A RADIOAUTOGRAPHIC STUDY OF GLYCERIDE SYNTHESIS IN VIVO DURING INTESTINAL ABSORPTION OF FATS AND LABELED GLUCOSE

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ABSTRACT

Radioautography was used to detect the synthesis of labeled glycerides in intestinal absorptive cells following injections of fatty chyme and glucose-6-H^3 into ligated segments of upper jejunum of fasting rats. Absorption intervals ranged from 2 to 20 min. Labeling is evident throughout the cells in as short a time as 2 min. Most grains are present over droplets of absorbed fat beginning with those in the endoplasmic reticulum immediately subjacent to the terminal web. With longer absorption periods, frequent grains are present over accumulations of fat droplets in the Golgi cisternae and intercellular spaces. A similar pattern of grains is seen following absorption of either linoleic acid or safflower oil. By comparison, considerably less label is present in the cells when the fat is extracted with alcohol prior to radioautographic procedures, or when labeled glucose alone is absorbed. A significant incorporation of glucose label into newly synthesized glycerides is indicated and confirmed by scintillation counts on saponified lipid extracts. The grain distribution implies an involvement of the extreme apical endoplasmic reticulum in this synthesis.

INTRODUCTION

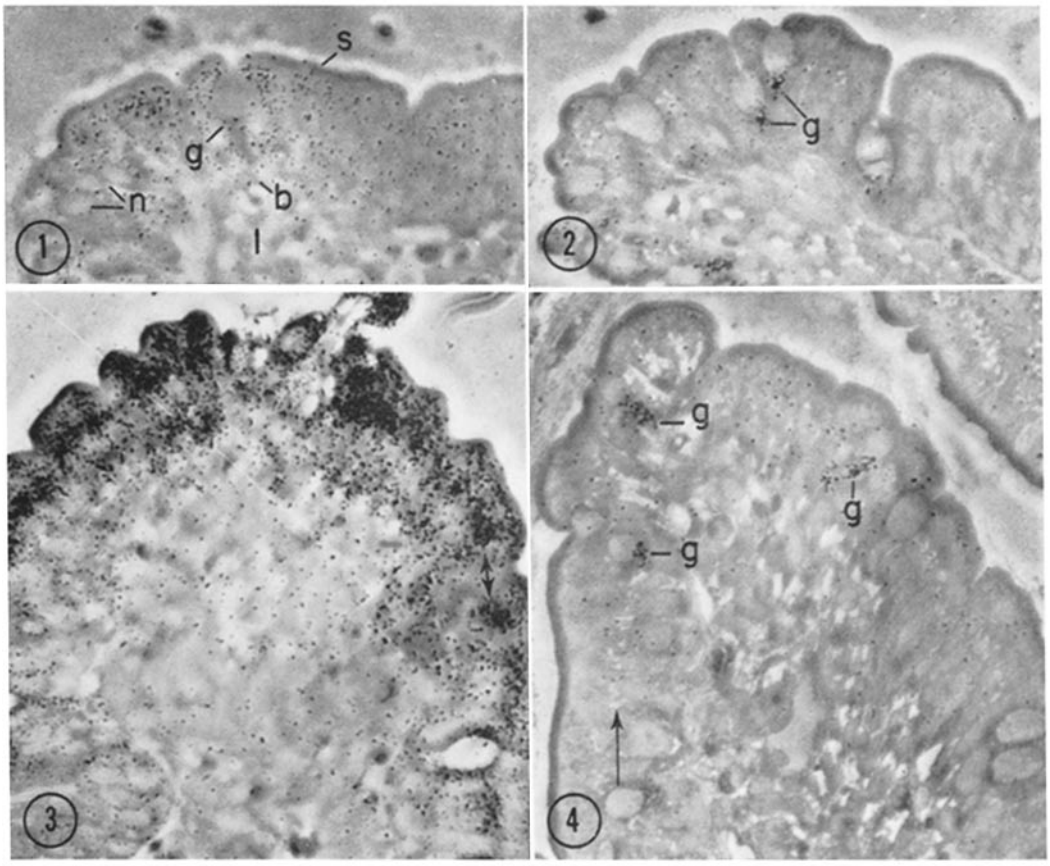
Current concepts of intestinal fat absorption hold that dietary fats are absorbed primarily as monoglycerides and free fatty acids (16, 25). In the intracellular resynthesis of triglycerides which follows, two major metabolic pathways exist: (*a*) esterification of absorbed fatty acids with absorbed monoglycerides (11, 12, 23, 34), and (*b*) esterification of absorbed fatty acids with endogenous *a*-glycerophosphate (11, 12).

