The ultrastructure of lipid absorption and transport in bovine intestinal mucosa

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THE ULTRASTRUCTURE OF LIPID ABSORPTION AND
TRANSPORT IN BOVINE INTESTINAL MUCOSA

by

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A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subjects: Cell Biology
Biochemistry

Approved:

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Ames, Iowa

1969
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INTRODUCTION

The development of the electron microscope in the 1930's, and its subsequent rapid innovations and refinements, has today provided the field of cellular biology one of its most important tools for scientific investigation. Not only is the instrument important in its own right for investigation of structure and function at the cellular and macromolecular level, but also it is important as a complementary technique to the modern and equally sophisticated biochemical procedures.

Although studies of cellular metabolism have been dominated by the numerous biochemical methods available to researchers today, precise localization of metabolic events in the cell requires visualization at high resolution. To be sure, cell fractionation techniques are constantly being refined to provide greater precision in identification of function relating to organelles and other subcellular components; however, correlation of function with structure, in vitro as well as in vivo, necessitates supplementation by electron microscopy.

The use of the electron microscope in cellular localization assumes even greater validity when the investigation concerns cell types which exhibit polarity in organization and function within the tissue and organ. Here, biochemical techniques, involving whole tissues and cells as well as cell fractions, remove the parameters of vectoral and compartmentalized metabolic events, while ultrastructural procedures retain such parameters. The subject of this investigation, the intestinal epithelial cell, constitutes such a polar cell possessing vectoral and compartmentalized metabolic events.
The absorptive epithelial cell of the bovine intestine provides a novel cell type for study owing to differences between the calf and the adult animal in regard to exogenous substrates presented to the small intestine. Dietary lipids ingested by the adult are initially metabolized in the rumen (fore-stomach) by a variety of microorganisms, in contrast to non-ruminating animals (including the young calf) in which lipids reach the small intestine essentially unchanged. Consequently, the bovine as an experimental animal allows a comparison of a single species under different conditions of lipid assimilation, as well as a comparison of this species with other experimental subjects including man.

This investigation was undertaken to gain insight into the problem of neutral lipid absorption and transport by the bovine intestinal epithelial cell, and to attempt to identify the cellular localization of events brought about by such absorption and transport. The general fine structure of the epithelial cell, the in vivo and in vitro transport of neutral lipid through the cell, and the possible significance of cholesterol in transport will be discussed.
REVIEW OF LITERATURE

The classic treatment of Verzar and McDougall (1936), and the more recent review works of Wilson (1962), Wiseman (1964), and Code and Heidel (1968), give extensive coverage to most of the aspects of intestinal absorption, including absorption of lipids. Deuel (1955), Bergström and Borgström (1955), and Borgström (1960) have earlier reviewed the digestion and absorption of lipids, and these works provide an excellent historical background.

This literature review will attempt to present the more modern concepts of lipid absorption, particularly the absorption of glycerides of long-chain fatty acids by the small intestine. Therefore, the recent reviews of Senior (1964), Isselbacher (1965, 1966), Johnston (1968), and Strauss (1968), provide the background from which to begin.

Lipid Absorption and Transport - Biochemistry

In order to discuss both biochemical and morphological aspects of lipid absorption and transport by the small intestine, I shall consider the phenomenon in four parts: (1) physicochemical effects of bile (bile salts) and of pancreatic secretions (lipases) on the lipid substrate in the gut lumen (intraluminal phase); (2) absorption of lipid into the intestinal epithelial cell (absorptive phase); (3) intracellular metabolism and transport of the absorbed lipid (intracellular phase); (4) cellular exit and extracellular transport to the lacteals of the lymphatic system (extracellular phase).
Intraluminal phase

Pancreatic lipase Mattson et al. (1952) first demonstrated the action of pancreatic lipase on triglyceride substrates, and showed the hydrolysis products to be primarily 2-monoglycerides and free fatty acids, the latter originating from the 1 and 3 positions of the triglyceride molecule. Entressangles et al. (1966) confirmed the absolute specificity for the 1 and 3 positions, using a triglyceride prepared with tritium radioactive label in the 2 position (1,3 dipalmityl 2-[9,10-H^3]oleyl-glycerol), and a highly purified pig pancreatic lipase. These authors showed specific hydrolysis at the 1 and 3 position, with the production of tritium labeled 2-monoglyceride. Isomerization of the 2-monoglyceride occurred to the extent of 20-30 moles of 1-monoglyceride formed per 100 moles of 2-monoglyceride; further hydrolysis of either the 1- or 2-monoglyceride was quite low. Some isomerization of the intermediate 1,2-diglyceride to 1,3-diglyceride was noted.

Mattson and Volpenhein (1964) fed rats with specifically labelled tri-, di-, and monooleins, and subsequently analyzed lymph lipids. They concluded that the triglycerides were hydrolyzed to 2-monoglycerides to the extent of 72%, with 6% 1-monoglyceride and 22% free glycerol being present. Little of the free glycerol was incorporated into lymph triglycerides. Mattson and Volpenhein (1968) found a second lipase (referred to by the authors as nonspecific lipase) capable of hydrolysis at the 2 position of mono-, di-, or triglycerides. They calculated the enzyme to be 10-60 times less active than pancreatic lipase. For a more complete review of pancreatic lipase, see Desnuelle (1961) and Desnuelle and Savary (1963).
Bile salts  The participation of bile salts in the intraluminal phase of fat digestion has largely been elucidated through the study of their physical and chemical properties in vitro (see reviews by Borgström, 1962; Hofmann and Borgström, 1962; and Hofmann, 1966). Briefly, the bile salts present in the intestine above a critical concentration function as detergents to form polymolecular aggregates called micelles. These aggregates can dissolve water-insoluble molecules in their interior yielding mixed micelles. The polar lipids such as monoglycerides and fatty acids have a higher solubility in micelles than nonpolar lipids such as triglycerides, diglycerides, and cholesterol. The dimension of the bile salt micelle formed with sodium taurodeoxycholate has been shown from gel filtration studies (Borgström, 1965) and from ultracentrifugational studies (Laurent and Persson, 1965) to be 40-50 Å in diameter.

Hofmann and Borgström (1962) demonstrated the involvement of bile salt micelles in fat digestion by analyzing human intestinal contents subjected to high speed centrifugation. They found two phases, the top oily phase which contained all of the triglyceride and most of the diglyceride with some free fatty acid, and the bottom micellar phase which contained most of the monoglyceride and fatty acid, and appreciable cholesterol. The bottom phase was 20-50 times the volume of the top phase. Borgström (1967), using millipore filters to measure partition values in vitro between the micellar and oil phases for various lipids, confirmed the results of Hofmann and Borgström (1962). Di- and triglycerides were present primarily in the oil phase, monoglycerides were distributed about equally between the two phases, and fatty acids favored the micellar phase at intestinal pH's where they exist in ionic form.
To summarize, pancreatic lipolysis in a closed *in vitro* system provides a steady state equilibrium between triglycerides and 1,2-di-glycerides, and between 1,2-diglycerides and 2-monoglycerides (Borgström, 1964). In an open *in vivo* system, bile salts form micelles which in turn can solubilize the hydrolysis products, fatty acids and 2-monoglycerides, thus shifting the equilibrium conditions observed *in vitro* toward reaction completion. Saturation of the bile salt micelles with the hydrolysis products results in two phases, an emulsified oil phase and a micellar phase. It is at the interface of these two phases that pancreatic lipase functions. The oil phase, therefore, generates the micellar phase, employing pancreatic lipase as a catalyst. The formed micelles present in the micellar phase are then sequestered by the intestinal mucosal cell, and intraluminal digestion proceeds toward completion.

**Absorptive phase**

**Absorption of higher glycerides** Feldman and Borgström (1966) showed that small quantities of di- and triglycerides were absorbed *in vitro* by hamster intestinal rings. The uptake of triglycerides was 6% in the presence of bile salts, but only 3% in their absence; absorption of diolein from bile salt emulsions was 7%. The small amount of absorption of these two lipids, compared to 36% uptake of oleic acid and monoolein from a micellar preparation, was interpreted to reflect the lesser ability of di- and triolein to enter a micellar phase in the *in vitro* system.

**Absorption from the micellar phase** The concept of the micellar phase being the primary vehicle for transporting digested glycerides to the surface of the mucosa has presented the problem of how cellular entry
of these digestion products is accomplished. Whether the micelle enters the cell intact, or whether the contained lipid is released at the membrane surface has not been determined.

The absorption of monoglycerides and fatty acids from bile salt mixed micelles was first shown in vitro by Johnston and Borgström (1964) using hamster intestinal slices. Employing C\(^{14}\)-oleic acid and H\(^{3}\)-l-monoolein, these workers demonstrated penetration of label into the intestinal mucosa and, from the C\(^{14}\)/H\(^{3}\) ratios, they concluded that the monoglyceride and fatty acid were absorbed intact. Further, they were able to show that micellar solutions of the fatty acid and monoglyceride were more effective than albumin solutions or triglyceride emulsions in promoting penetration, but that intracellular reesterification to triglyceride (see below) was not dependent on the substrate physical state. Consequently, the authors concluded that penetration was not a rate limiting step. In regard to the mechanism of penetration, Johnston and Borgström (1964) studied uptake in slices incubated at 0°C, heat inactivated, or incubated at 37°C in the presence of metabolic inhibitors. These treatments had no effect on uptake of oleic acid and l-monoolein, and the authors concluded that the transport was enzymatically and energetically independent, suggesting a passive molecular diffusion. These results have been more recently confirmed by the morphological data of Strauss (1966, 1968), and will be discussed later.

Gordon and Kern (1968) have provided new insight into the role of bile salts in micellar absorption. In looking at the in vitro uptake of labeled oleic acid and sodium taurodeoxycholate by hamster intestinal rings, they found a 1:1 relationship in the absorption of these two
substrates in both jejunal and ileal sections, independent of incubation time. Seventy-five percent of the oleic acid absorbed by jejunal tissue was esterified to higher glycerides, leaving an intracellular ratio of bile salt to free fatty acid at 4:1. This ratio was the same as that in the initial incubation medium, and in ileal tissue, although the latter esterified only 30% of the absorbed oleic acid. After experimentally eliminating the parameters of adsorption and membrane disruption as possible explanations for bile salt transport, the authors concluded that during fat absorption, bile salt mixed micelles diffuse across the cell membrane, and the bile salts themselves provide a shuttle mechanism for transport from the oil-micellar interface to the site of intracellular reesterification.

In addition to the above study, Dawson and Isselbacher (1960) had previously shown that conjugated bile salts stimulate the incorporation of C\(^{14}\)-palmitic acid into higher glycerides. Also, Knoebel and Ryan (1963) had shown that in biliary fistulated dogs there was an impaired intracellular esterification of oleic acid, while absorption of the acid was similar to controls. Kern and Borgström (1965a), also using biliary fistulated animals, obtained evidence that triglyceride formation was enhanced in the presence of bile salts, independent of their function in fatty acid absorption. All of these studies, consequently, supported an intracellular function for bile salts in addition to their participation in micellar formation. However, the precise mechanism for cellular entry of the bile salt and lipid as a mixed micelle or as molecular entities remains to be demonstrated.
**Intracellular phase**

**Triglyceride synthesis** That the intestinal mucosal cell can synthesize higher glycerides from free fatty acids and/or monoglycerides is well known, and involves two pathways (Senior, 1964; Johnston, 1968). The first, the monoglyceride pathway, involves the direct acylation of 2-monoglyceride by fatty acids activated to their CoA (coenzyme A) derivatives; 1,2-diglycerides are intermediate. Utilization of 1-monoglycerides has also been shown, with a 1,3-diglyceride intermediate. [In contrast to hamsters, rats are unable to convert 1,3-diglycerides to triglycerides (Johnston *et al.*, 1965; Gallo *et al.*, 1968).] The second, the α-glycerophosphate pathway, involves the diacylation of L-α-glycerophosphate by fatty acyl CoA derivatives forming a phosphatidic acid, hydrolysis of the phosphatidic acid to a diglyceride, and further acylation of the diglyceride to triglyceride. The source of L-α-glycerophosphate as the triglyceride backbone has been shown to be glucose from glycolysis, free exogenous glycerol from intraluminal lipolysis, or glycerol from monoglycerides following hydrolysis by the intracellular enzyme monoglyceride lipase (Senior, 1964).

Kern and Borgström (1965b) evaluated the quantitative participation of the two pathways in triglyceride biosynthesis and showed that in the hamster, 80-100% of the higher glycerides were synthesized through the monoglyceride pathway when monoglyceride was available. In the absence of monoglyceride, the α-glycerophosphate pathway produced a similar amount of triglyceride. Paris and Clement (1968) reported triglyceride biosynthesis in the rat to the extent of 85% via the monoglyceride pathway, while Kayden *et al.* (1967) confirmed the utilization of 2-mono-
glyceride as the major pathway in man.

Brindley and Hübscher (1965), using subcellular fractionation procedures on cat intestinal mucosa, found that enzymes of both the \( \alpha \)-glycerophosphate and monoglyceride pathways were associated mainly with the microsomal fraction. This supported earlier findings of microsomal localization of enzymes of the monoglyceride pathway (Senior and Isselbacher, 1962; Brown and Johnston, 1964). Subfractionation of the microsomal fraction by Brindley and Hübscher (1965) resulted in localization of the enzymes of the two pathways (as well as the fatty acid activating enzyme, acid:CoA ligase) primarily in the rough-surfaced vesicle fraction. However, the activity present in the smooth-surfaced fractions could not be explained by contamination alone. Phospholipid biosynthesis in the rough-surfaced fraction had been previously suggested (Gurr et al., 1965), and led Brindley and Hübscher (1965) to speculate that chylomicrons were formed (see below) in the RER, since triglyceride, phospholipid, and protein were available.

The particle free supernatant from the fractionation procedures by Brindley and Hübscher (1965) stimulated glyceride biosynthesis via the \( \alpha \)-glycerophosphate pathway, but not the monoglyceride pathway. Hübscher et al. (1967) and Johnston et al. (1967a) demonstrated that the stimulatory factor was the enzyme L-\( \alpha \)-phosphatidate phosphohydrolase, which hydrolyzes phosphatidic acid to diglyceride in the \( \alpha \)-glycerophosphate scheme. Hübscher et al. (1967) also showed that unsaturated, long-chain fatty acids were involved in stimulation, but the authors offered no proposal for a mechanism.

