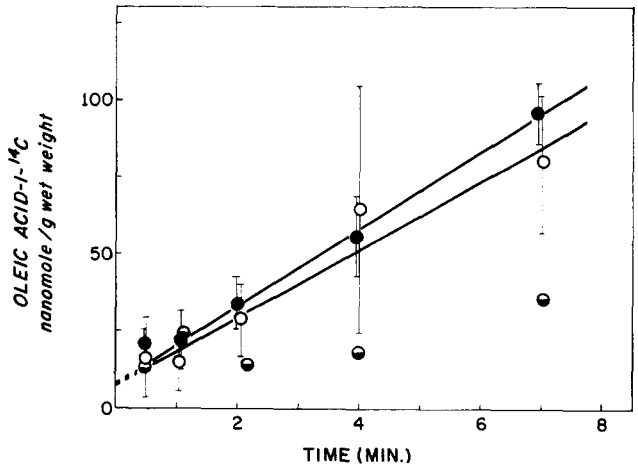

FIG. 3. Effect of dimethyl sulfoxide on initial uptake of oleic acid-1-^{14}C by everted slices from micellar solution at 0°C. ●, controls; ○, 1.0 M DMSO. Means of nine slices ± SD; data from three animals in each group. Incubation media contained 0.33 mM oleic acid-1-^{14}C, 10 mM sodium taurocholate, pH 6.6 buffer. ◓, 1.0 M DMSO, no bile salt; means of three slices.

TABLE 1 EFFECT OF pH ON UPTAKE OF OLEIC ACID-^{14}C BY EVERTED SLICES

	pH 5.8			pH 6.8			pH 7.8		
	5 min	10 min	20 min	5 min	10 min	20 min	5 min	10 min	20 min
	*nmoles FA-^{14}C/g (wet wt)**								
Total lipid-^{14}C	87	87	135	83	127	206	94	122	197
Fatty acid-^{14}C	54	46	46	49	41	54	34	34	43
Triglyceride-^{14}C	29	37	82	28	69	136	49	75	138

* Values are the mean concentrations per 3 slices. FA, fatty acid.
Media contained 0.33 mM oleic acid-1-^{14}C and 10 mM sodium taurocholate in phosphate buffer at 37°C.

TABLE 2 EFFECT OF OCTANOIC ACID ON UPTAKE OF OLEIC ACID-^{14}C BY EVERTED SLICES AT 0–3°C*

Medium	Lipid-^{14}C Uptake Rate	
	3 Slices ca. 50 mg each	1 Slice ca. 150 mg
	nmoles FA-^{14}C/min/g (wet wt) †	
Oleic acid-^{14}C, 0.33 mM	26.2 ± 8.7 (19)	13.1 ± 3.1 (37)
Oleic acid-^{14}C, 0.33 mM + Octanoic acid, 1.65 mM	25.7 ± 6.7 (19)	13.6 ± 4.7 (35)

* Flasks contained 10 mM sodium taurocholate in pH 6.6 phosphate buffer. Incubation times were between 1–15 min.
† Mean ± SD; number of experiments in parentheses. FA, fatty acid.

After 10 min and 20 min, incorporation of oleic acid into triglyceride at pH 5.8 was markedly less than at pH 7.8 (Table 1), and total lipid-^{14}C uptake was also less. Thus, when triglyceride synthesis was inhibited, oleic acid uptake was reduced, but no relation was found between the concentration of unionized oleic acid in the medium and its initial rate of uptake of mucosa.

Initial Uptake of Oleic Acid in the Presence of Octanoic Acid. Uptake of oleic acid at 0–3°C was linear for 15 min both with and without octanoic acid in the bathing medium. The presence of octanoic acid in a molar ratio of 5:1 did not affect the uptake of oleic acid when esterification was blocked at 0°C (Table 2). At 37°C, octanoic acid markedly inhibited the uptake of oleic acid by small everted slices (Fig. 5). Tissue levels of fatty acid-^{14}C were, however, not affected; only incorporation of oleic acid into triglyceride was reduced by octanoic acid, and this paralleled the reduced uptake of oleic acid.

Effect of Octanoic Acid on Initial Incorporation of Oleic Acid into Triglyceride. Since inhibition of uptake of oleic acid had occurred only at temperatures at which there was active synthesis of triglyceride, and since inhibited uptake was always accompanied by reduced incorporation