The absorbed lipids first become visible with the electron microscope as dense, osmiophilic droplets within the endoplasmic reticulum of the intestinal absorptive cells (28). The precise chemical nature of the lipids within these droplets is presently not known. It is possible that the droplets may be composed entirely of resynthesized triglycerides or, alternatively, that esterification continues as the droplets are transported through the endoplasmic reticulum (1, 29).

Recently improved radioautographic techniques (10) coupled with the use of labeled glyceride precursors offer a method with which to examine the component parts of the cell involved in glyceride synthesis. The present work demonstrates the utilization of dietary-supplied labeled glucose in the formation of glyceride-glycerol during triglyceride synthesis in vivo.

MATERIALS AND METHODS

In order to govern the length of the absorptive period and to insure the availability of dietary glucose in the areas active in fat absorption, the test mixture was



FIGURES 1 to 4 are light microscope radioautographs of the tips of villi exposed to linoleic acid chyme + glucose-H^3. Micrographs taken with phase contrast. × 600.

FIGURE 1 2 min absorption of chyme-glucose. OsO_4 fixation. 14 days' exposure of the emulsion. Dark photographic grains are distributed over the cytoplasm of the epithelial absorptive cells. In some cells the grains are more abundant over the apical cytoplasm. Labeling is also present in the Golgi region of a goblet cell (*g*). The nuclei (*n*) and striated border (*s*) are relatively free from label by comparison. Few grains are visible over the lamina propria (*l*). Basement membrane (*b*).

FIGURE 2 Villus from the same intestinal segment as shown in Fig. 1, but fixed in glutaraldehyde and the fat extracted with alcohol. 14 days' exposure of the emulsion. The number of grains over the cytoplasm in these cells is considerably less than in OsO_4-fixed specimens (cf. Fig. 1). Labeling is pronounced, however, in the Golgi region of the goblet cells (*g*).

FIGURE 3 20 min absorption of chyme-glucose. OsO_4 fixation. 7 days' exposure. Labeling of the cytoplasm is intense. The greatest grain concentration is over the Golgi regions which appear as a continuous dark band along the epithelium (arrow). The nuclei stand out as relatively unlabeled areas. Little labeling of components in the lamina propria is present.

FIGURE 4 20 min absorption of chyme-glucose followed by glutaraldehyde fixation and extraction of the fat. 14 days' exposure. In comparison with that in Fig. 3, the amount of label retained in the absorptive cells is markedly reduced with only scattered grains present over the cytoplasm. The Golgi apparatus is visible in these cells as a negative image (arrow). Labeling of the goblet cells (*g*) is heavy.