Johnston et al. (1967b) obtained evidence for the independence of
the two glyceride biosynthetic pathways. When isolated microsomal-bound C\(^{14}\)-diglycerides, prepared via the \(\alpha\)-glycerophosphate route, were incubated along with \(2-{H}^{3}\)-monopalmitin in the presence of palmityl CoA and the necessary cofactors, the authors found that the amounts of triglycerides synthesized from each was similar to the amounts synthesized by either substrate alone. Consequently, the diglyceride intermediates formed did not equilibrate, supporting the conclusion that separate pathways were involved. Johnston (1968) has presented evidence that a "triglyceride synthetase" complex of enzymes exists for the monoglyceride pathway, indicating a single cellular site for this route. The work of Brindley and Hübscher (1965) and Hübscher et al. (1967) also demonstrated that enzymes of the \(\alpha\)-glycerophosphate pathway were associated with a single site (the microsomal fraction); but, there was a requirement for the particle free supernatant (cytoplasmic ground substance) for synthesis of triglyceride.

Intracellular transport of triglyceride Newly synthesized triglycerides are transported to the lymph as very low density lipoproteins called chylomicrons. Chylomicrons originate within the epithelial cell (Zilversmit, 1968; Salpeter and Zilversmit, 1968), but the intracellular site of formation remains to be precisely determined. Several authors have speculated that chylomicron formation is associated with the endoplasmic reticulum system in the apical region of the mucosal cell, based on biochemical evidence for synthesis of triglyceride, phospholipid, cholesterol, and protein by this system (Senior, 1964; Brindley and Hübscher, 1965; Cardell et al., 1967; Johnston, 1968; Strauss, 1968).
Zilversmit (1967) has reviewed the chemical and physical properties of chylomicrons, and has defined them to be "primarily those lipid particles found in excessive amounts in the lacteals during the process of fat absorption and possessing a flotation coefficient greater than 400 (Sf>400)", and "particles in plasma which can be traced to their origin in chyle". The chemical composition of chylomicrons varies with size, approximating 88-96% triglyceride, 3-10% phospholipid, 1-2% cholesterol and cholesterol ester, and 1-2% protein. Median particle size is 200-300 μm, with an overall range of 70-500 μm. Structurally, the chylomicron consists of an envelope of phospholipid plus small amounts of protein and cholesterol, and a core of triglyceride, cholesterol, and cholesterol ester (Zilversmit, 1967, 1968). For a complete discussion of the metabolism of lipoproteins including chylomicrons, see Fredrickson et al. (1967).

While intestinal synthesis of triglyceride, phospholipid, and cholesterol has been demonstrated, synthesis of chylomicron protein has not been well defined. [Both synthesis (Wilson and Reinke, 1968) and absorption (Treadwell and Vahouny, 1968) of cholesterol by the intestine are known. Sjöstrand and Borgström (1967) showed that exogenous cholesterol was incorporated into the SER of intestinal cells at the same time free fatty acids were incorporated and resynthesized to triglycerides at this site. Biosynthesis of cholesterol involving the microsomal fraction was suggested by the finding of similar results for other cell types (see Cardell et al., 1967)]. Isselbacher and Budz (1963) initially showed that the intestinal mucosa incorporated C¹⁴-leucine into various protein classes as well as into chylomicrons and other lipoproteins.
Hatch et al. (1966) confirmed these findings, and further isolated the protein moiety from the class of lipoproteins having the lowest density. Similarities in peptide maps among the isolated labeled protein, soluble residual protein, and lymph chylomicron protein, led Hatch et al. (1966) to speculate that intracellular lipids become associated, during transport, with some of the predominant soluble proteins, forming chylomicrons.

Contrary to Hatch et al. (1966), the protein moiety of \( \beta \)-lipoproteins has been demonstrated to be required for chylomicron formation. The rare metabolic disorder, abetalipoproteinemia, in which humans are unable to produce \( \beta \)-lipoprotein, was characterized by inability to transport triglycerides from both the intestine and liver (Isselbacher, 1965; Levy et al., 1966). In this regard, Isselbacher and Budz (1963), and Sabesin and Isselbacher (1965) tested the effect of known protein synthesis inhibitors (puromycin, acetoxycycloheximide, and ethionine) on rats in vivo. Results were similar to those of the abetalipoproteinemia disorder in that there occurred an accumulation of triglycerides in intestinal cells, and a decrease in production of \( \beta \)-lipoprotein and in incorporation of \( {}^{14} \)C-leucine into protein.

Windmueller and Levy (1968) observed the production of \( \beta \)-lipoprotein by the intestine following inhibition of liver secretion of \( \beta \)-lipoprotein by orotic acid. Triglyceride secretion by the liver was impaired, but triglyceride transport from the intestine into the lymph was not inhibited. The authors concluded that production of \( \beta \)-lipoprotein was necessary for triglyceride transport from both liver and intestine.
Extracellular phase

Biochemical processes occurring during the transport of newly synthesized triglyceride (as chylomicrons) to the lacteals are not well defined. However, Zilversmit (1967) has suggested that the chemical composition of chylomicrons is altered through exchanges with proteins and lipids present in extracellular fluid compartments. The route of transport from the mucosal cell to the lacteals has been demonstrated through light and electron microscopic studies, and will be covered in the next section.

Summary

The metabolic events during triglyceride digestion, absorption, and transport appear to be as follows: (1) in the intestinal lumen, triglycerides are hydrolyzed by pancreatic lipase to 2-monoglycerides and free fatty acids, and these, in the presence of conjugated bile salts, form small polymolecular aggregates called micelles; (2) the hydrolysis products, either via the micelle or as molecular entities, are taken up by the intestinal mucosal cell by a process of passive diffusion; (3) the absorbed 2-monoglycerides and fatty acids are resynthesized to triglycerides either by the α-glycerophosphate pathway or the monoglyceride pathway, the site of resynthesis involving elements of both smooth and rough endoplasmic reticulum; (4) the resynthesized triglycerides along with phospholipid, cholesterol, cholesterol ester, and protein are assembled into low density lipoproteins called chylomicrons, and are transported as such out of the cell and to the lymphatic system. It should be noted that intracellularly, 2-monoglycerides and fatty acids
are available for other metabolic events such as cholesterol esterification and phospholipid synthesis in the case of free fatty acids, and further hydrolysis and integration of the hydrolysis products into various metabolic pathways in the case of 2-monoglycerides.

**Lipid Absorption and Transport - Electron Microscopy**

Electron microscopic studies as they relate to biochemical events concerning fat absorption will be discussed in the following paragraphs, while coverage of general intestinal fine structure will be left for the Results and Discussion sections of this dissertation. For a review of the ultrastructure of the intestinal epithelial cell under conditions of fasting, see Trier and Rubin (1965) and Trier (1968); for a review of this cell type during lipid absorption, see Cardell *et al.* (1967) and Strauss (1968).

The classic electron microscope study of the intestinal mucosal cell by Palay and Karlin (1959a, 1959b) provided the directional sequence of events for fat transport. Their results led to the concept of absorption of small droplets of fat (50-240 μm) by the mechanism of pinocytosis, transport of these membrane-enclosed droplets via the endoplasmic reticulum to the Golgi apparatus, and movement laterally from the Golgi region to the lateral plasma membrane where the droplets enter the intercellular spaces, possibly by reverse pinocytosis. The general picture of transport events within the cellular organelles as proposed by these authors has not been altered (Strauss, 1968), but the mechanism of absorption has been extensively questioned (Cardell *et al.*, 1967; Strauss, 1968).
Absorptive phase

Biochemical evidence for the absorption of digested triglycerides supports the concept of free diffusion across the cell membrane from the micellar phase. Consequently, the picture of pinocytosis of triglyceride as proposed by Palay and Karlin (1959a, 1959b) seems unlikely as a major mechanism for lipid absorption.

Cardell et al. (1967) presented additional and perhaps the best evidence against pinocytosis of lipid. Using an electron-opaque marker (metallic silver) which was suspended in corn oil and yielded particle sizes in the range of 70-100 Å, they were unable to detect the marker in either apical vesicles (present in the terminal web region just below the microvilli), lysosomes, smooth endoplasmic reticulum (SER), or Golgi vacuoles, after intestinal intubation. Further, injection of colloidal ferritin, mixed with intestinal contents of a donor fat-fed rat, into the intestine of a fasted rat resulted in uptake of ferritin by the apical pits at the base of the microvilli. However, no ferritin was observed in the SER containing fat droplets, indicating that the apical pits and vesicles did not discharge their contents into the SER system. Such a discharge would have been expected if pinocytosis of lipid was functioning.

Ultrastructurally, Cardell et al. (1967) found no difference in the microvilli and the terminal web region in absorptive cells from fasted and fat-fed rats. The incidence of pinocytotic vesicles containing morphologically detectable lipid was extremely rare even during the most rapid phases of fat absorption.

Ashworth and Lawrence (1966) observed osmium fixed micelles, prepared in vitro, to be particles 30-200 Å in diameter. They observed similar
particles in the intraluminal contents of fat-fed rats, and demonstrated (in thin sections) particles between the microvilli and adherent to the plasma membrane and its filamentous coat. These authors were unable to find particles in the matrix of the microvilli or terminal web, and consequently ruled out movement of the intact micelle across the membrane, or the pinocytotic uptake of the micelle. They concluded that there must be either an incorporation of the micelle into the plasma membrane and subsequent movement of the lipid through the intracellular matrix, or an intraluminal breakdown of the micelle followed by passive diffusion of lipid across the membrane.

Strauss (1966), using hamster intestine, studied absorption in vitro from micellar solutions at various temperatures. The morphological picture at 37°C paralleled that observed by other workers during fat absorption in vivo (Palay and Karlin, 1959b; Ladman et al., 1963; Strauss, 1963). However, at 0°C, no morphologically detectable lipid was present, either as droplets or small particles, until the tissue was post-incubated in a lipid-free medium at 37°C. Consequently, it was concluded that the absorptive phase was separate from intracellular triglyceride resynthesis, and that absorption occurred via diffusion of the micelles or their molecular entities. Strauss and Ito (1965) showed that following incubations at 0°C, lipid was present intracellularly. Using a C^{14}-labeled fatty acid and autoradiography, these authors demonstrated label to be present diffusely throughout the cytoplasm. Recently, Strauss (1968) presented data showing that 95% of the activity in tissue at 0°C was attributed to free fatty acid, while after a 37°C post-incubation, 45% of the activity was triglyceride and only 40% was fatty acid. This data
supported the previous conclusion that lipid absorption occurred by passive diffusion.

Dermer (1967a, 1967b, 1967c) reported high resolution studies of the rat microvillus membrane during the fasting and fat-absorbing states. During fat absorption, both in vivo, and in vitro in the presence of micellar solutions, a change in the unit membrane from an asymmetric, trilaminar structure (fasted state) to a symmetric, trilaminar structure was observed. The layer adjacent to the intestinal lumen (peripheral layer) appeared more dense and thicker in fat-fed conditions, while the cytoplasmic layer remained the same; the intermediate layer became slightly more electron dense. The total thickness of the unit membrane approximated 95 Å in both cases. Following micellar incubations at 0°C, the peripheral layer was found to be even more dense and thicker than in fat-fed states in vivo or in vitro at 37°C, while the intermediate layer showed no density change. Also, globular fat was not observed in the subjacent cytoplasm as it was in the previous cases. It was concluded that ultrastructural changes in the plasma membrane represented uniform molecular incorporations of the fatty acids and monoglycerides present in the micelle, and that these incorporations constituted the first cellular step in fat absorption. At 0°C, the conclusion was that membrane incorporation prevailed, but that further transport was blocked. This latter conclusion is contrary to Strauss and Ito (1965) and Strauss (1966), who showed that molecular transport at 0°C was not blocked.
Intracellular phase

Jersild (1966a) studied the intracellular transport of fat in rats following injection of $\text{H}^3$-oleic acid (mixed with fatty-chyme) into ligated segments of the jejunum, and analysis by electron microscope autoradiography. Within one minute following injection, 40-70 μm droplets appeared within the SER, and diffusely within the Golgi apparatus. At 2-5 minutes, the SER droplets became larger (60-130 μm), and were more abundant in the Golgi. At 5 minutes, labeled intercellular droplets were quite apparent, with occasional labeling observed as early as 2 minutes. At the 8-30 minute period, SER droplets were larger (120-300 μm) and had begun to aggregate, while droplet size reduction was apparent in the Golgi; labeling was quite low infranuclearly. The short times needed for droplet formation and transport suggested to the author that a high degree of organization for controlling sequential reactions within the cell was present. Furthermore, droplet coalescence in the apical SER, and reduction of droplet size in the Golgi, led to the conclusion that the Golgi functions as a rate-limiting organelle in fat transport.

Jersild (1966b, 1968) showed the incorporation of labeled glucose and glycerol into triglyceride using electron microscope autoradiography. Grain location and analysis of the labeled products following lipid extraction demonstrated the synthesis of triglyceride by the apical SER, such triglyceride being morphologically as well as autoradiographically detectable.

The experiments of Strauss (1966), in which intestinal tissue was incubated in micellar solutions at various temperatures, demonstrated that triglyceride resynthesis (but not lipid absorption) was energy
dependent, and confirmed the biochemical data of Johnston and Borgström (1964). Triglyceride synthesis at 0°C was inhibited, while at 10°C some synthesis occurred as was shown by very small droplets within the SER. At 10°C, no lipid appeared within Golgi vacuoles, even after long incubations (60 minutes). At 37°C, triglyceride synthesis proceeded normally, and SER droplets were morphologically similar to droplets in the extracellular spaces (chylomicrons). Strauss (1966) concluded that at 37°C, lipid droplets within the SER were chylomicrons, and that the absence of lipid in the Golgi at 10°C could be explained by a block in chylomicron formation in the SER. Dobbins (1966) also observed the absence of lipid droplets in the Golgi apparatus of cells from humans having abetalipoproteinemia. The characteristic failure to synthesize \( \beta \)-lipoprotein, resulting in the inability to form chylomicrons and transport lipid out of the intestinal cell, led the author to conclude that the block resided either in the SER membranes, or in the Golgi apparatus.

Strauss (1966) also observed large lipid droplets in the cytoplasmic matrix at 10°C. These appeared at very short incubation times, and were not enclosed within the SER membranes, but were external to them. These observations suggested that the cytoplasmic ground substance played an important role in initial triglyceride synthesis, possibly at the outer SER membrane surface.

Cardell et al. (1967) emphasized the importance of the SER, and pointed out its function in sequestration of the fatty acid and monoglyceride substrates to form triglycerides, setting up a continued inward diffusion gradient. Observations on the relative abundance of SER and RER during the fasted and fat-absorbing states showed a shift in favor of SER
during fat absorption, probably due to ribosome loss from RER. Consequently, instead of the triglycerides moving through the RER to pick up the protein coat for chylomicron formation, the authors suggested that the necessary enzymes and other proteins are provided by the RER, and that these in turn await the systematic events occurring in triglyceride synthesis and chylomicron assembly in the SER. In regard to the Golgi apparatus, Cardell et al. (1967) offered no new information, other than the observation that droplets from the SER coalesced to form larger droplets in this region. The possibility of lipid storage in the Golgi during periods of fasting was mentioned.