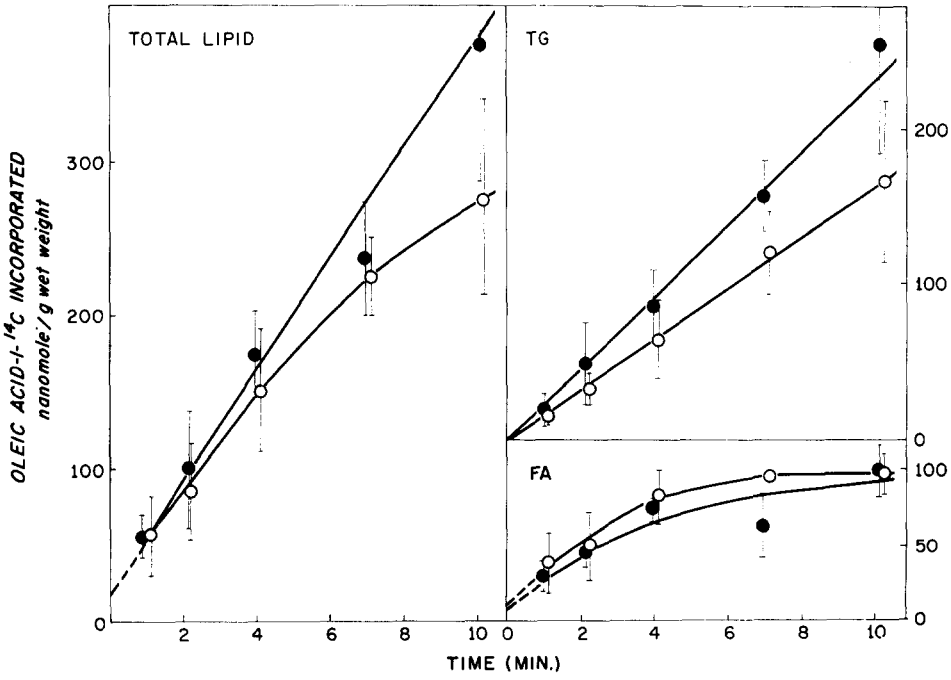

FIG. 4. Effect of dimethyl sulfoxide on uptake of oleic acid-1-^{14}C and its incorporation into triglyceride by everted slices. ●, controls; ○, 1.0 M DMSO. Means of nine slices ± SD; data from three animals in each group. Incubation media contained 0.33 mM oleic acid-1-^{14}C, 10 mM sodium taurocholate, pH 6.6 buffer; temp 37°C.

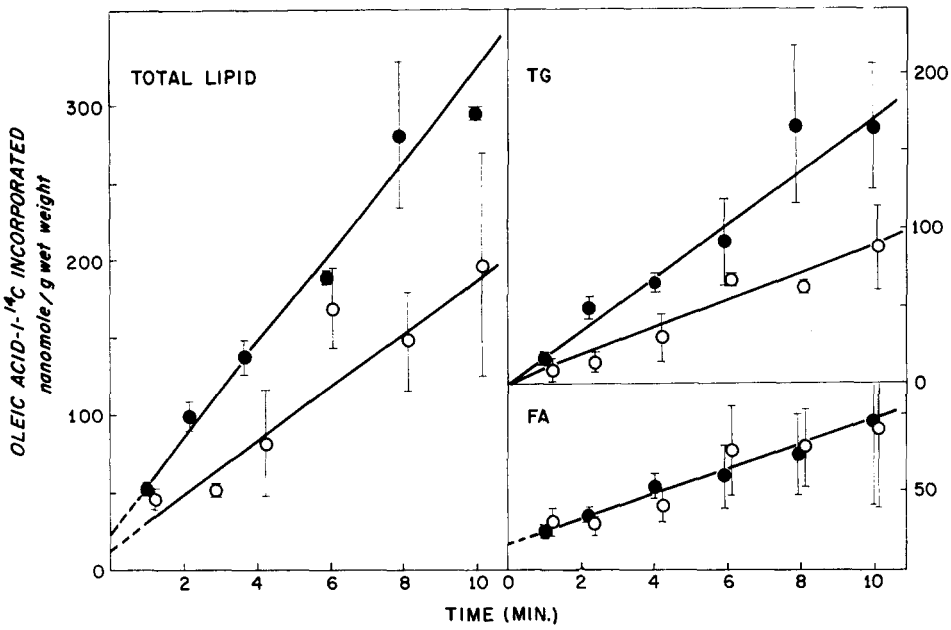

FIG. 5. Effect of octanoic acid on uptake of oleic acid-1-^{14}C and its incorporation into triglyceride by everted slices. ●, controls; ○, 1.65 mM octanoic acid. Means of six slices ± SD; two animals in each group. Incubation media contained 0.33 mM oleic acid-1-^{14}C, 10 mM sodium taurocholate, pH 6.6 buffer; temp 37°C.