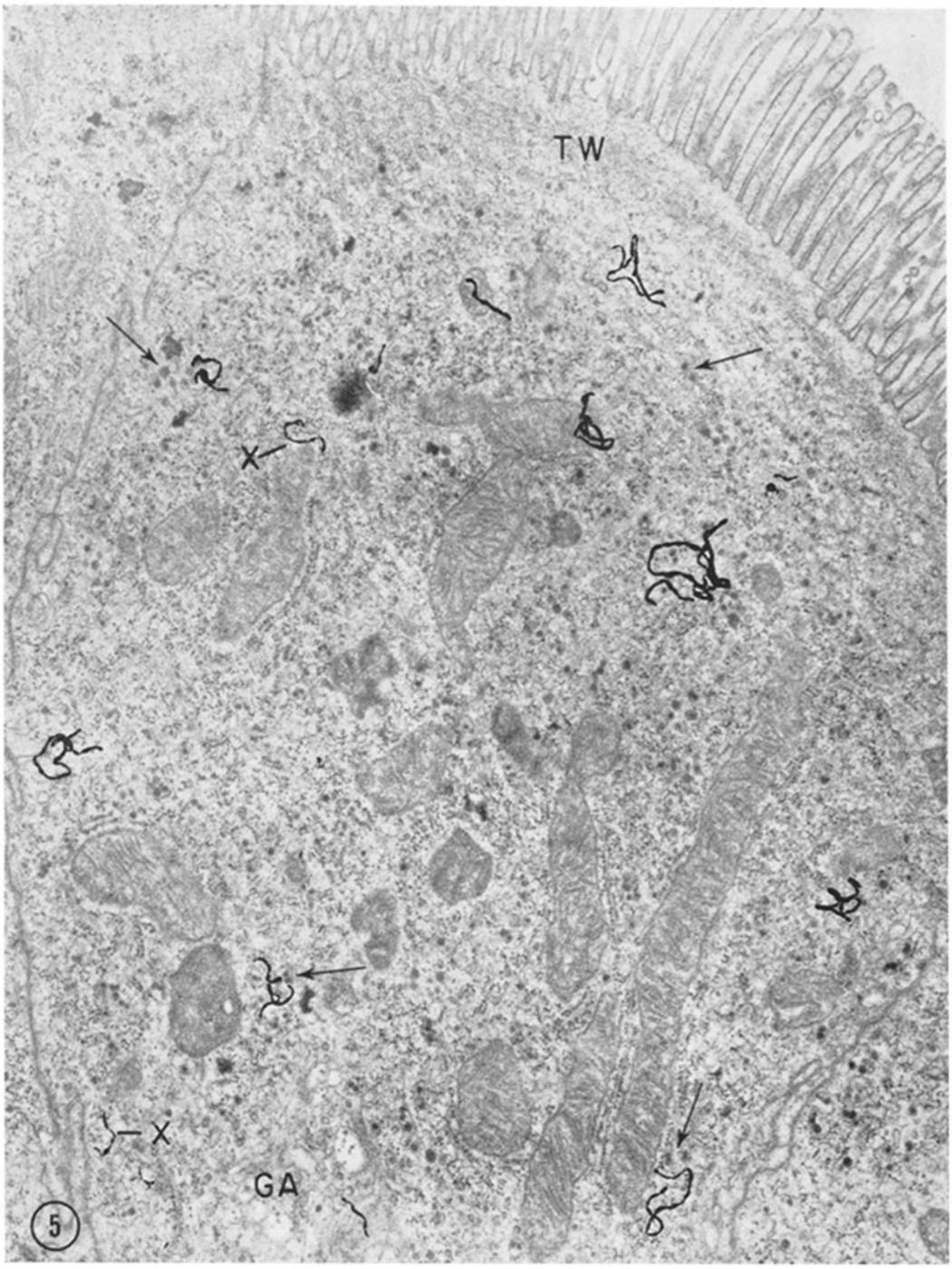


FIGURE 5 Electron microscope radioautograph of the apical cytoplasm of an absorptive cell. 2 min absorption of linoleic acid chyme + glucose-H^3. Small, dense droplets of fat (arrows) are present within the lumen of the endoplasmic reticulum. These droplets extend from the area subjacent to the terminal web (*TW*) through the Golgi zone of this cell. The Golgi cisternae (*GA*) contain only a few droplets. The photographic grains are distributed over much the same area and lie in the vicinity of the droplets. Two grains lie in apparently fat-free areas (X). × 22,000.

injected directly into ligated segments of upper jejunum with intact vasculature, as described in a previous communication (20). The procedure involved the collection of fatty chyme from the upper jejunum of rats fed linoleic acid (75% pure) or safflower oil. Glucose-6-H^3 (specific activity 260 mc/mmole) was mixed with the chyme at a concentration of 1 mc/cc.

Anesthesized female Wistar rats (85 to 200 gm), previously fasted for 24 hr, served as recipients. Following the injection of the chyme–glucose-H^3 mixture (varying from 60 to 200 μc) into the ligated segments, absorption was allowed to proceed for 2, 5, 10, or 20 min. Portions of the segments were fixed in 1% phosphate-buffered OsO_4, pH 7.2 (26). In some experiments additional pieces were fixed in 1% phosphate-buffered (14) glutaraldehyde, pH 7.2 to 7.6. The latter were fat-extracted by rapidly ascending to absolute alcohol for 10 or 30 min, and subsequently returned to phosphate buffer and postfixation in OsO_4. All pieces were then dehydrated and embedded in methacrylate.

In a second series, an aqueous solution of labeled glucose (1 mc/cc) was injected into ligated segments to compare glucose incorporation into cells without attending fat absorption over similar absorptive periods. These tissues were fixed in OsO_4 and embedded in methacrylate.

Sections were prepared for both light (0.5 μ thick) and electron microscope ($\sim$800 A thick) radioautography, with Ilford K-5 and L-4 emulsions, respectively. The radioautographic methods used were essentially as described by Caro and van Tubergen (10). Both chemical and physical developments of the emulsions were employed. Most electron microscope preparations were stained with lead citrate (32) prior to examination.