**Extracellular phase**

The final phase of intracellular lipid transport, the lateral exit of resynthesized triglyceride assembled into chylomicrons, is thought to occur by reverse pinocytosis (Senior, 1964; Cardell et al., 1967). No specific work has been done on this phase of transport, and no other mechanisms have been offered.

Palay and Karlin (1959b) showed that following cellular exit, newly synthesized lipid was moved through the inter-epithelial cell space, across the basement membrane and lamina propria matrix, and into the lacteals. Lipid was observed to enter the lacteals through fenestrations in the endothelium, or across the endothelial cytoplasm (Casley-Smith, 1962).

**Summary**

It has been shown that electron microscope observations have confirmed and extended the biochemical work presented in the previous section. Absorption of lipid from the micellar phase, as well as the involvement of
the endoplasmic reticulum in triglyceride resynthesis and transport, have been demonstrated ultrastructurally.

It has not been precisely determined whether absorption of fatty acids and monoglycerides from the micellar phase takes place in the form of intact micelles or individual molecules. Gordon and Kern (1968) presented biochemical evidence for the absorption of intact micelles, while Ashworth and Lawrence (1966), Dermer (1967a, 1967b, 1967c), and Strauss (1968) provided ultrastructural evidence for absorption of the molecular entities. However, none of these latter four authors rules out absorption of intact micelles.

Pinocytosis has been excluded as a major mechanism for lipid absorption (Cardell et al., 1967). Feldman and Borgström (1966) presented biochemical evidence for uptake of small quantities of di- and triglycerides, but showed that the amount absorbed paralleled the amount of di- and triglyceride present in the micellar phase. It was concluded that absorption of higher glycerides was from the micellar phase.

Synthesis of triglyceride by elements of the SER has been demonstrated ultrastructurally, and by autoradiography of labeled substrates. Chylomicron formation has been suggested to take place in the apical SER, based on both biochemical and morphological evidence (Strauss, 1966; Cardell et al., 1967). The importance of the Golgi as rate-limiting in intracellular transport has been suggested (Jersild, 1966a, Dobbins, 1966).

Lipid Absorption and Transport - Ruminants

Very little information has accumulated concerning the problem of lipid transport by the ruminant intestine, and no papers have reported
on intestinal fine structure. Garton (1965, 1967) has reviewed digestion and absorption of lipids in ruminant animals, and a brief survey of these works is presented in the following paragraphs.

The feature distinguishing animals classed as ruminants from other herbiverous animals is the presentation of ingested feedstuffs to fermentation in the rumen during gastric digestion. With reference to the lipid fraction of the diet, the domesticated ruminant receives a variety of fatty acids ingested as glycerides, predominately triglycerides and the mono- and digalactosyl derivatives of 1,2-diglycerides. Before these glycerides can be acted upon by intestinal enzymes and secretions, they are first subjected to action by a host of microorganisms present in the rumen.

**Metabolism in the rumen**

The rumen microorganisms, bacteria and protozoa, provide the exogenous metabolic processes of hydrolysis and hydrogenation to lipids before they move to the small intestine. They also add a small amount of their own structural lipids (branched-chain and odd carbon-numbered, straight-chain fatty acids), which are found in the digesta and subsequently in the depot fats of ruminants.

The principal hydrolysis products of dietary lipids in the rumen were shown to be free fatty acids, glycerol, and galactose (from galacto-glycerides). *In vitro* studies by Garton *et al.* (1958, 1959, 1961) provided evidence for the complete lipolysis of triglycerides to free fatty acids and glycerol, and Bailey (1962, 1964) similarly demonstrated hydrolysis of galactoglycerides to fatty acids, galactose, and glycerol. Both galactose
and glycerol are subsequently metabolized in the rumen to volatile fatty acids.

Bath and Hill (1967) confirmed that complete hydrolysis of exogenous glyceridic lipids was effected in the rumen. Using rumen-fistulated animals, they were unable to detect mono- and diglycerides following a fat diet. Hawke and Robertson (1964) had previously found hydrolysis intermediates (mono- and diglycerides) in the rumen after infusion of linseed oil. However, these results were explained in terms of overloading the rumen with triglyceride (Bath and Hill, 1967).

Concurrent with hydrolysis of dietary lipids by rumen microorganisms is the hydrogenation of unsaturated fatty acids, both free and esterified, to produce the corresponding saturated acids, and positional and geometrical (trans) isomers of unsaturated acids. Reiser (1951), Willey et al. (1952), and Reiser and Reddy (1956) provided direct evidence for hydrogenation from in vitro incubations of polyunsaturated oil with sheep rumen contents, and indirect evidence from the observation of a high degree of saturation in the depot fats of animals given diets rich in polyunsaturates. Since then, several workers have confirmed these observations (see review by Garton, 1967).

Ward et al. (1964) studied the hydrogenation of C14-labeled fatty acids (linolenic, linoleic, and oleic) using an in vitro technique simulating physiological conditions. All three acids were converted to stearic acid, with the intermediary formation of a significant amount of trans C18 monounsaturated acid from linolenic acid (the principal fatty acid of leaf lipids), and linoleic acid (the principal fatty acid of seed lipids). Oleic acid yielded a high percentage of stearic acid, with
significant amounts of trans C_{18} monounsaturated acid, and unchanged oleic acid. These in vitro results have been confirmed in vivo from the finding of similar lipids in depot fat of ruminants (Garton, 1967).

In summary, the hydrolytic and reductive events in the rumen provide a digesta containing predominately free fatty acids consisting of stearic acid and C_{18} monounsaturated acids as the major fraction, and of structural lipids of the rumen microorganisms. In addition, a significant amount of the 16-carbon saturated acid, palmitic acid, is present from the diet (Garton, 1967).

Metabolism in the small intestine

During passage of digesta from the rumen through the abomasum (true stomach) and into the upper part of the small intestine, little change is noted in the proportions of fatty acids present. The bacterial and protozoan lipids are subjected to digestion in the abomasum, but contribute only a small fraction of the lipid other than their addition of palmitic and C_{18} monounsaturated acids. Consequently, lipid of dietary origin reaches the upper small intestine largely as free fatty acids, with some contribution of esterified fatty acids and other neutral lipids from microorganisms, and of phospholipids from bile (Felinski et al., 1964; Lennox et al., 1968). Leat and Harrison (1967) showed that no mono-glycerides were present in the upper intestine of sheep following a fat diet.

Lennox et al. (1968) analyzed lipid fractions from the digesta and bile of slaughtered sheep, and lipid and conjugated bile acids from sheep fitted with jejunal cannulas at three different positions. In comparing
the digesta from the rumen, abomasum, and upper intestine, they found that the esterified fatty acid fraction of the upper intestine increased in proportion over that of the rumen. The increase resulted from a biliary contribution of phospholipids, mainly lecithin and lysolecithin. Concurrent with the increase in esterified fatty acids was an increase in the percentage of C₁₈ unsaturated fatty acids, both in the esterified and unesterified fractions. [This finding had previously been observed in sheep by Ward et al. (1964), Felinski et al. (1964), and Leat (1965).] Analysis of these fractions showed the increase in unsaturation resided in the phospholipid fraction, and consequently was of bile origin. Adams and Heath (1963), and Leat (1965) had shown that ruminant bile contained a large proportion of lecithin, which in turn contained a high percentage of C₁₈ mono- and diunsaturated fatty acids.

Analysis of digesta from the jejunum of cannulated sheep by Lennox et al. (1968) showed an increase in the content of conjugated bile acids with increasing distance from the bile duct, while unesterified fatty acids and neutral lipids remained unchanged. This indicated absorption of unesterified fatty acids, and hydrolysis and absorption of neutral lipids, with no absorption of bile acids. Moreover, the authors presented evidence that lecithin was hydrolyzed to lysolecithin, and that lysolecithin was absorbed as both lipids moved down the intestine. Nilsson and Borgström (1967) demonstrated that rat and hamster intestinal slices were capable of absorbing lysolecithin but not lecithin.

In their final analysis of the intestinal digesta, Lennox et al. (1968) fractionated the digesta into micellar and solid phases, and found only 7% of the free fatty acids in the micellar phase. Similar results
were obtained from the lower jejunum, even though the pH was 8.0 (compared to pH 2-3 for upper jejunum) and fatty acids existed largely in ionic form favoring micelle formation. The proportions of conjugated bile acids, lecithin, and lysolecithin in the micellar phase were 61%, 25%, and 38% respectively. The authors suggested the possibility of lysolecithin functioning in the micellar solubilization of free fatty acids similarly to the monoglycerides of non-ruminant animals, but pointed out the very small amount of these free fatty acids (7%) available to the intestine in micellar form. However, in light of the previous discussion of the state of intraluminal contents during the process of fat absorption in non-ruminant animals, it is readily conceivable that absorption from the micellar phase exists also in the ruminant, but with a slower production of micellar phase and a lower absorptive rate. Nevertheless, the formation of a micellar phase, and the partitioning of lipid and bile salts between the micellar and solid phases in ruminants are exceedingly complex.

Heath and Morris (1963) showed the absorption of triglyceride in sheep to be directly dependent upon the presence of bile, and to a much lesser extent upon pancreatic juice. A similar case was shown for lambs, although some absorption did occur compared to sheep. The authors studied the activity of pancreatic juice in vitro, demonstrating a pH optimum of 7.8, but with appreciable activity over the entire intestinal pH range. Additionally, the presence of Ca\(^{++}\) and bile increased in vitro hydrolysis. Leat (1965) and Leat and Harrison (1967) presented evidence which suggested that pancreatic lipase functioned primarily in the conversion of biliary lecithin to lysolecithin. The latter was then available to participate in micellar formation in place of monoglyceride.
Lennox and Carton (1968) placed re-entrant cannulas at two different portions of the small intestine in each of three sheep, and were able to determine the amounts of esterified and unesterified fatty acids in the digesta at various points during its passage down the intestine. From their results, they concluded that hydrolysis of esterified fatty acids and absorption of nonesterified fatty acids were complete by the time the digesta reached the ileum. Further, the absorption of C_{18} monounsaturated acid was somewhat preferred over C_{18} and C_{16} saturated acids, and other C_{18} unsaturated acids, although the saturated acids were quite efficiently absorbed.

Studies by Ogilvie et al. (1961) showed that introduction of linseed oil and bile directly into the duodenum of sheep over a six week period resulted in an increased percentage of C_{18} di- and triunsaturated esterified fatty acids in perinephric fat. Further, Heath et al. (1964) analyzed lymph lipids in sheep and lambs maintained on a standard diet, and subsequently subjected to maize oil added directly into the rumen or abomasum. In all cases, lymph lipids of lambs reflected the dietary proportions of the various fatty acids; even introducing maize oil into the undeveloped rumen of the lamb yielded an increase in C_{18} diunsaturated esterified fatty acids. However, ruminant intestinal lymph contained saturated and C_{18} monounsaturated fatty acids (esterified and nonesterified) to the extent of 75%, compared to 30% in the diet. Adding maize oil to the rumen did not increase the percentage of C_{18} di- and triunsaturated acids in the lymph. Addition of maize oil to the abomasum did, however, increase the percentage of C_{18} diunsaturated acids from 9% to 28%. These studies, as well as those of Lennox and Garton (1968),
indicate that the ruminant intestine exhibits the capacity to digest higher glycerides, and to absorb di- and triunsaturated fatty acids, as well as monounsaturated and saturated acids.

Recently, Leat and Cunningham (1968) have shown that sheep intestine can absorb monoglycerides intact and incorporate them directly into triglyceride. Using intestinal segments, as well as isolated loops in vivo, the authors demonstrated synthesis of triglyceride to the extent of 50% by way of the monoglyceride pathway. Similar results were obtained with lambs and rats, indicating that the adult ruminant was no different from the non-ruminant animal in the ability to incorporate monoglycerides into triglycerides. It should be noted, however, that physiologically, no monoglycerides are present in the intestinal tract of adult sheep (Leat and Harrison, 1967).

Summary

Reflecting on the above discussion of lipid absorption in the ruminant, it can be seen that very little work has been reported compared to that involving the simple-stomached animals. The types of lipids presented to the upper intestine of the adult, primarily the free and esterified fatty acids of the C16 and C18 saturated group and the C18 monounsaturated group, have been fairly well determined. And, it has been shown that the lipid content present in lymph and depot fat of ruminant can be altered to reflect the dietary lipid composition (as is the case for non-ruminant animals including the young, milk-fed calf) if metabolism in the rumen is bypassed. But the physicochemical state of lipids in the intestinal lumen, and the absorption, intracellular metabolism, and intracellular transport of these lipids remain to be elucidated.
MATERIALS AND METHODS

Experimental animals

Preliminary experiments on lipid absorption in the bovine were carried out on two male Holstein calves, age 14 weeks and 33 weeks. These animals were each surgically fitted with a single duodenal cannula, positioned internally approximately 15 cm posterior to the entrance of the pancreatic duct. The cannula was exteriorized between the eleventh and twelfth ribs about the level of the costo-chondral junction. A period of at least two weeks was allowed for recovery before experimentation was begun.

The initial results from experiments on the first two calves were sufficient to establish the techniques of intestinal cannulation and biopsy as feasible for the in vivo study of the bovine intestine and of lipid absorption. Subsequently, two female Holstein calves were similarly cannulated and used for all further trials.

One Holstein female, 6115-2, was cannulated at age 7 weeks and was biopsied at various periods from age 9 to 40 weeks. A second calf, 7087-4, was cannulated at age 29 weeks and was biopsied until age 40 weeks. Prior to cannulation, and for the period of tissue sampling thereafter, both animals received milk (12% of body weight) twice daily, and were maintained on shavings.

For studies on the adult bovine intestine, a one-year-old Holstein steer, having a re-entrant duodenal cannula (Nelson, 1968), was employed.

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1Surgery was performed by Dr. A. D. McGilliard, Dept. of Animal Science, Iowa State University, Ames, Iowa.
This animal had been fed a diet containing ground corn, ground corn cobs, dehydrated alfalfa pellets, molasses, urea, dicalcium phosphate, salt, a vitamin-trace mineral premix, and an antibiotic premix. The diet was fed at a level of two times the digestible energy required for maintenance.