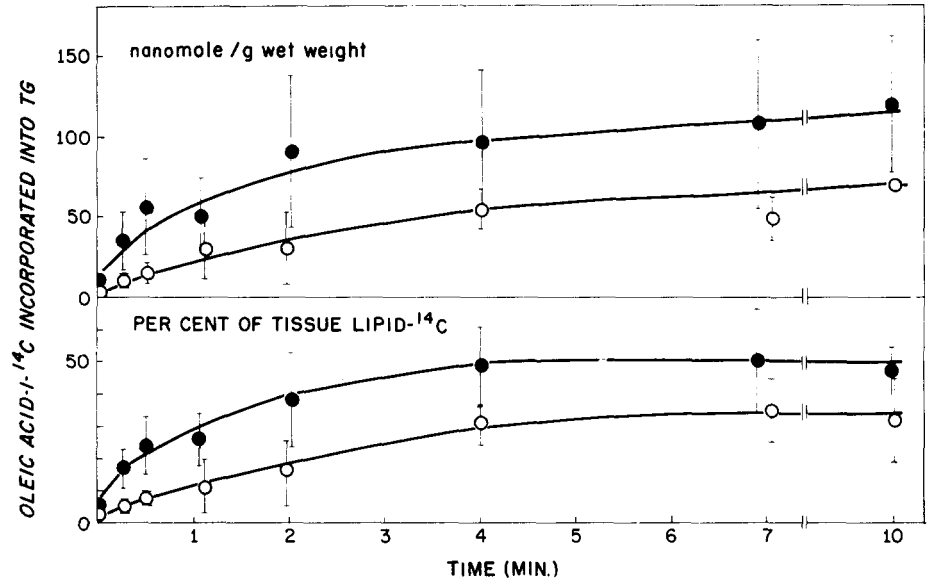

FIG. 6. Effect of octanoic acid on initial incorporation of oleic acid into triglyceride by everted slices. ●, controls; ○, octanoic acid. Preincubation for 15 min at 0–1°C in media containing 0.33 mM oleic acid ± 1.65 mM octanoic acid, 10 mM sodium taurocholate, pH 6.6 buffer. Triglyceride-^{14}C levels after warming to 37°C. Means of 7–8 slices ± SD; 7–8 animals in each group.

of oleic acid into triglyceride, it was not clear whether the reduced uptake was the result of, or was independent of, the inhibition of triglyceride synthesis. To approach this problem, the initial rates of triglyceride synthesis were measured at 37°C, after 15-min preincubation periods at 0°C in micellar oleic acid-^{14}C media with and without octanoic acid. Fig. 6 shows the results of such experiments. Octanoic acid, taken up at 0°C and therefore already present in the mucosa when the slices were warmed to 37°C, strongly inhibited incorporation of oleic acid into triglyceride. Thus, the reduced uptake of oleic acid by everted slices noted at 37°C in the presence of octanoic acid (Fig. 5) was probably due to the decrease in the inward diffusion gradient for oleic acid

when triglyceride synthesis was inhibited, and unesterified oleic acid was allowed to accumulate intracellularly.

Experiments Using Everted Sacs

In this preparation, the increase in serosal fluid during incubation may be used as an index of viability of the mucosa. Serosal fluid volumes increased at rates between 15 and 70 μl/g wet wt per min in all sac experiments, except in the presence of DMSO where substantial decreases were observed, probably due to its osmotic effect.

Uptake Kinetics of Oleic Acid at 26°C. The initial rate of uptake of oleic acid by everted sacs from a micellar solution of oleic acid-1-^{14}C was slower than the uptake by everted slices under the same conditions, although both were linear up to 10 min (Fig. 7). Incorporation of oleic acid into triglyceride in everted sacs also increased linearly with time. In contrast with slices, however, the level of unesterified fatty acid-^{14}C in sac tissue remained constant after the first minute.

Effect of Mucosal Oleic Acid Concentration on its Uptake and Incorporation into Triglyceride. Linear relationships were obtained between the micellar concentration of oleic acid-^{14}C in the mucosal fluid and the initial (1–3 min) uptake rate, the incorporation into triglyceride, and the tissue level of fatty acid-^{14}C, respectively (Fig. 8). These data are also consistent with a diffusion mechanism for oleic acid uptake under the conditions studied.

Effect of DMSO on Oleic Acid Uptake and its Incorporation into Triglyceride. Uptake of oleic acid by everted sacs was not significantly decreased by the presence of 1.0 M DMSO during a 20-min incubation period at 37°C (255 ± 71 and 182 ± 21 nmoles fatty acid-^{14}C/g wet wt