In a third series, the radioactivity incorporated into the fat fraction of intestinal segment homogenates was measured with a liquid scintillation counter. Lipid extraction was based on the method of Folch et al. (15). In each of two rats, two segments were ligated, about 2 cm apart. Chyme–glucose-H^3 (100 μc) was injected into one segment and aqueous glucose-H^3 (100 μc) into the second segment of each animal. After 20 min all segments were removed, rinsed in saline, and homogenized in chloroform-methanol extract. Water was then added at $\frac{1}{5}$ the original volume; the mixture was shaken and allowed to stand until the resulting aqueous, chloroform, and residual layers separated. An aliquot of each layer was counted. The remaining chloroform phase was dried, then saponified by adding alcoholic KOH and heating in a water bath. Nonsaponifiable lipids were removed with two extractions in pentane and counted. The free fatty acids were removed and counted by acidification and pentane extraction. The radioactivity remaining in the aqueous residue was severely quenched and, therefore, calculated by subtraction from the original chloroform phase.

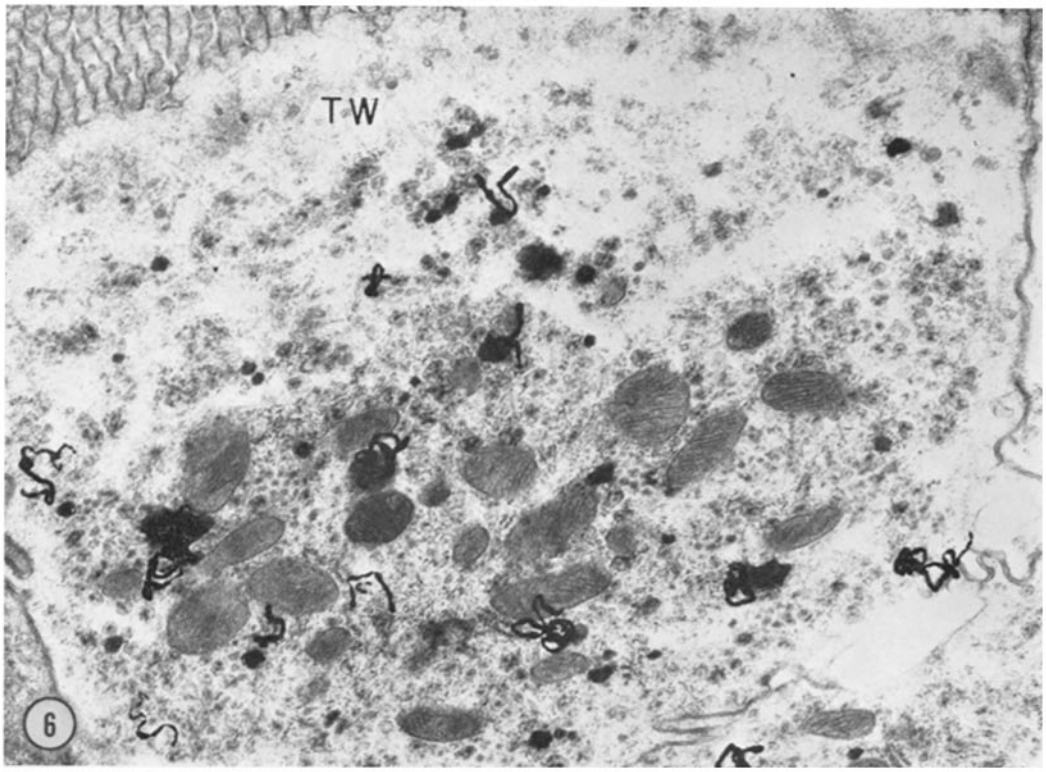
OBSERVATIONS

Radioautographs of intestinal absorptive cells exposed to fatty acid chyme and glucose-H^3 show that label is rapidly incorporated into these cells. Light microscope radioautography demonstrates that in as short a time as 2 to 5 min following the injection, label is distributed throughout the cell cytoplasm (Fig. 1). In general, the grains are greater in concentration over the apical cytoplasm than over the subnuclear area, and they decrease in number in the cells toward the base of the villus. Very few grains are present over the lamina propria. In electron micrographs of these specimens abundant fat droplets of small size can be seen throughout the apical cytoplasm) (Figs. 5 to 7) and can be seen in fewer numbers beneath the nucleus. For the most part, these droplets are enclosed by profiles of endoplasmic reticulum. Here most of the radioautographic grains lie over areas containing such droplets. It is to be noted that these grains are predominant from the area immediately beneath the terminal web, i.e. over the droplet-containing subjacent endoplasmic reticulum (Figs. 6 and 7), to the nuclear level (Fig. 5).