**Intestinal tissue**

Tissue biopsies from the upper jejunal section of the small intestine were obtained using a hydraulically operated suction biopsy tube¹ (Quinton et al., 1962; Loder et al., 1964). The tube was threaded via the cannula to a level 1-2 m distal to its opening, and the desired number of tissue sections were excised (Figure 1). Tyrodes fluid (Long, 1961) was employed to drive the hydraulically operated knife, and also served as the medium which delivered the biopsied tissue externally. The tissue samples were collected in a beaker within 10 seconds after separation from the intestine at the muscularis mucosae, and were washed by the hydraulic fluid. From the beaker, the tissue was removed by means of a stainless steel "netted" dipper, blotted gently on a paper towel to remove the small amount of collecting fluid adhering to the tissue and dipper, and placed in the desired solutions according to experimental design. The tissue pieces measured 2-3 mm in diameter (Figure 2), and following osmium fixation and complete dehydration, weighed 1-2 mg.

¹Quinton Instrument Company, Seattle, Washington.
Figure 1. The hydraulic biopsy instrument is shown inserted into the intestinal tract of a milk-fed Holstein calf. Experimental animals were surgically fitted with duodenal cannulas through which the biopsy capsule was passed.

Figure 2. An intestinal biopsy is shown (arrow) following excision at the level of the muscularis mucosae in the bovine jejunum.
In vivo absorption

For studying the in vivo absorption of fat in both calves and the adult animal, an emulsion of lard oil mixed 1:1 (w/w) with 0.154 M NaCl at room temperature was employed. The lard oil was supplied to the Iowa State University Dairy Nutrition Farm for use in milk replacer formulas, and the typical lot analysis supplied by the distributor was given as follows:

Specifications

<table>
<thead>
<tr>
<th>Specification</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free fatty acids</td>
<td>4-5%</td>
</tr>
<tr>
<td>Pour point</td>
<td>40-45°F</td>
</tr>
<tr>
<td>Saponification no.</td>
<td>194-200</td>
</tr>
<tr>
<td>Iodine no.</td>
<td>70-75</td>
</tr>
<tr>
<td>Specific gravity (60°F)</td>
<td>0.910-0.920</td>
</tr>
</tbody>
</table>

Fatty acid composition

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lauric</td>
<td>trace</td>
</tr>
<tr>
<td>Myristic</td>
<td>2%</td>
</tr>
<tr>
<td>Myristoleic</td>
<td>1%</td>
</tr>
<tr>
<td>Palmitic</td>
<td>20%</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>6%</td>
</tr>
<tr>
<td>Stearic</td>
<td>8%</td>
</tr>
<tr>
<td>Oleic</td>
<td>54%</td>
</tr>
<tr>
<td>Linoleic</td>
<td>9%</td>
</tr>
<tr>
<td>Linolenic</td>
<td>trace</td>
</tr>
</tbody>
</table>

The lard oil as supplied also contained an emulsifier, polyethylene glycol mono- and dioleate, and mixed with physiological saline as described above, provided a reasonably stable and fluid emulsion.

The lard oil emulsion was introduced into the small intestine via a separate section of 1.5 x 2.3 mm (S-1 formulation) polyvinyl chloride

\[1\] Milk Specialties, Inc., Dundee, Illinois.
tubing\textsuperscript{1} attached adjacent to the hydraulic biopsy tube and opening 5-15 cm prior to the biopsy aperture. The auxiliary tubing was attached to a gravity-flow dropping bottle, enabling a continuous infusion of fat. Initially, 100 g of the lard oil-saline emulsion was instilled into the intestine, followed by a continuous flow of approximately 200 g per 30 minutes. The use of an auxiliary tube for introduction of substrate offered the advantage of excising a control biopsy, followed by flooding the same area with lipid as it passes distally from the tube opening and past the biopsy aperture.

Tissue samples were excised at desired times, usually 15, 30, and 60 minutes following the introduction of fat into the intestine. Control samples were taken following a 36-hour fast in the case of the milk-fed calves, and 72 hours in the case of the adult ruminant. Water was given ad libitum during fasting.

\textit{In vitro} absorption

Studies on the \textit{in vitro} uptake of lipid were carried out employing a micellar solution of oleic acid (0.6 \textmu moles/ml), monoolein (0.3 \textmu moles/ml), and sodium taurodeoxycholate (2.4 \textmu moles/ml). To a 50 ml erlynmeyer flask were added 30 \textmu moles oleic acid\textsuperscript{2} and 15 \textmu moles monoolein\textsuperscript{2} both as benzene solutions. The mixture was warmed to 40°C and evaporated under a stream of nitrogen. To the residue was then added a solution of sodium

\textsuperscript{1}\textit{Surpreant Manufacturing Company, Clinton, Massachusetts.}

\textsuperscript{2}\textit{Proctor and Gamble Company, Cincinnati, Ohio.}
taurodeoxycholate\textsuperscript{1} in modified Krebs-Ringer phosphate buffer (Umbreit et al., 1964) (NaCl 7.26 g, KCl 9.37 g, Na\textsubscript{2}HPO\textsubscript{4}·7H\textsubscript{2}O 4.32 g, glucose 2 g, distilled water to 1000 ml, pH 6.3). The final micellar preparation was allowed to stir for 5 minutes or until completely clear. It was then stored overnight at room temperature, and used the following day.

Incubations were carried out in flat-bottomed test tubes (127 × 16 mm) equipped with Morton stainless steel culture tube closures.\textsuperscript{2} The closures were fitted with 16-gauge needles cut off to reach within 1-2 mm of the bottom of the test tube, and were hooked up to a manifold supplied with a gas mixture of O\textsubscript{2} and CO\textsubscript{2} (95:5). All incubation solutions were gassed 30-45 minutes at 37°C prior to introduction of the tissue. Incubations were carried out at 37°C in a Dubnoff incubator-shaker.

A comparison of uptake from an emulsion as opposed to a micellar solution was effected using a mixture of lard oil and modified Krebs-Ringer phosphate buffer. From the information on the lard oil supplied by the distributor, the concentration of free fatty acid was calculated, and the total amount of lard oil added to the buffer was equal with respect to the fatty acid content in the micellar preparation (0.6 μmoles/ml). Consequently, the total amount of lipid available in the emulsion was greater than that in the micellar solution, but the free fatty acid concentrations were approximately equal.

Biopsies were collected from calf 6115-2, age 6 months (fasted 36

\textsuperscript{1}Calbiochem, Los Angeles, California.

\textsuperscript{2}Bellco Glass Inc., Vineland, New Jersey.
taurodeoxycholate\(^1\) in modified Krebs-Ringer phosphate buffer (Umbreit et al., 1964) (NaCl 7.26 g, KCl 9.37 g, Na\(_2\)HPO\(_4\)·7H\(_2\)O 4.32 g, glucose 2 g, distilled water to 1000 ml, pH 6.3). The final micellar preparation was allowed to stir for 5 minutes or until completely clear. It was then stored overnight at room temperature, and used the following day.

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\(^1\)Calbiochem, Los Angeles, California.

\(^2\)Bellco Glass Inc., Vineland, New Jersey.
hours), and placed in 2 ml volumes of the micellar solution and lard oil emulsion. Incubations were carried out for 30 minutes at 37°C.

Results from the comparison of the micellar solution and lard oil emulsion were integrated into a time study performed on calf 6115-2, age 8 months. Two biopsies were each placed in a 2 ml volume of micellar solution, and allowed to incubate for 2 and 5 minutes without returning to the incubator-shaker. Careful agitation by hand was employed at frequent intervals, and exact times were adhered to. At the end of the incubation time the tissue was removed from the medium by means of the "netted" dipper, blotted, and placed directly into fixative. Two other biopsies were incubated in the micellar solution for 15 and 45 minutes respectively. These tissue pieces were returned to the incubator-shaker and gas manifold, allowed to agitate for the specified times, and placed in fixative. A fifth biopsy was allowed to incubate in the micellar solution for 15 minutes, but was followed by a second incubation at 37°C in lipid-free, modified Krebs-Ringer phosphate buffer. Control samples in all cases were taken from the fasted animal and placed directly into fixative.

A further experiment was carried out to determine whether tissue from the adult animal could absorb and metabolize lipid from a micellar solution. Experimental conditions were identical to those described above for the calf. Incubation of a biopsy from the one-year-old steer was performed for 30 minutes at 37°C.
Electron microscopy

Routine procedures Intestinal biopsies, obtained directly from the animal or following incubations, were immediately transferred into a fixation medium of freshly prepared 3.5% glutaraldehyde\(^1\) buffered to pH 7.3 with 0.1 M phosphate. A portion of the glutaraldehyde stock solution was filtered over 1-2 g of activated charcoal\(^2\) prior to addition of the buffer. Fixation in glutaraldehyde was carried out for 2-5 hours at room temperature, with one solution change after the first half-hour. During this time period the tissue was cut under a dissecting microscope into smaller pieces suitable for electron microscopy.

Following glutaraldehyde fixation, tissue pieces were rinsed with three changes of 10% sucrose in 0.1 M phosphate buffer, pH 7.3, over a 30-45 minute period, and post-fixed in 1% osmium tetroxide (Millonig, 1961). Post-fixation was for 2 hours at room temperature.

Tissue dehydration was effected using a graded series of ethanol: 50%, 75%, 95% for 5 minutes each; 100% for 30 minutes (two changes). Propylene oxide (two 15-minute changes) was employed as the transitional solvent, and as a vehicle for infiltration. Final embedding was carried out in a mixture of Epon-Araldite (Anderson and Ellis, 1965), which was polymerized for 60 hours at 60°C. The tissue was flat embedded to allow proper orientation.

\(^1\)Biological grade, Fisher Scientific Co., Fair Lawn, New Jersey.

In order to select the most desirable cells with respect to lipid absorption, i.e. those most highly differentiated toward the villous tip (Ladman et al., 1963), thick sections of 1.5 μ were examined under phase-contrast. Groups of cells lying just below the villus tip and having a longitudinal orientation with respect to the sectioned villus were selected, and blocks were trimmed for thin-sectioning accordingly (Figure 3). Sections having silver-to-gray interference colors were cut using a Reichert Om U2 ultramicrotome and a diamond knife, and were mounted on 200 or 300 mesh copper grids coated with parlodion and carbon. The sections were double stained with 2% aqueous uranyl acetate, and lead citrate (Reynolds, 1963).

Electron microscopy was carried out on an RCA EMU-3F electron microscope at 100 kv. Photographs were taken at original magnifications of 3,000-18,000 with Cronar \(^1\) film. Negatives were enlarged using Kodak Kodabromide F4 or F5 papers, or Agfa Grade 6.

**Cholesterol localization** The use of digitonin as a marker for free β-hydroxysterols was modified from that reported by Ökrös (1968). Tissue biopsies either from *in vivo* or *in vitro* procedures were fixed at room temperature in 3.5% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.3) for 5-6 hours. After the first 2 hours, the tissue and fixative were placed in the refrigerator at 4°C, and subsequent processing was carried out at this temperature through the second propylene oxide transition stage. Following glutaraldehyde fixation, the tissue was rinsed

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\(^1\) Cronar Ortho S Litho, 4 x 10 in, E. I. DuPont de Nemours and Co., Inc., Wilmington, Delaware.
in 10% sucrose and placed in a 1.2% solution of digitonin\(^1\) in 0.1 M phosphate buffer, pH 7.3, for 2-3 hours. The tissue was next exposed to two 1 1/2 hour changes of a 0.5% solution of digitonin in 35% ethanol, followed by a 5 minute rinse each in 35% ethanol and phosphate buffer (Millonig, 1961). Post-fixation was in 1% osmium tetroxide for 2 hours. Dehydration was completed at 4°C in a graded series of ethanol. The tissue was infiltrated with a mixture of propylene oxide and Epon-Araldite, and was embedded in Epon-Araldite. Electron microscopy was performed routinely.

**Isolated chylomicrons**

Intestinal lymph was collected from a female Holstein calf, age 10 weeks, surgically fitted with an intestinal lymph-duct cannula. The animal had been maintained on whole milk, 12% of body weight.

Chylomicrons were prepared from lymph by the method of Jones and Price (1968). Lymph was collected into chilled centrifuge tubes approximately 2 hours following feeding. Samples were centrifuged to precipitate the clot, and a 0.5 ml aliquot of the supernatant was added to 25 ml of buffered osmium tetroxide fixative (Millonig, 1961). Following a 5 hour fixation, chylomicrons were precipitated at 20,000 × g for 30 minutes in a Spinco model L ultracentrifuge, dehydrated rapidly in an ethanol series, and embedded in Epon-Araldite. Sections were examined unstained.

\(^1\)Sigma Chemical Company, St. Louis, Missouri.
OBSERVATIONS AND RESULTS

General structure

Light and electron microscopic observations on biopsies from the jejunum of either the milk-fed calf or the mature, grain-fed ruminant reveal an organizational pattern similar to that of intestinal mucosae of other mammalian systems (Cardell et al., 1967; Strauss, 1968; Trier, 1968). Numerous finger-like projections (villi) extend into the intestinal lumen, and are comprised of a single layer of columnar cells (referred to as intestinal epithelial, intestinal mucosal, or intestinal absorptive cells) that line a connective tissue matrix termed the lamina propria (Figure 3). A variety of cell types (macrophages, fibroblasts, lymphocytes, eosinophils, plasma cells), as well as blood- and lymph-vascular elements, and unmyelinated nerve and smooth muscle fibers, are found in the lamina propria.

Between and at the base of villi, the epithelial cell layer lines invaginations called the crypts of Lieberkühn. Cells characterized as undifferentiated, goblet, enterochromaffin, and Paneth are found here, and function in production of intestinal secretions and in cellular proliferation.

A smooth muscle cell layer is found below the epithelium and lamina propria, and with these two layers comprises what is called the intestinal mucosa. This muscle layer (muscularis mucosae) divides the mucosa from the submucosa. (No micrographs are presented for the crypts of Lieberkühn or the muscularis mucosae, but these regions are always observed in thick sections which are cut for tissue orientation.)
Figures 3-37. Micrographs of bovine intestinal epithelial cells fixed in 3.5% glutaraldehyde and post-fixed in 1% osmium tetroxide (both phosphate buffered). Tissue was dehydrated in a graded series of ethanol, and was embedded in an Epon-Araldite resin mixture. Thin sections were double stained in 2% aqueous uranyl acetate, and lead citrate.

All micrographs are of intestinal epithelial cells (or regions thereof) unless otherwise designated. Descriptions of the cells are given by regions: the apical region, or that portion above the nucleus and Golgi apparatus; the Golgi region; the nuclear region; and the basal region, or that part of the cell below the nucleus. Experimental procedures relating to the individual figures are described.