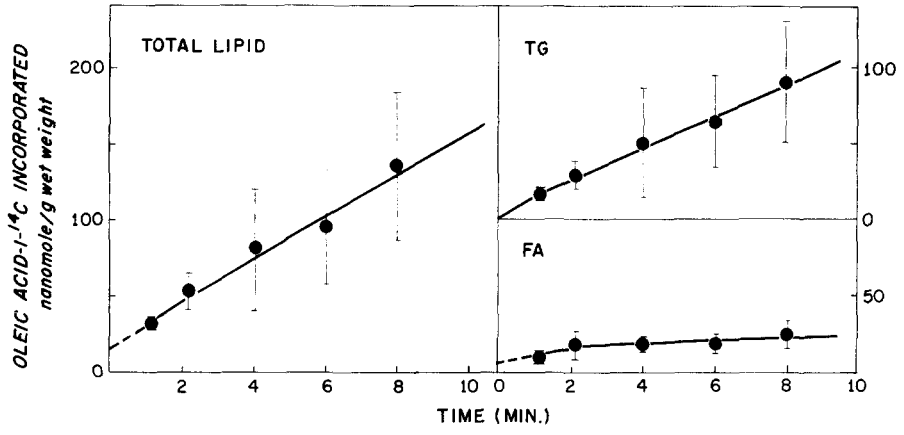

FIG. 7. Uptake of oleic acid-1-^{14}C and its incorporation into triglyceride by everted sacs. Means of 4 sacs ± SD; each point includes data from two animals. Incubation medium contained 0.33 mM oleic acid-1-^{14}C, 10 mM sodium taurocholate, pH 6.6 buffer; temp 26°C.

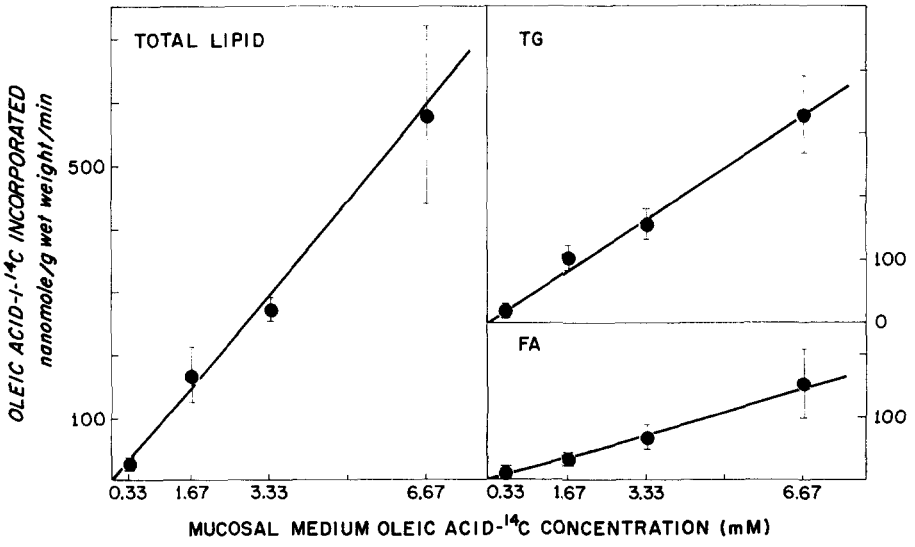

FIG. 8. Effect of oleic acid-1-^{14}C concentration on its uptake and incorporation into triglyceride by everted sacs. Means of two sacs ± SD; each point includes data from two animals. pH 6.6 buffer; temp 26°C.

in four control and four experimental sacs, respectively; $P>0.10$). Incorporation of oleic acid into triglyceride was also not significantly decreased ($P>0.30$). The conditions used were identical with those that produced significant effects when everted slices were used instead of sacs.

Effect of pH on Oleic Acid Uptake. Sacs were filled with 1% albumin buffer at pH 7.7. Incubations were carried out at 26°C in buffered media containing 0.33 mM oleic acid-^{14}C and 10 mM sodium taurocholate. The results agreed with those obtained from everted slices insofar as low pH did not increase oleic acid uptake. The average uptakes in 5 min at pH 7.7 were, in fact, faster than at pH 5.7, although the difference was not significant (110 ± 48 nmoles fatty acid-^{14}C/g wet wt compared with 149 ± 14). Reduced incorporation of oleic acid into triglyceride again accounted quantitatively for the slight change in uptake (50 ± 7 compared with 75 ± 12, $P = 0.1$). Qualitatively similar results were obtained after 10 min and 20 min; in all instances the level of fatty acid-^{14}C in the tissues was unaffected by the pH of the mucosal medium.

Oleic Acid Uptake and its Incorporation into Triglyceride in the Presence of Octanoic Acid. Octanoic acid did not inhibit either uptake or incorporation of oleic acid into triglycerides in sacs (Fig. 9) under conditions which produced significant inhibition in everted slices (Fig. 5). Rather, sacs showed an effect opposite to that obtained in slices, with octanoate causing stimulation instead of inhibition. Even preincubation for 20 min in mucosal media containing octanoic acid did not significantly reduce subsequent initial (3 min) uptake and incorporation of oleic acid into triglyceride in sacs ($P>0.2$ and >0.3, respectively, Table 3).

TABLE 3 EFFECT OF PREINCUBATION WITH OCTANOIC ACID ON INITIAL UPTAKE OF OLEIC ACID-^{14}C BY EVERTED SACS*

	Total Lipid-^{14}C	Fatty Acid-^{14}C	Triglyceride-^{14}C
	nmoles FA-^{14}C/g (wet wt) †		
Control‡	58 ± 8	14 ± 5	38 ± 8
With octanoic acid§	50 ± 6	16 ± 6	30 ± 11

* Incubations were performed in micellar oleic acid-^{14}C (0.33 mM) in 10 mM sodium taurocholate in pH 6.6 phosphate buffer for 3 min at 37°C.