By 10 and 20 min, the absorptive picture has

FIGURE 6 2 min absorption of linoleic acid chyme + glucose-H^3. The section is a nearly transverse cut which passes through only the extreme apical cytoplasm of the absorptive cell. Numerous grains are located over areas containing small droplets of fat. These droplets are surrounded by, and are abundantly distributed throughout, circular profiles of endoplasmic reticulum beneath the terminal web (*TW*). × 22,000.

FIGURE 7 5 min absorption of linoleic acid chyme + glucose-H^3. Prominent droplets of fat are present throughout the endoplasmic reticulum. The grains are evenly distributed over these areas beginning immediately beneath the terminal web and extending deeper into the cell. 2 months' exposure of the emulsion. × 22,000.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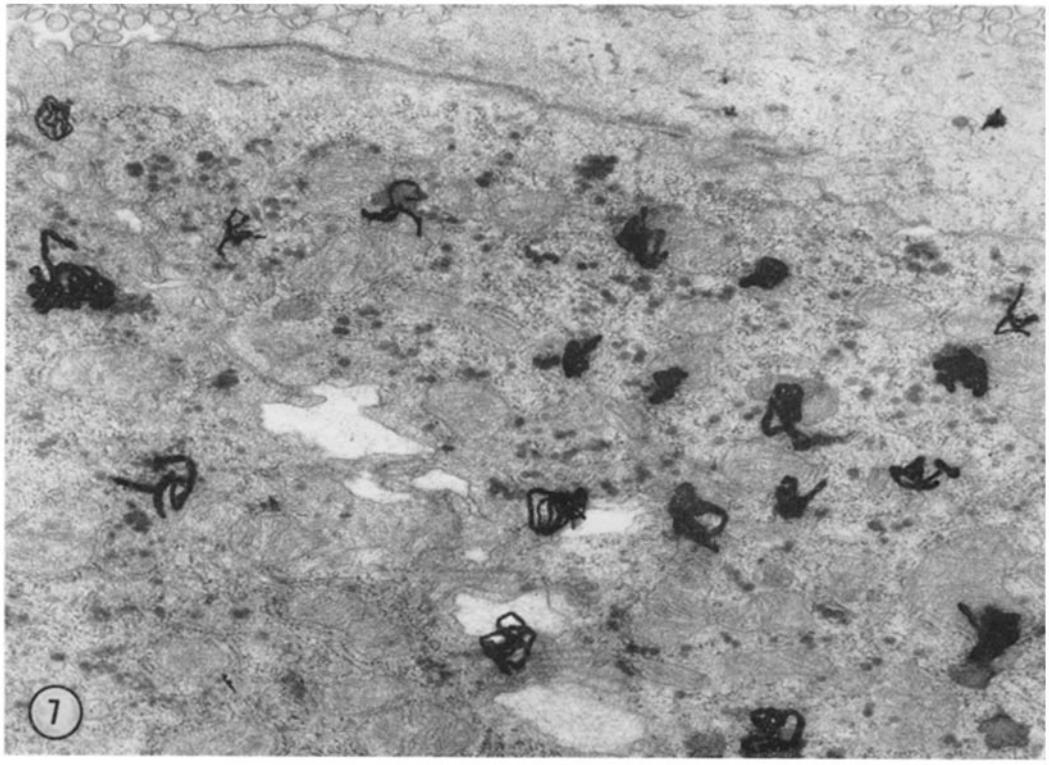
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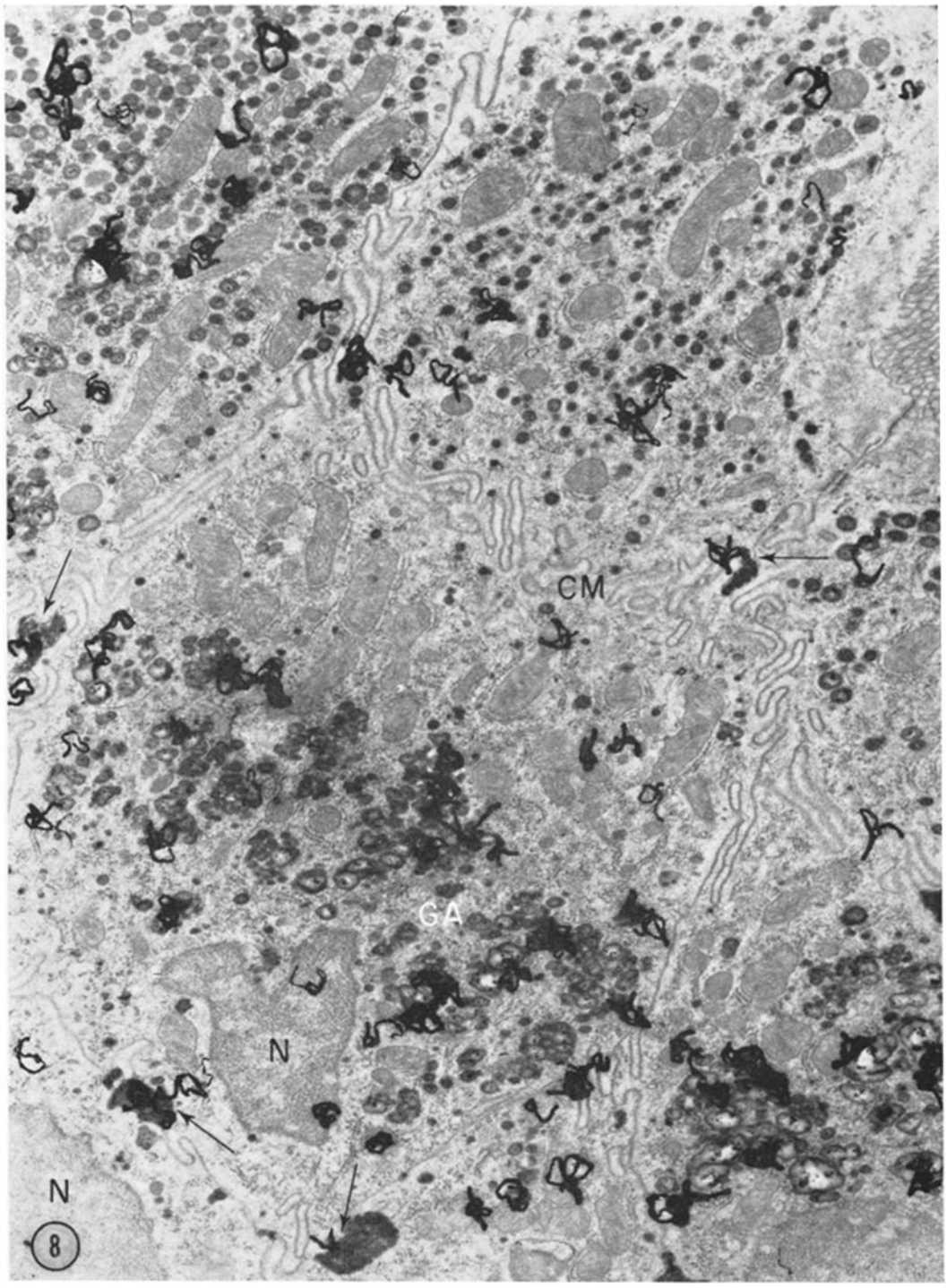