Figure 3. Light micrograph of a portion of a single villus, sectioned longitudinally just below the villus tip (lower left). The region within the rectangle represents that area which is routinely oriented for thin sectioning. The lamina propria (LP), and the epithelial cell layer (E) with its striated border (microvilli, M), are shown. X 1000

Figure 4. Oblique section through the intestinal epithelium at the level of the nuclei (N). Basal (BMi) and apical (AMI) mitochondria, and the Golgi apparatus (G) are demonstrated. The cells were fixed directly in 1% osmium tetroxide, phosphate buffered. X 6000
Figure 5. Low power micrograph of the apical portion of several cells from the fasted, milk-fed calf. The following characteristic features are labeled: intestinal lumen (IL); microvilli (M); terminal web (TW); junctional complex (JC); desmosome (D); endoplasmic reticulum (ER); mitochondria (Mi); Golgi apparatus (G); nuclei (N); lateral plasma membrane (LM); lysosomes (LY). X 7000
Figure 6. The region of the Golgi apparatus (G), located immediately above the level of the nucleus (N). Lipid is present intercellularly (IS), and is suggested intracellularly by an apparent Golgi packet (GP). However, this latter structure also resembles the neighboring lysosomes (LY) which contain scattered droplets. Note the desmosome (D), and the typical endoplasmic reticulum profile (ER). X 20,500

Figure 6A. Golgi cisternae during fasting, showing the association of a lipid-like structure. X 25,000
Figure 7. The region immediately below and associated with the epithelial nucleus (N) is often observed to contain an unknown structure characteristic of annulate lamellae. Observation of this complex is dependent upon the area and plane of section (see also Figure 8). X 35,500
Figure 8. Low power micrograph of several mucosal cells (from the one-year-old steer) following a 40 minute exposure to lard oil in vivo. Lipid is apparent as apical droplets (AD), Golgi packets (GP), and intercellular aggregates (IS). A few lipid droplets (LD) are observed at the level of the nucleus and in the basal part of the cells. The "annulate lamellae" structures are evident in this section (AL), as is a portion of a goblet cell (Go). The epithelial cells are separated from the lamina propria by a basement membrane (BM). X 7000
Figure 9. Apical portion of a cell from the fasted calf. This micrograph represents the control for the time study in vitro, the results of which are illustrated in Figures 9-16. Lipid droplets are not apparent in the apical or Golgi (G) regions. X 22,500
Figure 10. Apical region of a cell from tissue incubated 2 minutes in a micellar solution. Very little evidence for droplet formation (AD) is apparent at this time. X 32,500
Figure 11. Apical regions of two cells from tissue exposed to a micellar solution for 5 minutes. Numerous small droplets (AD) are evident throughout this region. Distension of the Golgi apparatus is suggested. Note the presence of numerous microtubules (arrows). X 11,5000
Figure 12. Apical regions of two cells from tissue incubated for 15 minutes in a micellar solution. Apical droplets are aggregated and measure 85-150 μm. X 15,000
Figure 13. Golgi region of a cell from tissue exposed to a micellar solution for 15 minutes. Golgi vacuoles (GV) are evident and appear to contain lipid droplets. However, the distinct Golgi packets which are observed in vivo are not present. There is no evidence for intercellular lipid. Microtubules (Mt) are indicated. X 20,000
Figure 14. Apical region of two cells from tissue incubated 45 minutes in a micellar solution. Intercellular spaces (IS) are widened, but do not contain lipid. X 25,000

Figure 15. Golgi region of two cells from tissue exposed to a micellar solution for 45 minutes. There is a faint suggestion of lipid droplets within Golgi vacuoles (GV), but the distinction is far from obvious compared to the situation in vivo. X 25,000
Figure 16. Apical region of a cell from tissue incubated 30 minutes in buffered lard oil. There is no striking appearance of lipid (as droplets) in the apical cytoplasm or Golgi apparatus. X 20,000
Figure 17. Apical region of a cell from tissue exposed an estimated 6 minutes to lard oil \textit{(in vivo)} in the milk-fed calf. A few lipid droplets (AD) are apparent. X 20,000

Figure 18. Golgi regions of two cells from tissue exposed an estimated 6 minutes to lard oil \textit{(in vivo)} in the milk-fed calf. An increase in activity is apparent compared to the control (cf. Figure 6). X 20,000
Figure 19. Microvilli of an epithelial cell from the fasted calf. The limiting unit membrane appears symmetrical, and exhibits a layer of "fuzz" on the external surface (arrows). Note the invagination (apical pit, AP) at the base of the microvilli, and the microvillus filaments (Mf). X 54,000
Figure 20. Apical portion of a cell from tissue exposed to lard oil in vivo (calf) for 60 minutes. The surface unit membranes (black arrows) of the microvilli are symmetrical and appear no different from those limiting the microvilli in the fasted state. Filaments are evident (Mf). One of the lipid droplets (white arrow) illustrates the limiting membrane and "peripheral zone" which are characteristic of apical droplets following absorption and metabolism. X 41,500

Figure 20A. Invagination (apical pit) at the base of microvilli of a cell from tissue exposed to lard oil (calf) for 60 minutes. The unit membrane of the pit is continuous with, and is of the same dimension as that of the microvilli. X 65,000
Figure 21. Apical region of a cell from tissue (calf) incubated for 15 minutes in a micellar solution. No change is noted in the microvillus membrane (arrows) from that of control cells (see also Figure 21B). Apical pits (AP) are evident. X 34,500

Figure 21A. Terminal web vesicles, resembling apical lipid droplets, in a cell from tissue (calf) incubated 30 minutes in a micellar solution. Note the presence of ribosomes (R) in this region, closely associated with a vesicle. X 63,500

Figure 21B. A single vesicle in the terminal web of a cell from tissue (calf) incubated 15 minutes in a micellar solution. The limiting membrane of the vesicle appears to be trilaminar (arrow), and of similar size to that of the nearby microvilli. X 65,600
Figure 22. Apical region of a cell (sectioned obliquely) from tissue exposed to lard oil \textit{in vivo} (calf) for 30 minutes. SER profiles are apparent in this region, and lipid droplets associated with SER are evident (arrows). X 31,500

Figure 22A. Apical region of a cell from tissue incubated for 15 minutes in a micellar solution. Lipid within SER channels, some of which exhibit scattered ribosomes (R), is demonstrated. The specific droplet indicated by the "R" is similar to that described in Figure 22B. X 58,000

Figure 22B. Another area of the cell described in Figure 22A. A portion of the membrane enclosing apical droplets is shown to be trilaminar (arrows). In one droplet (white arrow), lipid does not completely fill the membrane enclosure. This observation invites speculation that lipid synthesis is taking place at the smooth membrane surface, while the attached ribosomes oriented at the opposite side are available to contribute protein for chylomicron formation. X 87,000
Figure 23. Apical region of a cell from tissue incubated in a micellar solution for 30 minutes. Note the presence of ribosomes (R) in the terminal web and associated with apical droplets. X 36,500

Figure 23A. Lipid droplet (arrow) at the end of a RER channel, following tissue incubation in a micellar solution for 15 minutes. X 41,000

Figure 23B. Lipid within the RER (arrows) following a 15 minute exposure of the tissue to lard oil \textit{in vivo}, and a dehydration (ethanolic) between the glutaraldehyde and osmium fixation steps. X 41,000

Figure 23C. Apical droplets (arrows) apparently "coming off" RER channels, from tissue exposed to lard oil \textit{in vivo} for 60 minutes. X 41,000
Figure 24. Golgi region of a cell from tissue exposed 15 minutes to lard oil in vivo. Golgi packets (GP) are shown forming from the Golgi cisternae. Packets are bounded by a unit membrane (arrows). X 36,500

Figure 25. Golgi region of a cell from tissue exposed 30 minutes to lard oil in vivo. The definite demarcation of individual droplets is demonstrated within Golgi packets (GP). Note the single droplet apparently "coming off" a Golgi cisterna (arrow). X 25,000

Figure 25A. Golgi packets forming from Golgi cisternae (arrows) following a 30 minute exposure of the tissue to lard oil in vivo. The tissue was fixed in formaldehyde and post-fixed in osmium tetroxide. X 31,500
Figures 26-32. Intestinal epithelial cells from tissue treated with aqueous and ethanolic solutions of digitonin following glutaraldehyde fixation and prior to osmium tetroxide post-fixation.

Figure 26. Golgi region of a cell from the fasted calf. A heavy digitonin-cholesterol precipitate is associated with Golgi cisternae (GC), Golgi vesicles (GV), lateral plasma membranes (LM), and lysosomal membranes (LY). A heavy reaction is also noted at the edges of some mitochondria (Mi). X 46,000
Figure 27. Nuclear region of a cell from the fasted calf. Heavy digitonin-cholesterol precipitates are observed associated with lateral plasma membranes (LM), lysosomal membranes (LY), and some mitochondria (Mi). Note the short lamellar segments (arrows) associated with the intercellular space, which appears to contain lipid. X 46,000

Figure 27A. Apical region of the cell described in figure 27. Note the slight reaction at the mitochondrion (Mi), and at RER (arrows). X 50,500
Figure 28. Apical region of a cell from tissue (calf) exposed to lard oil for 30 minutes *in vitro*. A digitonin-cholesterol precipitate is apparent at the periphery of apical droplets (white arrows), and is suggested at the center of the majority of the droplets (black arrows). X 23,500

Figure 29. Apical region of a cell from tissue (calf) incubated in a micellar solution for 15 minutes. A digitonin-cholesterol reaction is also demonstrated at the peripheries (white arrows) and centers (black arrows) of apical droplets. X 38,500
Figure 30 and 31. Apical regions of cells from tissue (calf) incubated in a micellar solution for 15 minutes.

Figure 30. The 30 Å electron-dense lamellae at the peripheries of the apical droplets (arrows). X 69,500

Figure 31. Large apical droplet with an extensive lamellar precipitate at the periphery (white arrow). Note also the reaction associated with the smaller droplet (black arrow). X 69,500
Figure 32. Golgi region of a cell from tissue (calf) exposed to lard oil for 30 minutes \textit{in vivo}. A heavy digitonin-cholesterol reaction is observed at the lateral plasma membrane (LM). The reactions at the Golgi "vacuoles" and intercellular spaces are slight, although lamellar segments are indicated (arrows). X 32,000
Figure 33. Large Golgi packet above the nucleus (N) of a cell from tissue (calf) exposed to lard oil for 30 minutes in vivo. The packet appears to be in the process of entering the intercellular space by reverse pinocytosis. The limiting membrane of the packet has apparently fused with the lateral plasma membrane of the cell (LM1) while the lateral plasma membrane of the adjacent cell (LM2) encloses a portion of the mass. X 42,000

Figure 33A. Single droplet apparently in the process of entering the intercellular space by reverse pinocytosis. The unit membrane of the droplet is continuous with the lateral plasma unit membrane (arrows). X 55,000

Figure 33B-33D. A comparison of lipid droplet sizes.

Figure 33B. Golgi packet of a cell following a 15 minute exposure to lard oil. X 55,000

Figure 33C. Intercellular droplets under conditions similar to those of Figure 33B. X 55,000

Figure 33D. Lipid profiles (chylomicrons) isolated from the lymph of a milk-fed calf. The variation in size of the isolated particles is indicated (arrows). Generally, the size of isolated chylomicrons is slightly larger than that of inter- and intracellular droplets. X 55,000
Figure 34. Intercellular lipid droplets at the level of the nucleus (N) in cells from tissue (calf) exposed to lard oil in vivo for 15 minutes. X 30,500
Figure 35. Basal region of cells from tissue (calf) exposed to lard oil in vivo for 60 minutes. Lipid droplets in the intercellular spaces (IS) as well as in the lamina propria matrix (LP) are apparent. Note the openings (arrows) at the base of the epithelium, which are continuous with the intercellular spaces and are adjacent to the basement membrane (BM). X 20,000
Figure 36. Portion of a capillary (CP) in the lamina propria; tissue was exposed to lard oil for 60 minutes in vivo. Note the appearance of droplets, characteristic of lipid, in the capillary endothelium (arrows). Lipid is evident in the lamina propria matrix (LP). X 21,000

Figure 37. Portion of a lacteal (LAC) in the lamina propria; tissue was exposed to lard oil for 60 minutes in vivo. Lipid droplets are present in the lacteal lumen (arrows). X 21,000
The vast majority of cells found in the villus epithelium are of the absorptive type, while goblet cells are frequently present. This dissertation is concerned only with the absorptive cells located in the region just below the villus tip (Figure 3). For a discussion of the remaining cell types present in the mucosa, see Trier (1968).

**Epithelial cell fine structure - fasted state**

The intestinal epithelial cell is classed as a simple columnar cell, possessing an elongated rectangular geometry when sectioned longitudinally (perpendicular to the cell surface) (Figures 3, 5), and a polygonal geometry when sectioned transversely (parallel to the cell surface) (Figure 4). The nucleus is elliptical in shape with its long axis parallel to the long axis of the cell, and is located slightly toward the basal region of the cell from center (Figure 5).

**Microvilli** The mucosal cell surface is characterized by numerous finger-like projections termed microvilli (also brush border or striated border) measuring 1.5-2.5 μ in length, and 0.1-0.15 μ in width (Figure 5). The microvilli are bounded by a unit membrane which is continuous with the surface plasma membrane, and measures approximately 100 Å. Internally, 50 Å filaments extend from the microvillus tip (immediately below the limiting membrane) to the subjacent cytoplasm, running parallel to the microvillus long-axis (Figures 19, 20). An electron-dense coat of "fuzz" is present on the outer surface of the membrane, and has been characterized as mucopolysaccharide (Ito, 1965) (Figures 19, 20A). Its function is not yet known.
**Terminal web**  Immediately below the microvilli is a zone devoid of cellular organelles and referred to as the terminal web (Figure 5). This zone is made up generally of fibrous material, and the filaments of the microvilli extend into it (Figure 20). Occasionally, invaginations (apical pits) at the base of the microvilli, and vesicular profiles (terminal web vesicles) apparently originating from these invaginations, are observed in the terminal web (Figure 19).

**Lateral and basal plasma membrane**  The lateral and basal boundaries of the epithelial cell are of 90 Å unit membrane structure. The lateral membrane is observed to form extensive convolutions which parallel the membranes of adjacent cells (Figure 5). Lateral cell attachments are effected by the junctional complex located immediately below the cell surface as described by Farquar and Palade (1963), and by randomly located desmosomes (Figures 5, 6). The basal membrane rests on the lamina propria, and is separated from the connective tissue matrix by a continuous mucoprotein substance called the basement membrane (Figures 8, 35).

**Endoplasmic reticulum**  A well developed system of smooth (SER) and rough (RER) endoplasmic reticulum is present throughout the cell beginning just below the terminal web (Figure 5). The relative abundance of the two forms in the fasting and fat-fed states is difficult to assess, and continuity between the two is quite frequent. Long channels of RER are common, but the reticular system is best described as a network of smooth, membrane-bound channels dotted intermittently with strings of ribosomes, such strings not always appearing parallel on both sides of the channel (Figure 6).
Mitochondria  Mitochondria are located throughout the cell. In the apical region they appear randomly dispersed, while basally they appear more numerous and tightly packed (Figures 4, 5).