† Numbers are means ± SD of four sacs, two sacs in each group from each of two animals. FA, fatty acid.

‡ The serosal fluid was 1% albumin in phosphate buffer, pH 6.6. Preincubation was in mucosal medium containing 10 mM sodium taurocholate in pH 6.6 buffer, for 20 min.

§ Serosal fluid and preincubation medium contained, in addition, 1.65 mM octanoic acid.

In an attempt to "force" an inhibition of oleic acid uptake and incorporation into triglyceride by octanoic acid in sacs, similar to that observed in everted slices, the concentration of oleic acid-^{14}C in the mucosal medium was reduced to 7.7 μM and different amounts of octanoic acid were added to produce concentrations of up to 33 mM. A sodium taurocholate concentration of 20 mM was sufficient to give a clear solution at all except the highest lipid concentrations, where opalescence persisted. It was

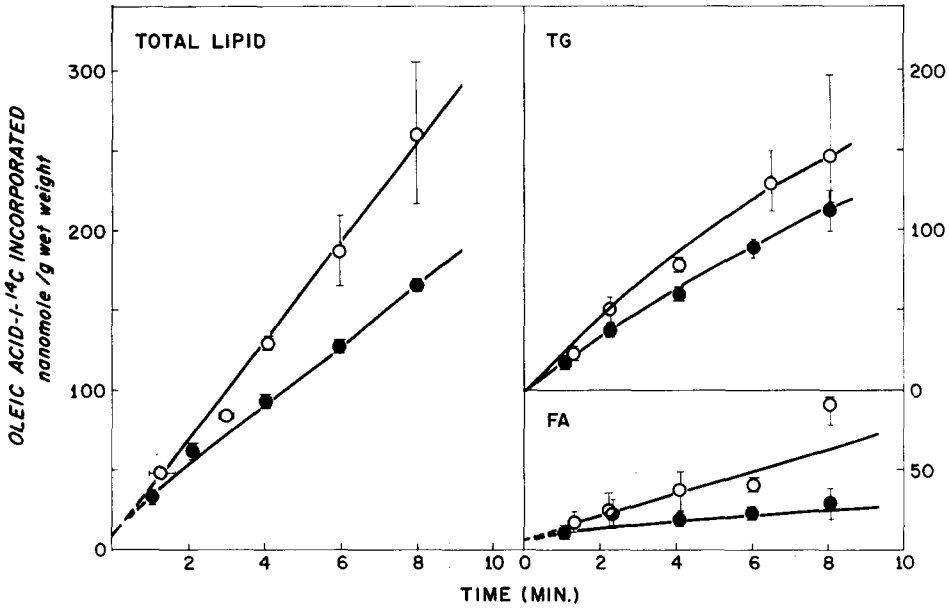

FIG. 9. Effect of octanoic acid on uptake of oleic acid-^{14}C and its incorporation into triglyceride by everted sacs. ●, controls; ○, 1.65 mM octanoic acid. Means of two sacs ± SD; each point includes data from two animals. Incubation media contained 0.33 mM oleic acid-1-^{14}C, 10 mM sodium taurocholate, pH 6.6 buffer; temp 37°C.

necessary to increase the ratio of octanoic acid to oleic acid to above 435 before incorporation of oleic acid into triglyceride by everted sacs was inhibited, and even then oleic acid uptake was not measurably reduced (Table 4).

Finally, when everted sacs filled with albumin buffer containing 1.65 mM octanoic acid and preincubated for 20 min at 37°C in lipid-free mucosal medium were placed in 0.33 mM micellar oleic acid-^{14}C in buffer for 3 min, there was no significant reduction in oleic acid uptake or its incorporation into triglyceride compared with control sacs filled with lipid-free albumin buffer.

Significance of Intracellular Binding and Esterification of Oleic Acid in the Regulation of its Uptake