Golgi apparatus  The Golgi system is evident in the supra-nuclear region of the cell, with distinguishing profiles generally observable laterally as well as centrally (Figures 5, 6). Metabolic activity during the fasting state is suggested by distended Golgi vesicles, some containing morphologically identifiable lipid. Lipid present in the intercellular spaces is frequently observed, further suggesting that fasting conditions fail to completely deplete lipid absorption and transport (Figure 6). Endogenous sources, such as sloughed cells and gastrointestinal secretions, best explain the presence of lipid.

Lysosomes  Lysosomal bodies comparable to those observed in rat intestine (Cardell et al., 1967) are located primarily in the apical cytoplasm and the Golgi region (Figures 5, 6). No involvement of these organelles in fat absorption and transport is demonstrated.

Microtubules  The appearance of microtubules in sections from fasted or fat-fed animals is infrequent. However, they are well demonstrated following tissue incubations (Figures 11, 13). A difference in ionic environment (concentration) set up under in vitro conditions offers a tentative explanation for this observation. The involvement of microtubules in fat transport is questionable.

Infra-nuclear region  An organized structure of unknown function is sometimes observed at the base of the nucleus (Figures 7, 8). This structure has been observed in both milk-fed calves and the adult animal, and is characteristic of annulate lamellae (Kessel, 1968). The appearance
of these organized structures is dependent upon the plane of section as well as the precise area of section. Several cells in a single section may reveal only one or two "lamellae" (cf. Figure 8), while the remaining cells are devoid of them. Morphology does not indicate an obvious function, but location, along with the presence of numerous mitochondria, suggests some type of involvement in basal transport.

Epithelial cell fine structure - fat-absorbing state

Electron-transparent circular profiles are readily observed in sections from intestinal tissue exposed to lard oil in vivo (Figure 8), or to a micellar solution in vitro (Figure 12). Droplets measuring 85-150 μ are present singly in the apical cytoplasm of the cell (apical droplets), and as aggregates in the region of the Golgi apparatus (Golgi packets) (Figure 8). The definite prominence of aggregated droplets in the Golgi (in vivo) suggests a typical packaging function for this organelle, such packages being enclosed by Golgi-derived membranes. Aggregated droplets also appear in the intercellular spaces, suggesting that Golgi packets are moved intact to the lateral plasma membrane where they are extruded by reverse pinocytosis (see below).

The method of tissue preparation for electron microscopy results in electron transparency of the apical, Golgi, and intercellular droplets, indicating that the majority of the lipid is extracted. The exact composition of the droplets as they exist before extraction is not known, and is difficult to evaluate (by analysis of extracts) since all lipid in the absorptive cell, whether from exogenous or endogenous sources, is not morphologically detectable (Strauss, 1968). Nevertheless, the ability of
the intestine to synthesize triglyceride, and the physical properties of triglycerides in aqueous media, suggest that these droplets represent newly synthesized triglyceride.

In vitro The dynamics of absorption and metabolism of digested glycerides (glycerides of long-chain fatty acids) by the intestinal epithelial cell are best studied using in vitro incubations. Under these conditions substrate composition and time sequence can be carefully controlled.

Monoolein and oleic acid were employed as lipid substrates and were presented to the intestinal villus (of the milk-fed calf) in micellar form. In a single case, higher glycerides in emulsified form (lard oil) were incubated with tissue biopsies. The morphology of cells exposed to lipid at various times was compared to that of cells obtained from the fasted animal, as well as to that of cells exposed to lipid in vivo. (A 30 minute incubation of tissue from the adult animal was also carried out in the presence of a micellar solution. The result was the same as that for the milk-fed calf as described below.)

Controls Sections from control biopsies (from the fasted animal) show little evidence for lipid absorption and metabolism. The apical cytoplasm as well as the Golgi region is free of lipid droplets (Figure 9).

2 minutes At 2 minutes, a few isolated droplets are observed in the apical region of the cell (Figure 10). There is some indication that droplets are beginning to form, but detection is difficult against the cytoplasmic background. No lipid was apparent in sections which showed the Golgi apparatus.
5 minutes Following a 5 minute incubation, the apical cytoplasm contains numerous lipid droplets of 60-90 μm diameter (Figure 11). The Golgi region is slightly distended, but no aggregation of droplets to form Golgi packets is observed. No lipid is present intercellularly.

15 minutes Numerous 80-150 μm droplets are observed after 15 minutes (Figure 12). Apical droplets generally are aggregated at this time, although some cells do not yet exhibit such aggregation (cf. Figure 21). This difference most likely reflects the degree of differentiation (maturity) with respect to cell location on the villus, some cells being able to metabolize lipid faster than others. The Golgi apparatus contains a large number of what appear to be lipid aggregations (Golgi vesicles), but the precise droplet demarcation that is observed in vivo is not demonstrated (Figure 13). Intercellular lipid is still absent.

30 minutes The epithelial cells following a 30 minute incubation are morphologically similar to those seen after a 15 minute incubation, showing aggregated apical droplets (80-150 μm). A few droplets much larger than average are also observed (Figure 22). Golgi packets are indistinct, and intercellular lipid is still lacking.

45 minutes The appearance of the cell following a 45 minute incubation, or following an initial incubation for 15 minutes in a micellar solution and a subsequent incubation for 30 minutes in a lipid free buffer, is characterized by enlarged intercellular spaces, as well as by lipid aggregations and large single droplets (Figures 14, 15). Some packaging in the Golgi region is suggested, but is still not nearly as obvious as the in vivo situation. No lipid is demonstrated in the widened intercellular spaces (Figure 14). (This latter observation is supported by
micrographs of sections contaminated with lead precipitates. The electron-dense lead is uniformly associated with intracellular droplets, while no such density is observed intercellularly.)

**30 minutes - lard oil**   The use of a fat emulsion *in vitro* provided a substrate with a larger particle diameter (approximately 5000 Å) compared to the micelle (10-100 Å) (Johnston, 1968), and with a composition primarily of higher glycerides. Hydrolysis of the glycerides, followed by uptake by the mucosa, was not anticipated, in that pancreatic lipase and bile salts were absent *in vitro*. However, free fatty acids were present at a concentration similar to that in the micellar preparation, and uptake and metabolism of these lipids was expected.

Micrographs of the epithelial cell following a 30 minute incubation in lard oil reveal very little evidence for absorption and metabolism. In some sections, a few droplets are observed in the apical cytoplasm, while other sections exhibit no such droplets (Figure 16). Golgi vesicles are distended, but no lipid is detectable in the distentions.

**In vivo**   A time study of lipid absorption, metabolism, and transport *in vivo* is difficult to achieve. Such a study requires knowledge of the precise time that a lipid environment is present at the site of biopsy. Furthermore, maintenance of the lipid environment (saturated) over the desired time periods is necessary. The size of the substrate delivery-tube opening is relatively small compared to the size of the small intestine, making it difficult to saturate the entire intestinal surface. Similarly, the biopsy opening is quite small, increasing the error of excising tissue from an area not saturated with lipid.

Figures 17 and 18 show the apical cytoplasmic and Golgi regions,
respectively, following an estimated 6 minutes exposure to lard oil (*in vivo*). A few droplets are present in the apical cytoplasm, resembling the situation for a 2 minute incubation (*in vitro*) (Figure 10). In comparison to the control (Figure 6), there is some indication that Golgi activity has increased, although no Golgi packets are evident.

A complete morphological pattern for absorption and transport is achieved within 15 minutes following introduction of a large volume of fat into the jejunum. Apical droplets, Golgi packets, and intercellular aggregates are observed at this time, with the droplets measuring 80-150 μm. At later times (30 and 60 minutes) no structural changes are noted, with the exception of a greater amount of lipid in the intercellular spaces at the basal region of the epithelium. Lipid droplets also appear more numerous in the connective tissue matrix of the lamina propria and the lacteals at later times (60 minutes). However, extraction of lipid makes it difficult to identify droplets in these regions.

Figure 8 illustrates epithelial cells from the adult animal following a 40 minute exposure to lard oil. The lipid pattern is representative of that observed in milk-fed calves as well, and illustrates the morphology observed as early as 15 minutes following introduction of lard oil into the intestine.

During experimental procedures, the presence of lard oil in the intestine of the calf and the adult animal stimulated the secretion of bile. This stimulation was evidenced by a substantial loss of bile through the cannula, beginning shortly after the infusion of lipid. These results suggested that bile salts function in the assimilation of the lard oil, possibly through their participation in the formation of a micellar
phase for absorption (as has been shown for other mammalian systems).

Absorption and transport - absorptive phase

The bovine jejunum is capable of absorption and metabolism of lipid in vitro from micellar preparations. However, the lipid phase involved in absorption in vitro under experimental conditions used here, and in vivo under physiological conditions, is not known. The presence of bile in vivo (stimulated by lipid), as well as lipid uptake from micellar solutions but not from emulsions (lard oil) in vitro, strongly suggests that absorption in vivo is from the micellar phase.

No change in the structure of the microvillus unit membrane is observed during absorption, compared to the fasting state. Tissue biopsies fixed initially in glutaraldehyde and followed by osmium show a symmetrical unit membrane structure for the fasting state (Figure 19). (Similar results were obtained following direct osmium fixation or fixation in formaldehyde followed by osmium.) The same membrane structure is observed after glutaraldehyde and osmium fixation of biopsies exposed to lipid in vivo (Figure 20), or to micellar solutions in vitro (Figures 21, 21B).

Invaginations at the base of microvilli are frequently observed in both fasted and fed tissue (cf. Figures 19 and 20A). These "apical pits" (Cardell et al., 1967) are bounded by a unit membrane structure continuous with the plasma membrane, and are coated with "fuzz" on their external surface, similar to that on the microvilli (Figure 20A). The electron density of the "fuzz" also resembles that observed at the periphery of apical droplets. The apical pits are indicative of pino-
cytosis, and are quite prominent during the fasting state.

Circular profiles having a morphology identical with that of apical droplets are sometimes observed in the terminal web (terminal web vesicles). Figures 21A and 21B show two profiles in the terminal web during lipid absorption \textit{in vitro}. Both are enclosed by membranes (apparently trilaminar) having dimensions only slightly less than that of the microvillus membrane (approximately 90 Å compared to 95 Å). The identification of these vesicles as lipid droplets is suggested, based on morphology, and their origin via pinocytosis is possible. The presence of ribosomes adjacent to a terminal web vesicle (Figure 21A) suggests metabolic activity in this region.

**Absorption and transport - intracellular phase**

**Apical droplets**  
Apical lipid droplets are membrane enclosed, and possess an electron dense zone at the periphery ("peripheral zone"), adjacent to the membrane (Figure 20). Resolution of the external boundary as a unit membrane (trilaminar; 75 Å) is evident for only a small segment of certain droplets (Figure 22B), yet the existence of such a structure is strongly suggested on this basis. The close association of droplets with the limiting membrane, as well as the requirement for plane of section (through the droplet center), makes resolution of a trilaminar structure difficult.

Droplets appear throughout the cytoplasm above the level of the nucleus (apical region), beginning just below the terminal web (Figure 23). As shown previously, profiles closely resembling apical droplets appear in the terminal web region. Whether these are lipid droplets, either newly
synthesized in this region or "backed-up" from the apical cytoplasm, or whether these are lipid or non-lipid profiles from pinocytosis, is not known.

The limiting membrane of apical droplets is observed to be part of the endoplasmic reticulum system (Figures 22, 22A). The majority of droplets are free of ribosomes, but association with RER is quite apparent. Figures 22A and 22B illustrate two lipid profiles having attached ribosomes oriented toward one side. Lipid synthesis appears incomplete in that the "electron-transparency" does not entirely fill the membrane enclosed area. This latter observation invites speculation that synthesis of triglyceride is taking place at the smooth membrane surface, while the rough membrane surface elaborates protein for chylomicron formation (see below).

Further association of droplets with RER is shown in Figures 23-23C. Figures 23A and 23C show droplets at one end of a segment of RER, suggesting that they are in the process of completing passage through RER channels. Figure 23B is from a section of tissue which was treated with a graded series of ethanol following glutaraldehyde fixation and prior to osmium fixation. Such a procedure results in extensive extraction of lipid, including that present in membranous structures. Lipid is shown to be within RER channels, and this observation is consistent throughout the entire apical part of the cell.

Golgi region  Lipid droplets aggregate within the Golgi cisternae, forming Golgi packets (Figures 24, 25). Individuality of the packaged droplets is maintained, and is demarcated by electron dense lines having
a morphology similar to the "peripheral zone" of the apical droplet. The size of the droplets within the packages is similar to that of the apical droplets (80—150 μm). These results indicate that individual droplets enter Golgi cisternae and become aggregated or packaged, the final product being a unit membrane-enclosed structure which is "pinched off" at the lateral regions of the cisternae (Figure 24). Figure 25A illustrates a similar "pinching off" phenomenon in a cell fixed in formaldehyde followed by osmium.

Figure 25 shows an individual droplet apparently "coming off" the end of a Golgi channel. This observation is compatible with the hypothesis that intercellular droplets, singly or aggregated, pass through the Golgi apparatus before cellular exit (see below). The entrance of individual droplets into Golgi channels (followed by packet formation) is not apparent, but profiles of endoplasmic reticulum are occasionally observed oriented toward the Golgi region, suggesting a possible continuity between the two systems.

Chylomicron formation Apical and Golgi droplets can be tentatively identified as chylomicrons on the basis of their size and appearance, as has been shown for the rat (Cardell et al., 1967) and the hamster (Strauss, 1968). However, identification on the basis of precise chemical characterization within the cell has not been accomplished.

An approach to localizing chylomicron formation involves the determination of where the additional components, phospholipid, cholesterol, cholesterol ester, and protein, are added to newly synthesized triglyceride. The intracellular region in which one or more chylomicron components is shown (morphologically) to be associated with lipid droplets would be
indicative of a formation site.

Initially, it was decided to localize the protein component by means of a labelled marker (H\(^3\)-leucine) and autoradiography at the light and electron microscope level. Preliminary observations, however, showed that incorporation of label into protein was similar in both faster and fat-fed tissue, and that detection of labelled protein representing only chylomicron protein would be quite difficult. Furthermore, since autoradiographic resolution was at best 0.1 \(\mu\), specific localization of label to lipid droplets, especially their thin (<100 \(\AA\)) electron-dense envelope of which the protein is a part, would have been highly subject to error.