Present understanding of fatty acid movements within mucosal cells is extremely limited since little is known of the concentrations and chemical activities of fatty acid molecules in different regions inside the cell. Preliminary experiments[8] have shown that a significant fraction of fatty acid taken up by everted sacs at 0°C is freely mobile. Fatty acid-^{14}C was detected in serosal fluid after 15-min incubation periods in mucosal solutions of micellar oleic acid-^{14}C. When the sacs were then washed in a mucosal solution of 1% albumin, much of the radioactivity in both the tissue and the serosal fluid was lost. However, even prolonged washing did not remove all the labeled fatty acid in the tissue, suggesting some form of intracellular binding even at low temperatures. It was therefore of interest to compare the initial rate of fatty acid uptake with that of triglyceride incorporation. Jejunal slices absorbed fatty acid linearly with time at 0°C at an average rate of 13.4 nmoles fatty acid-^{14}C/g wet wt per min from a bathing medium containing 0.33 mM oleic acid (150-mg slices, Table 2). After 15 min, the fatty acid-^{14}C concentration in the whole tissue was about 0.2 mM, or approximately 0.4 mM in the mucosa. When the slices were warmed for 15 sec, oleic acid was initially converted to triglyceride at an average rate of about 90 nmoles/g wet wt per min (Fig. 6). These data support the suggestion that fatty acid molecules which enter the cell attach rapidly to esterification binding sites and produce high local concentrations of fatty acid by energy-independent binding. Since the binding itself should constitute a "sink" for the uptake process, the extent to which triglyceride incorporation per se is necessary to promote further fatty acid uptake requires reevaluation.

Presently available evidence concerning the effect of inhibition of mucosal esterification on fatty acid uptake is contradictory. Rodgers, Riley, Drummey, and Isselbacher (15) have suggested that the slight decrement in oleic acid absorption shown by adrenalectomized rats might be causally related to the somewhat lower level of mucosal esterifying enzymes found in these animals. Kessler, Mishkin, and Stein (16) showed a relationship between the degree of inhibition of mucosal esterification after ethionine administration to rats and the reduction in fatty acid absorption compared with untreated controls. The changes were not great, however. Experiments by Hyun, Vahouny, and Treadwell (17) suggested that 2-ethyl-*n*-caproic acid suppressed esterification of oleic acid in lymphatic- and portally-cannulated rats and diverted unesterified fatty acids to the portal route; mucosal uptake did not decrease. Evidence available from intestinal preparations in vitro is equally confusing. Vahouny, Nelson, and Treadwell (18) found that a high (20:1) ratio of 2-ethyl-*n*-caproic acid was needed to block oleic acid esterification by everted sacs of rat jejunum, yet oleic acid uptake during a 10-min incubation period was reduced only slightly. In the present experiments, the similarity in uptake kinetics at 0°C, 23°C, and 37°C suggests not only that mucosal absorption of fatty acid is energy-independent, but also that triglyceride synthesis is not the major mechanism controlling uptake.

TABLE 4 EFFECT OF HIGH MUCOSAL OCTANOATE CONCENTRATIONS ON UPTAKE AND INCORPORATION INTO TRIGLYCERIDE OF OLEIC ACID-^{14}C BY EVERTED SACS

Octanoic Acid: Oleic Acid-^{14}C Ratio	5 min			10 min		
	Total Lipid-^{14}C	FA-^{14}C	TG-^{14}C	Total Lipid-^{14}C	FA-^{14}C	TG-^{14}C
	nmoles oleic acid-^{14}C incorporated/g wet wt of tissue					
0	3.7	1.6	1.6	4.3	1.1	2.6
43.5	2.2	1.0	1.0	2.2	0.8	3.5
435	3.4	1.6	1.6	4.3	1.4	2.6
2175	5.3	4.9	0.3	5.3	4.8	0.3
4350	3.9	3.5	0.3	5.0	4.7	0.3

Numbers are means of two sacs. Mucosal medium oleic acid-^{14}C concentration was 7.7 μM. FA, fatty acid; TG, triglyceride.

TABLE 5 pKa's of Mixed Micellar Solutions

Na Taurocholate	Oleic Acid	Monoolein	Octanoic Acid	Monooctanoin	pKa_1*		pKa_2*	
		concentration, mM						
10					2.37		—	
10		5			2.40		—	
10	10				2.35	6.84		
10	10	5			2.38	6.88		
10			50		2.34		5.07	
10			50	10	2.31		5.39	
10	10	5	50	10	2.35			5.61
20					2.40		—	
20	10	5			2.32	6.33		
20	5				2.47	7.00		
20	5		25					5.49
30					2.36		—	
30	10	5			2.50	6.47		
40					2.33		—	
40		5			2.43		—	
40	10	5			2.30	6.57		
40	40	20			2.38	7.37†		
40	10	5	100	20	2.34			5.63
40	20	10	200	50	2.30			5.86†
40	30	15	300	100	2.28			5.80†

* Means of 1–3 replicate estimations.
† Solution remained opalescent.

Fatty Acid Ionization in Micellar Bile Salt Solution

Single, sharp end points were obtained when solutions of bile salt alone were titrated with dilute NaOH. The pH of half-ionization (pKa) of a micellar solution of sodium taurocholate was 2.3–2.4 (Table 5). This pKa was unchanged when fatty acids were added to the micellar solutions, but a second end point was now observed; the pKa's of the fatty acids under these conditions were 6.6–6.9 for oleic acid and 5.1–5.4 for octanoic acid. When both oleic and octanoic acids were present in sodium taurocholate solution the second (fatty acid) pKa was between 5.5 and 5.8. This result implies that the negative charges derived from the fatty acids in the micelle might be spread over the surface of the micelle, and not remain discretely associated with individual fatty acid anions. Moreover, it follows that the proportion of ionized oleic acid molecules must be less at a given pH in a sodium taurocholate–oleic acid micelle than in a taurocholate–octanoic acid–oleic acid micelle at the same pH. Monoglycerides did not affect the pKa's of the fatty acids or the bile salt.

Significance of the pH of Micellar Phase in the Kinetics of Fatty Acid Absorption

The question, "Can the intestinal mucosa be considered functionally as a simple lipid interface?" has been raised repeatedly since Danielli and Davson (19) proposed a bimolecular lipid leaflet model for plasma membranes. So far no single study has answered this question, and the accumulated evidence remains controversial. For example, in vivo, during continuous duodenal infusion of a single triglyceride species (5), unanesthetized rats maximally absorbed an emulsified medium-chain triglyceride, trioctanoin, 4–5 times faster than an emulsified long-chain triglyceride, triolein. The concentrations of unesterified fatty acids in the aqueous luminal phase during maximal absorption were approximately 200 mM and 35 mM for octanoic and oleic acids, respectively, and the corresponding luminal pH's were 5.9 and 6.7. In the present experiments the pH's of half-ionization of the same fatty acids in simulated intestinal contents were about 5.0 and 6.7, respectively, so that the fractions of aqueous fatty acid ionized at the pH of the lumen should have been about 90% for octanoic acid during maximal trioctanoin infusion, and 50% for oleic acid during maximal triolein infusion. The aqueous concentrations of the unionized forms of the medium- and long-chain fatty acids should therefore have been approximately equal under conditions of maximal absorption. If the mechanism of fatty acid uptake were simple molecular diffusion of unionized fatty acids, their relative uptake rates should be inversely proportional to the square roots of their molecular weights (i.e., 1.0:0.7) and directly proportional to their activities (approximately proportional to concentration) in the luminal aqueous phase. Since the absorption rate was in fact four times faster for the medium-chain lipid, simple diffusion of unionized fatty acid does not seem to explain fully the findings in vivo.

In the same series of experiments (5) apparent inhibition of maximal absorption of triolein by trioctanoin was demonstrated. The relatively acid luminal pH (5.9) developed during infusion of mixed lipids at high rates was considered a possible factor contributing to the reduced triolein absorption, since it seemed reasonable that oleic acid ionization might have been suppressed. However, it has now been shown that micellar solutions containing bile salt, oleic acid, monoolein, octanoic acid, and monooctanoin show only a single end point for fatty acid, at a pH of 5.4–5.7. The result suggests that the degree of ionization of oleic acid might not have changed greatly during the intraduodenal infusion of the mixed lipids. Furthermore, LCT output by lymphatic-cannulated rats was also inhibited by MCT, without a change in luminal pH. Taken together, these experiments demonstrate that the competition between long- and medium-chain fats during absorption is not dependent on the pH of the micellar phase to any great extent.

The present in vitro experiments are in agreement with the results obtained in vivo. Moreover, the data suggest that the brush border membrane did not behave as a lipid interface. Partition of oleic acid between micellar and oil phases favors the oil phase at low pH (20), yet uptake of oleic acid from micellar solution by jejunal slices and sacs was not increased at low pH. If fatty acid nevertheless entered the cell membrane by diffusion, then both ionized and unionized molecules must have diffused equally rapidly. The uptake of whole micelles containing differing proportions of ionized fatty acid molecules depending on the pH would be one possible explanation for these findings. Available evidence both supports (21) and denies (22) such a mechanism. No explanation can be offered for the discrepancy between the present results and those of Webb, Hamilton, and Dawson (23), who obtained an increase in oleic acid uptake at low pH in sheets of rat jejunum.

Thus, information derived from differing model systems pertaining to the uptake of fatty acid and to the interaction between medium- and long-chain lipids during intestinal absorption remains incomplete. Evidence obtained both in vivo and in vitro indicates that the intestinal brush border membrane does not function as a simple lipid interface, and that diffusion of unionized fatty acid molecules is not the rate-limiting step in fatty acid uptake. Information from different model systems is often contradictory, however, and realistic in vitro models of mechanisms operating in vivo have yet to be developed. Experiments designed to characterize some of the binding properties of lipids to mucosal tissues have been begun (21, 22, 24), and should provide helpful information. A clearer evaluation of binding affinities between lipids and mucopolysaccharides under different conditions of pH and solubilization would seem to be of prime importance in future investigations.

The technical assistance of Miss Michele Moore, Miss Renee Zerinsky, and Miss Jill Martin is gratefully acknowledged. Frequent discussion with Dr. P. R. Holt was most helpful. The work was supported by grants from the National Institutes of Health, HE 10055 and AM 13436, supplemented by the New York Heart Association, Inc.

Manuscript received 12 June 1970; accepted 15 October 1970.