The identification of cholesterol as a part of chylomicrons was made attractive after digitonin was adapted as a marker for free 3-\(\beta\)-hydroxy-sterols at the electron microscope level (Ökrös, 1968). The electron microscope picture of the 1:1 reaction complex was reported as cylinders with slightly tapering ends, the cylinders being "made up of electron-dense and electron-transparent coaxial lamellae", 600-700 \(\mu\) in length, 40-100 \(\mu\) central width, and 30-80 \(\mu\) end width; the lamellar thicknesses approximated 20-30 \(\AA\). More recently, Scallen and Dietert (1968) used a fixative containing 2.5% glutaraldehyde, 2% formaldehyde, and 0.2% digitonin in 0.1 M cacodylate buffer, pH 7.2; post-fixation was in 1% oxmium tetroxide. They reported a 27-44% retention of unesterified cholesterol in liver tissue, whereas the same method devoid of digitonin retained virtually no cholesterol. Ultrastructurally, a high quality of structural detail was achieved, and the cylinders observed by Ökrös (1968) were reported to occur primarily at membrane surfaces.

The method used here for cholesterol localization is based on that of
Ökrös (1968). In the fasted calf, an extensive reaction is observed as extremely electron-dense lines and myelin figures, and is located in the Golgi region (Figure 26), lateral plasma membrane (Figures 26, 27), lysosomal membranes (Figures 26, 27), and intercellular spaces (Figure 27). Figure 27A shows evidence for a slight localization at one mitochondrion and at RER segments.

In the Golgi region (Figure 26), electron-dense lines measuring 30 Å in width follow Golgi cisternae, and in many instances form myelin-like figures or figures representing various geometrical sections of the coaxial lamellae observed by Ökrös (1968). Heavy precipitations are apparent in the vesicular regions at the ends of the cisternae.

A strong electron-density is observed at the lateral plasma membrane, indicating that cholesterol in membranes is free to complex with digitonin. However, other membrane structures such as mitochondria and SER fail to demonstrate a strong reaction. In the intercellular space (apparently containing lipid) (Figure 27), short segments of electron-dense lamellae are observed, indicating the presence of cholesterol.

Figures 28 and 29 illustrate the apical cellular regions following absorption of lipid in vivo and in vitro, respectively. Apical droplets are bounded by electron-dense lamellae (30 Å), the reaction appearing more strongly in the regions below the terminal web. Reactions are observed at the center of droplets, although in most cases they are not as strong as the reactions at the periphery. The microvillus and surface plasma membrane, as well as droplets in the terminal web, do not indicate cholesterol localization. Control tissue, treated with solutions devoid of digitonin, also does not exhibit a characteristic reaction, and is
morphologically similar to tissue fixed routinely.

The origin of cholesterol observed intracellularly was not determined. However, the in vitro techniques eliminated the presence of exogenous cholesterol (with the exception of exogenous cholesterol stored during fasting).

Figures 30 and 31 further illustrate the digitonin-cholesterol reaction at the periphery of apical droplets. The larger droplet in Figure 31 demonstrates a heavy reaction oriented toward one side. (The reaction is not always oriented in the same direction.) Some of the larger droplets within a single cell do not show a strong reaction, suggesting that the plane of section did not pass through the precipitate.

Figure 32 shows a washed out appearance of the lipid packets in the Golgi region. Droplet demarcation is not at all evident, and the appearance of these areas is similar to that of the intercellular space (cf. Figure 27). The possibility of a digitonin-cholesterol reaction is indicated, but the presence of fragments of the electron dense "peripheral zone" from lipid droplets cannot be ruled out as the source of lamellar segments.

**Absorption and transport - extracellular phase**

**Cellular exit** Lipid droplets packaged in the Golgi region are extruded into the intercellular spaces by reverse pinocytosis. Figure 33 presents evidence for this mechanism, showing the presence of a large group of droplets at the level of the upper part of the nucleus. The unit membrane encompassing the Golgi packets has apparently fused with the lateral plasma membrane, forming a continuous membrane system and
placing the droplets within the intercellular space. Figure 33A similarly shows a single droplet in the process of cellular exit.

**Intercellular transport** The appearance of lipid in the intercellular spaces resembles that in the Golgi packets, with the exception of the presence of a greater amount of electron-dense material (Figure 34). The origin and function of this dense material are unknown. Intercellular lipid initially appears near the Golgi region, and is observed at various levels toward the base of the cell. Often, single droplets appear intercellularly, consistent with the observation of single droplets within the Golgi cisternae as well as within profiles suggesting the process of reverse pinocytosis (cf. Figures 25 and 33A). Channels open at the base of the epithelium, and lipid is moved out of these channels across the basement membrane and into the lamina propria (Figure 35).

**Appearance in lacteals** From the base of the epithelium, droplets apparently move at random through the connective tissue matrix and into the lacteals (Figures 36, 37). However, detection of lipid in this region is difficult, due to extraction during specimen preparation. Figure 37 illustrates lipid in the lumen of a lacteal, with profiles similar in size to those in intercellular spaces. Figure 36 shows lipid in the connective tissue matrix below the epithelium, and demonstrates what appears to be lipid droplets in the endothelium of a capillary.

**Isolated chylomicrons** Particles similar to those isolated and identified as chylomicrons by Casley-Smith (1962) were prepared from intestinal lymph of a milk-fed calf and examined by electron microscopy (Figure 33D). Size variation ranges from 90 to 360 μm, generally being larger than the apical or Golgi droplets observed in fixed tissue.
The outer rim of the particles is electron-dense, resembling the periphery of apical and Golgi droplets, but is not of unit membrane structure. Internally, there is no structure, indicating lipid extraction.

Figure 33D compares the dimensions of chylomicrons with that of intercellular droplets (Figure 33C), and Golgi droplets (Figure 33B). The frequent appearance of larger particles in the chylomicron preparation suggests some aggregation in the lymphatics or other extracellular compartments.
DISCUSSION

The morphology of the bovine intestinal epithelial cell following absorption, metabolism, and transport of glycerides of long-chain fatty acids follows a pattern similar to that existing for other mammalian systems including man (cf. Dobbins, 1966; Rubin, 1966; Cardell et al., 1967; Strauss, 1968). The route of intracellular and extracellular transport, and the participation of the endoplasmic reticulum and Golgi apparatus in metabolism and transport are substantiated. Furthermore, intracellular localization of cholesterol adds support to the hypothesis that chylomicrons are formed by the endoplasmic reticulum system in the apical region of the cell. However, the mechanism of lipid absorption, the involvement of the RER in metabolism, and the importance of the Golgi apparatus as an organelle for transport control, remain speculative.

The majority of the experiments presented in this dissertation were performed on milk-fed calves, classed as non-ruminants. Identical results, regarding transport patterns in vivo, and in vitro from micellar solutions, were achieved with a fully ruminating animal. Therefore this discussion is most likely applicable to both animal groups.

Absorption and transport - absorptive phase

Micellar absorption  The bovine intestinal epithelial cell is capable of absorption and metabolism of lipid from a micellar solution in vitro. Whether this micellar phase is required for absorption in vivo is not yet known.

Physiological conditions existing in the calf are similar to those in other non-ruminant animals, in that triglycerides (in the milk diet)
are available to the small intestine. Furthermore, the requirement of bile salts for fat absorption in calves appears to be similar to that for other animals, especially the lamb (Heath and Morris, 1963). This latter conclusion is based on the observation of bile secretion following introduction of fat into the small intestine.

When bile and pancreatic secretions are not available to the intestinal tissue (calf), as in the case of biopsies handled in vitro, the tissue is unable to absorb and metabolize emulsified lipid (lard oil). There is morphological evidence for a small amount of metabolism under these conditions, but the level is extremely low compared to that following incubation in a micellar solution (even though the emulsion and micellar solution contain an equal concentration of free fatty acids). In opposition, Strauss (1963) demonstrated lipid intracellularly following one-hour incubations of hamster intestine with a cottonseed oil emulsion, containing lecithin and an ethylene oxide polypropylene glycol polymer as emulsifiers. Free fatty acids were not reported as a component of the emulsion, but their presence with lecithin would have been highly favorable to formation of a micellar phase. A longer incubation time and the possible existence of a micellar phase could therefore account for the positive results of Strauss (1963), in contrast to the negative results presented for the calf.

The experimental work presented for the bovine adult did not simulate physiological conditions in regard to intraluminal contents. Higher glycerides (lard oil) are normally not present in the ruminant small intestine, yet these glycerides can apparently be digested, absorbed, and metabolized as shown morphologically. Similarly, Ogilvie et al. (1961)
and Heath et al. (1964) showed that sheep were able to digest and absorb glycerides introduced into the abomasum or intestine (bypassing the rumen). These results, as well as the presence of bile during lipid absorption in the sheep (Heath and Morris, 1963) and the steer (this dissertation), suggest that glycerides are assimilated in the ruminant in a manner similar to that in calves and other non-ruminating animals, involving the micellar phase. However, the participation of the micellar phase under physiological conditions (absence of glycerides) has not been precisely shown (Lennox et al., 1968).

Assuming that the micellar phase is required for absorption in vivo, the question of whether lipid transport across the surface plasma membrane occurs via intact micelles or by passive diffusion of molecular entities from the micellar phase must be raised. Movement of intact micelles was discounted by Ashworth and Lawrence (1966), but was supported by Gordon and Kern (1968). Moreover, Dermer (1967a) presented a high resolution study which supported molecular diffusion based on the observation that the rat microvillus unit membrane changed from an asymmetric, trilaminar structure during the fasted state to a symmetric, trilaminar structure during the fat-absorbing state. The micrographs presented in this dissertation demonstrate no ultrastructural changes in the surface plasma membrane or microvillus interior following absorption in vivo or in vitro, and therefore fail to support either transport mechanism. The microvillus unit membrane is observed to be symmetric in the calf during both the fasted and fat-absorbing states.

Difficulties in ultrastructural identification of lipid transport mechanisms approaching the molecular level, as well as evidence for partial
lipid extraction during specimen preparation for electron microscopy, complicate interpretation of lipid absorption. Buschmann and Taylor (1968) reported as preliminary observations that newly absorbed lipid in the microvillus and terminal web regions was extensively extracted. Several papers have reported on lipid losses during specimen preparation, and results range from 10-25% loss of unsaturated lipid from intestinal tissue (Buschmann and Taylor, 1968; Dermer, 1968; Saunders et al., 1968), to 100% loss of neutral lipid in Acanthamoebae (Korn and Wiseman, 1966). Free cholesterol and palmitic acid were retained in intestinal tissue to the extent of only 50% (Saunders et al., 1968), while approximately 75% of the palmitic acid in liver tissue was retained (Stein and Stein, 1967a). The morphology of lipid following bovine tissue preparation exhibits extensive electron transparency, and it is concluded that significant extraction has occurred.

**Pinocytosis** The identification of invaginations at the base of microvilli, and of circular profiles located in the terminal web region, has necessitated consideration of the participation of pinocytosis in lipid absorption. The resemblance between the terminal web vesicles and the apical droplets suggests the former are lipid, but their origin is uncertain. Several points of origin are possible: (1) pinocytosis of triglyceride from the emulsified oil phase; (2) synthesis of triglyceride at the surface membrane or within the terminal web; (3) pinocytosis of triglyceride precursors concurrent with triglyceride synthesis at the surface membrane; (4) intracellular "backup" of lipid from the endoplasmic reticulum channels; (5) intracellular triglyceride synthesis at the extreme apical portion of the SER extending into the terminal web;
(6) unspecific pinocytosis of metabolites, including lipid.

Pinocytosis of lipids was largely discounted by the electron-opaque marker experiments of Cardell et al. (1967), and by the differences in unit membrane size enclosing microvilli and apical profiles containing lipid (Cardell et al., 1967; Dermer, 1967a). In the bovine intestine, however, membrane size differences are not very large, and pinocytosis cannot be excluded on this basis. Napolitano and Kleinerman (1964) reported no difference in membrane dimensions for microvilli and apical lipid vesicles in the cat.

The frequent observation of invaginations suggestive of pinocytosis occurs in cells absorbing lipid in vivo or in vitro, as well as in cells during fasting conditions. Although fasting conditions imply negative conclusions, some evidence for continuing metabolism and transport of lipid (probably of endogenous origin) has been presented. Moreover, indirect evidence against pinocytosis of triglyceride from the emulsified oil phase (as a major physiological mechanism) is given by the fact that both triglycerides and an emulsified oil phase are absent under in vitro conditions, while the frequency of pinocytotic profiles does not change.

The results of Forstner et al. (1965) lend support to the idea of synthesis of triglyceride at the surface membrane. These workers showed that brush borders, isolated either from hamster or rat, were capable of incorporating C\textsuperscript{14}-palmitate into higher glycerides and pholpholipids. Specific association of the enzymes with the brush border plasma membrane, or with the fibrillar components, was not obtained.

It is possible that glyceride synthesis also occurs at the microvillus membrane of the calf mucosa. However, direct synthesis at a
surface membrane site would probably yield a droplet absent of a limiting membrane, contrary to what is observed. In this regard, membrane synthesis concurrent with pinocytosis appears as a favorable explanation, although it is difficult to imagine a highly developed mammalian system hydrolyzing triglyceride in the intestinal lumen followed by immediate resynthesis at the initial membrane barrier.

Lipid droplets "backed-up" into the terminal web, or synthesized in SER channels extending into the terminal web are two other alternatives. However, no identification of SER cisternae or evidence for an extensive "back-up" of lipid droplets in this region are observed. Also, it is clear that epithelial cells are highly specialized for movement of materials in one direction, and it seems unlikely that much "counter-current" movement occurs.

Finally, the possibility remains that the terminal web profiles are not lipid. The only basis for suggesting their being lipid is the morphological similarity to lipid droplets in the apical cytoplasm. The fact that the profiles are transparent suggests that their original contents could have been a variety of low molecular weight metabolites (including lipid) which were extracted during specimen preparation.

**Conclusion** Passive molecular diffusion from the micellar phase, whether via intact micelles or single molecules, most likely represents the major route for lipid absorption by the bovine intestinal epithelial cell. The situation is evident in vitro, and can be related to both the adult and young animals when higher glycerides (lard oil) are presented directly to the small intestine. Physicochemical events in the ruminant intestine during normal dietary intake were not studied, and have not
been precisely determined in the literature.

Based on morphological observations, pinocytosis of triglyceride as a mechanism for absorption is probably minor, and is difficult to explain in the presence of a micellar environment. The possibility exists that the presence of surface invaginations and terminal web profiles represents pinocytosis of triglyceride precursors concurrent with triglyceride synthesis at the surface membrane. However, a more favorable explanation is that pinocytosis is a remnant of that mechanism utilized by the absorptive cells of the newborn animal, and that in the calf and adult animal, it functions unspecifically and to a limited extent. In this regard, a number of small molecular weight metabolites, including lipid, could be taken up (by pinocytosis) and subsequently extracted (or rendered impervious to the specific stain utilized) during processing for electron microscopy.