References

1. Bennett Clark, S., and P. R. Holt. 1969. Inhibition of intestinal absorption of triolein by trioctanoin: studies of diffusion kinetics of fatty acids in vitro. *Clin. Res.* **17:** 300.
2. Bennett Clark, S. 1970. Oleic acid uptake and metabolism by everted slices and sacs of rat jejunum. *Fed. Proc.* **29:** 595.
3. Korn, E. D. 1968. Structure and function of the plasma membrane: a biochemical perspective. *In* Biological Interfaces: Flows and Exchanges. Little, Brown and Co., Boston. 257–274.
4. Ling, G. N. 1969. A new model for the living cell: a summary of the theory and experimental evidence in its support. *Int. Rev. Cytol.* **26:** 1–61.
5. Bennett Clark, S., and P. R. Holt. 1969. Inhibition of steady-state intestinal absorption of long-chain triglyceride by medium-chain triglyceride in the unanesthetized rat. *J. Clin. Invest.* **48:** 2235–2243.
6. Lyon, I. 1968. Studies on transmural potentials in vitro in relation to intestinal absorption. V. Kinetic characteristics of lipid interactions with rat gut. *Biochim. Biophys. Acta.* **163:** 75–84.
7. Smyth, D. H., and C. B. Taylor. 1958. Intestinal transfer of short-chain fatty acids in vitro. *J. Physiol.* (*London*). **141:** 73–80.
8. Barry, R. J. C., M. J. Jackson, and D. H. Smyth. 1966. Transfer of propionate by rat small intestine in vitro. *J. Physiol.* (*London*). **182:** 150–163.
9. Bennett, S. 1964. Intestinal absorptive capacity and site of absorption of fat under steady state conditions in the unanaesthetised rat. *Quart. J. Exp. Physiol.* **49:** 210–218.
10. Shepherd, P., and W. J. Simmonds. 1959. Some conditions affecting the maintenance of a steady lymphatic absorption of fat. *Aust. J. Exp. Biol. Med. Sci.* **37:** 1–9.
11. Bollman, J. L., J. C. Cain, and J. H. Grindlay. 1948. Techniques for collection of lymph from liver, small intestine, or thoracic duct of rat. *J. Lab. Clin. Med.* **33:** 1349–1352.
12. Cohen, M., R. G. H. Morgan, and A. F. Hofmann. 1969. One-step quantitative extraction of medium-chain and long-chain fatty acids from aqueous samples. *J. Lipid Res.* **10:** 614–616.
13. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1:** 279–285.
14. Patterson, M. S., and R. C. Greene. 1965. Measurement of low energy beta-emitters in aqueous solution by liquid scintillation counting of emulsions. *Anal. Chem.* **37:** 854–857.
15. Rodgers, J. B., E. M. Riley, G. D. Drummey, and K. J. Isselbacher. 1967. Lipid absorption in adrenalectomized rats: the role of altered enzyme activity in the intestinal mucosa. *Gastroenterology.* **53:** 547–556.

16. Kessler, J. I., S. Mishkin, and J. Stein. 1969. The effect of *dl*-ethionine on the intestinal absorption and transport of palmitic acid-1-^{14}C and tripalmitin-1-^{14}C. Role of intramucosal factors in the uptake of luminal lipids. *J. Clin. Invest.* **48:** 1397–1407.
17. Hyun, S. A., G. V. Vahouny, and C. R. Treadwell. 1967. Portal absorption of fatty acids in lymph- and portal vein-cannulated rats. *Biochim. Biophys. Acta.* **137:** 296–305.
18. Vahouny, G. V., J. Nelson, and C. R. Treadwell. 1968. Inhibition of triglyceride synthesis in everted intestinal sacs. *Proc. Soc. Exp. Biol. Med.* **128:** 495–500.
19. Danielli, J. F., and H. Davson. 1934. A contribution to the theory of permeability of thin films. *J. Cell. Comp. Physiol.* **5:** 495–508.
20. Borgström, B. 1967. Partition of lipids between emulsified oil and micellar phases of glyceride–bile salt dispersions. *J. Lipid Res.* **8:** 598–608.
21. Gordon, S. G., and F. Kern, Jr. 1968. The absorption of bile salt and fatty acid by hamster small intestine. *Biochim. Biophys. Acta.* **152:** 372–378.
22. Hoffman, N. E. 1970. The relationship between uptake in vitro of oleic acid and micellar solubilization. *Biochim. Biophys. Acta.* **196:** 193–203.
23. Webb, J. P. W., J. D. Hamilton, and A. M. Dawson. 1969. A physicochemical study of fat absorption in rats. Limitations of methods in vitro. *Biochim. Biophys. Acta.* **187:** 42–52.
24. Mishkin, S., and J. I. Kessler. 1970. The uptake and release of bile salt and fatty acid by hamster jejunum. *Biochim. Biophys. Acta.* **202:** 222–224.