Absorption and transport - intracellular phase

Apical region Intracellular lipid from exogenous sources is present as 80-150 μm droplets, membrane enclosed, in the apical cytoplasm. Adjacent to the limiting membrane and encircling the periphery of the droplet is a zone of electron density, the chemical composition of which is unknown. Its density suggests a protein-phospholipid structure, yet it resembles somewhat the mucopolysaccharide "fuzz" of the surface cell membrane. However, since pinocytosis has been excluded as a major mechanism for lipid absorption (see above), origin of the "peripheral zone" from the microvillus "fuzz" is discounted. Based on the generally accepted view that intracellular events involve triglyceride resynthesis
followed by chylomicron formation, it can be speculated that the "peri-
pheral zone" represents the phospholipid-protein-cholesterol component of
the finished chylomicron (cf. Zilversmit, 1967).

Droplets are frequently observed within or associated with channels
of RER, contrary to evidence presented by Cardell et al. (1967) for the
rat. Their observations discounted synthesis and transport of lipid by
RER, and suggested that the enzymes and protein necessary for triglyceride
synthesis and chylomicron formation were supplied by the RER during
periods of fasting or minimal lipid absorption. During active absorption,
the authors suggested that SER was derived from RER that had lost ribo-
somes, such SER retaining its triglyceride synthesizing enzymes and
chylomicron protein.

In the calf, the presence of lipid is indicated within RER channels,
especially in tissue that has been treated with lipid solvents before
osmium fixation. Lipid droplets are present at the end of short segments
of RER, indicating that they are in the process of completing passage
through the channels. Although droplets enclosed by smooth membranes are
well in the majority, the involvement of RER in both synthesis and trans-
port cannot be discounted from the evidence obtained. Dispersed lipid
within RER and SER channels suggests that triglyceride synthesis is taking
place, while the presence of droplets within and associated with RER
segments invites speculation that chylomicron protein is being or has been
provided. In fact certain lipid-containing profiles have been observed
which are suggestive of synthesis at the smooth membrane surface, while
ribosomes are available on the opposite side to possibly provide chylo-
micron protein.
**Golgi region** Apical droplets of newly synthesized triglyceride appear to be transported to, and systematically packaged within the Golgi apparatus. Golgi packets vary in size and number of droplets, but micrographs indicate that formation involves a coalescence of single droplets within the Golgi cisternae, followed by a "pinching off" of the package at the peripheral vesicular region of the apparatus. No structural change in the individual droplets is observed, implying that they are not chemically altered. The electron dense "peripheral zone" is maintained, allowing identification of single droplets, and the entire package is encompassed by a unit membrane derived from the Golgi cisternae.

The importance of the Golgi apparatus in lipid transport has not been well emphasized in the literature. Golgi packets were not demonstrated for the rat or hamster intestine; rather, some coalescence of apical droplets to form slightly larger individual droplets was observed (cf. Cardell *et al.*, 1967; Strauss, 1968). Dobbins (1966) observed the formation of Golgi packets during absorption by the normal small intestine of humans, but did not evaluate their presence. Furthermore, the presence of packets intercellularly was not shown. However, Dobbins (1966) did conclude that the Golgi apparatus might be rate limiting in lipid transport, based on the absence of lipid in this organelle in patients with abetalipoproteinemia (inability to synthesize β-lipoprotein).

Rubin *et al.* (1967) also demonstrated Golgi packets in cells from patients exhibiting the pathological condition of intestinal heterotopia. These authors suggested that the Golgi functioned in elaborating an amorphous material which was subsequently involved in packaging of the droplets, and in formation of chylomicrons. This study is unique in
suggesting the formation of chylomicrons by the Golgi.

The results presented in this dissertation emphasize the importance of the Golgi apparatus in transport. Morphological evidence strongly suggests that newly synthesized triglyceride must first enter the Golgi system to be "packaged" before it can be exported to the lacteals. Furthermore, in vitro experiments show that removal of intestinal tissue from its intact environment results in failure of the Golgi to form packets, and in the absence of lipid in intercellular spaces.

The precise mechanism by which the Golgi apparatus appears to control transport (in the bovine mucosa) is not known. It is possible that communication between the Golgi-derived membranes (enclosing the packets) and the lateral plasma membrane is required for cellular exit (see below). However, no defect in the Golgi membrane system is apparent in vitro, in which case cellular exit is blocked. It is also possible that simple diffusion gradients exist in the transport scheme, whereby a block in removal of lipid (synthesized for export) by the lacteals (as would be the case when tissue is removed for in vitro experiments) would effect a feedback block in some other stage of transport, such as packet formation in the Golgi. Cardell et al. (1967) suggested that the SER sequesters fatty acids and monoglycerides to form triglycerides, setting up a diffusion gradient. It can further be hypothesized that a complete gradient of intra- and extracellular events operates, from the point of passive diffusion of molecules into the cell to the point of appearance of lipid in the lacteals. There is a sequestration of apical droplets by the Golgi, and in turn, a sequestration of Golgi packets by the lateral plasma membrane, extracellular spaces, and lacteals.
A third possibility for explaining Golgi control is that some type of chemical or physical alteration in the composition of the apical droplet is carried out by the Golgi apparatus. This is not apparent in the bovine case, in that no morphological changes are observed when apical and Golgi droplets are compared. However, Stein and Stein (1967b) point out that in the rat liver (as in the intestine), triglyceride is exported as very low density lipoprotein and passes through the Golgi apparatus before such export. In contrast, triglyceride exported from mammary tissue does not contain additional chemical components, and does not appear in the Golgi apparatus prior to export.

Finally, it is possible that some type of hormonal regulation prevails at the Golgi apparatus. Again, tissue biopsies which were employed in vitro had lost their blood and lymph supplies, and consequently, their homeostatic controls. Isselbacher (1967) pointed out that adrenal steroids were required for intestinal lipid metabolism (rat), although the control was localized with esterifying (triglyceride synthesizing) enzymes rather than transport events in the Golgi apparatus.

**Chylomicron formation**  The identification of apical and Golgi droplets as chylomicrons is speculative. However, biochemical evidence for the presence of the necessary components of chylomicrons in the apical cytoplasm, as well as size considerations, has led several workers to believe that individual lipid droplets in this region are the finished chylomicrons (Senior, 1964; Brindley and Hübscher, 1965; Cardell et al., 1967; Johnston, 1968; Strauss, 1968). Results presented in this dissertation, based on droplet size and morphology, association of the droplets with RER (discussed above), and localization of cholesterol, tend to
support this hypothesis.

The structures (chylomicrons) isolated from the intestinal lymph of a milk-fed calf are 90-360 μm in diameter. This range is slightly larger than that for intracellular lipid droplets, but both are of the order of that reported for rats by Zilversmit et al. (1966) (median diameter 150-200 μm with an upper limit of 500 μm), and by Casley-Smith (1962) (10-100 μm for lipoproteins and 100-1000 μm for chylomicrons).

The isolated chylomicrons (calf) are similar in fine structure to intracellular and intercellular lipid droplets. Both possess an electron-dense periphery and an electron-transparent center, suggestive of the morphological structures characterized chemically and reported by Zilversmit (1967).

Intracellular cholesterol localization was effected through the formation of digitonin-cholesterol precipitates after the method of Ökrös (1968). As expected, extensive reactions in the fasted cell are concentrated in the Golgi region, lateral plasma membrane, and lysosomal membranes. Less concentration is observed in intercellular spaces, in the mitochondrial membranes, and in the endoplasmic reticulum. Surprisingly, no reaction is observed at the surface plasma membrane or terminal web region. This latter observation is difficult to explain since cholesterol should be present in the brush border as shown by Forstner et al. (1968) for the rat. One explanation which seems possible is that this region possesses greater susceptibility to loss of free cholesterol and/or the cholesterol-digitonin complex during processing for electron microscopy. Cholesterol (as well as other lipids) is not fixed by aldehydes, and subsequent treatment with digitonin solutions could have resulted in a
leaching of the cholesterol from the external regions of the cell, followed by precipitation in the digitonin medium.

Cholesterol localization is evident at the periphery of apical droplets following absorption in vitro or in vivo, with some suggestion of a reaction at the droplet center. In the rat, Zilversmit (1967) showed cholesterol to be present at the periphery and in the interior of the chylomicron, in agreement with the localization observed at both sites in the calf.

Whether the digitonin reaction observed at the periphery of apical droplets is due to the presence of cholesterol associated with the limiting membrane or with the droplet itself is difficult to assess. In tissue which is not subjected to lipid absorption, the reaction at RER and SER membranes is slight compared to that at the periphery of apical droplets during absorption. Furthermore, the intensity of the reaction associated with droplets appears directly dependent upon droplet size. It is highly conceivable, therefore, that the reaction involves cholesterol of the droplet (chylomicron), and that during processing of the tissue, solvent action causes an orientation of the laminar structures toward the droplet periphery. It would follow that the less-dense reaction at the droplet center would represent incomplete solvent action. The orientation of digitonin-cholesterol complexes is random for droplets within a given cell.

The apparent lack of a strong reaction associated with lipid in the Golgi region and intercellular spaces is difficult to explain in view of a strong reaction with individual droplets. In fact, this observation lends indirect support to the conclusion that the reaction at the periphery of apical droplets is due to cholesterol in the limiting membrane.
However, the weak Golgi and intercellular reactions might be interpreted as follows: (1) the regions in question are quite large, requiring the proper plane of section to demonstrate the lamellar precipitate (sometimes a portion of a precipitate appears associated with a Golgi vacuole); (2) the short lamellar segments observed in these regions represent a digitonin precipitate broken up by solvent action; (3) the cholesterol concentration in the chylomicron could decrease (in favor of cholesterol ester) as the chylomicron moves toward the Golgi region.

The possibility exists that chylomicron cholesterol is too small in quantity to give a digitonin reaction. However, cholesterol present in isolated microsomal membranes is reported as 3-6% of total lipid (Fleischer and Rouser, 1965), while cholesterol makes up 1-2% of total lipid of chylomicrons. On a volume basis, chylomicrons contain a larger quantity of lipid, and therefore the amount of cholesterol would not be expected to be much less (if not more) than that in membranes.

**Conclusion** Lipid droplets present individually in the apical cytoplasm and as packets in the Golgi region are identified morphologically as chylomicrons. The localization of free cholesterol demonstrates this component of chylomicrons to be associated with apical droplets.

Whether the digitonin reaction identifies cholesterol associated with the limiting membrane of the droplet, or with the droplet itself, is not precisely known. Nevertheless, the reaction shows the immediate availability of cholesterol as a chylomicron component in the apical cytoplasmic region.

The Golgi apparatus is directly involved in the intracellular transport of newly synthesized lipid. It is suggested that this organelle
exerts some control over the transport scheme, but the mechanism of control remains speculative.

**Absorption and transport - extracellular phase**

**Cellular exit**  Lipid droplets packaged in the Golgi apparatus are emptied into the intercellular spaces by the mechanism of reverse pinocytosis. This mechanism is not frequently observed in thin sections (cf. Cardell et al., 1967) but micrographs suggesting reverse pinocytosis for a package of droplets as well as a single droplet have been presented (this dissertation). Intercellular droplets occur initially at the level of the Golgi apparatus, and are morphologically identical with intracellular droplets.

The mechanism of reverse pinocytosis implies membrane fusion between the Golgi membrane of the packaged droplets and the lateral plasma membrane. Such an implication tends to suggest a transport control, in that proper information between the two membrane systems must exist before fusion is permitted and the lipid droplets are released. It would follow that the exit of single droplets requires passage through the Golgi region, a phenomenon which is observed.

**Extracellular transport**  No new information has been obtained regarding the transport of lipid from the intercellular spaces to the jejunal lacteals. Droplets are moved as aggregates toward the base of the epithelium and across the basement membrane, where they are discharged into the connective tissue matrix (lamina propria) and eventually into the lacteals. Profiles resembling lipid droplets are observed in capillary endothelium, but the significance of this observation is un-
known. Droplet composition consisting of medium-chain triglycerides offers an explanation, but exogenous sources of medium-chain fatty acids were lacking in the experimental procedures. McKay et al. (1967) observed lipid in capillary endothelium in rats fed long-chain triglycerides while Palade (1959) demonstrated "small fat particles" in the endothelial cells of starving animals.
The ultrastructure of absorption, metabolism, and transport of glycerides of long-chain fatty acids by the bovine small intestinal mucosa was investigated. Jejunal biopsies were obtained from non-ruminating calves and a mature ruminant, using a hydraulically operated suction biopsy tube passed internally via a duodenal cannula. Biopsies were excised from animals following introduction of a large volume of emulsified fat (lard oil) directly into the intestine, or from fasted animals for in vitro incubations.

The morphology of the bovine intestinal epithelial cell following assimilation of the neutral lipid was shown to follow a pattern similar to that existing for other mammalian systems including man. Lipid droplets were present individually in the apical cytoplasm, and as aggregates in the Golgi apparatus and intercellular spaces. Evidence was presented which indicated that the lipid droplets, formed by the endoplasmic reticulum system in the apical cell region, were packaged within Golgi cisternae before export to the intercellular spaces.

The bovine intestinal mucosa was demonstrated to be capable of absorption and metabolism in vitro of oleic acid and monoolein presented as a micellar solution. Lipid droplets were present in the apical cell region and were morphologically identical to those observed in vivo. However, the formation of distinct aggregates by the Golgi apparatus, and the appearance of aggregates intercellularly was not evident. The significance of this observation with respect to control of transport by the Golgi apparatus was discussed.
Absorption and metabolism of lipid in vitro from an emulsified fat preparation (lard oil) was tested. In contrast to the results following micellar incubations, the bovine epithelium did not demonstrate morphological evidence for lipid uptake and triglyceride resynthesis.

Apical lipid droplets, appearing after absorption and metabolism in vivo or in vitro, were identified as chylomicrons based on size and morphology. Additionally, free cholesterol was localized as electron-dense digitonin-cholesterol precipitates associated with apical droplets. This observation supported the conclusion that cholesterol is available as a chylomicron component in the apical cell region, and that chylomicron formation probably takes place in the ordered sequence of events associated with triglyceride resynthesis.


I wish to extend my most sincere appreciation to the following people for their contributions during my candidacy for the Ph.D. degree: to Dr. R. S. Allen for his assistance and guidance in planning and evaluating my research program; to Dr. D. E. Outka for his careful evaluation of ultrastructure techniques and results, and of the final presentation and assembly of this dissertation; to Dr. A. D. McGilliard for his invaluable assistance in carrying out experimental procedures and performing the necessary surgery; and to my wife, Barbara, for her devotion and encouragement throughout.

The research presented was supported in part by NASA Fellowship NsG(T) 35.1, NIH Fellowship 1-F1-GM-37, 917-01, and PHS Grant HE-04969.