FIGURE 8 20 min absorption of linoleic acid chyme + glucose-H^3. Oblique section through the epithelium with irregular contours of cell margins (*CM*) crossing the center of the figure. Cell apex at the upper right. Large fat droplets fill the endoplasmic reticulum. Those droplets within the enlarged Golgi cisternae (*GA*) vary in size and density. Most grains show a relationship to fat-containing areas and are especially abundant over the Golgi cisternae. Single grains lie over small clusters of fat droplets in intercellular spaces (arrows). 3 grains are visible over nuclei (*N*). 2 months' exposure. × 15,000.

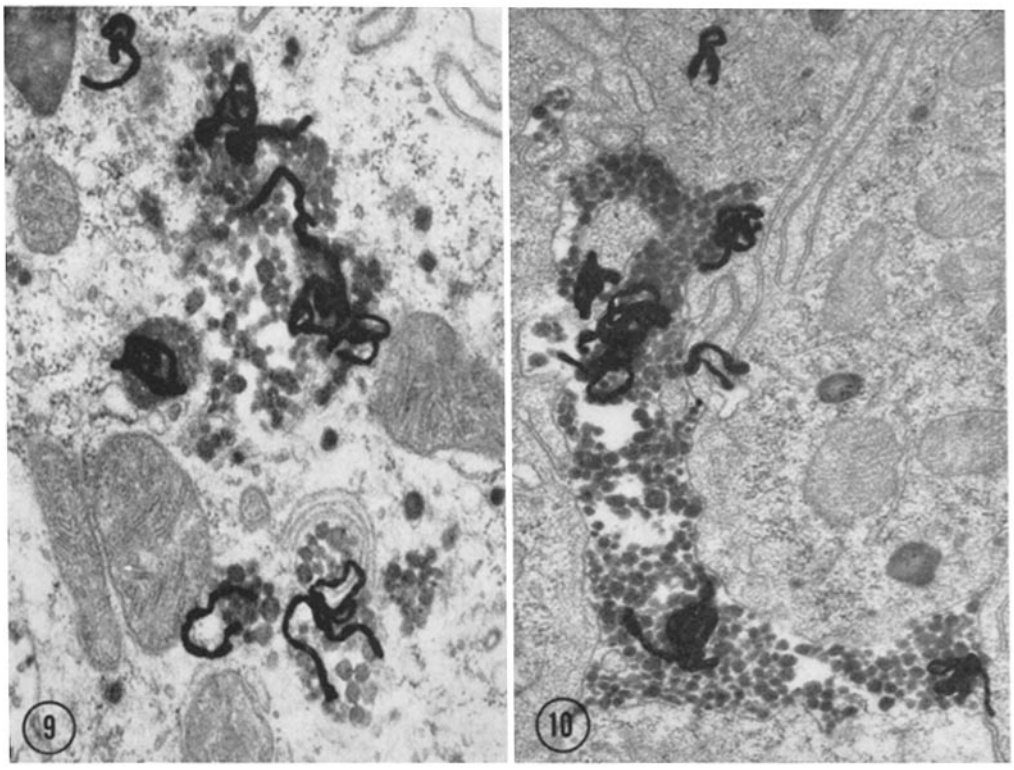


FIGURE 9 Portion of the Golgi apparatus of a cell following 20 min absorption. Several grains are clustered over dilations of the cisternae which contain numerous droplets of fat. 2 months' exposure. × 30,000.

FIGURE 10 An enlarged intercellular space at the subnuclear level which contains a large number of fat droplets or chylomicrons. The grains are closely associated with this accumulation of droplets. A small droplet within the endoplasmic reticulum is all but covered by the single grain at the top of the figure. 20 min absorption. 2 months' exposure. × 27,000.

progressed considerably. The fat droplets enclosed by the endoplasmic reticulum are larger in size. The Golgi cisternae are extensively dilated with fat, and the intercellular spaces show droplet accumulations. Grains are numerous over such fat-laden areas (Fig. 8) and often are visible in clusters over the Golgi cisternae (Fig. 9). The latter are most striking in light microscope radioautographs (Fig. 3). Grains are also frequently grouped over the droplet aggregations present in expanded lateral intercellular spaces (Fig. 10). The use of a physical developer, which produces grains of smaller size, provides additional support for the relationship between most grains and the fat droplets. Fig. 11 illustrates the results of physical development on a 20 min absorption specimen. Staining of this specimen was omitted in view of possible grain displacement during gelatin removal in alkaline staining solutions (10).

A relatively small number of grains show no apparent relationship to the fat droplets. Occasional grains are to be found over the microvilli, the terminal web zone, and nuclei, as well as over areas of the cytoplasm in which absorbed fat is not visible.

Radioautographs of absorptive cells exposed to glucose-H^3 and chyme prepared from safflower oil show a grain distribution and concentration similar to that seen with linoleic acid absorption during the shorter absorptive periods. At 20 min, however, the over-all grain concentrations are less than the maximum achieved with linoleic acid (Fig. 12).

It becomes necessary to determine whether or

not the labeling described above is related to an incorporation into glucose-H^3–derived glyceride-glycerol. The comparison of adjacent pieces of tissue, in which the fat has been preserved in one (OsO_4-fixed) and removed in the other (glutaraldehyde-fixed, alcohol-extracted), demonstrates a marked reduction in the number of developed grains in the fat-extracted specimens. Exposures of the photographic emulsions are identical or longer for the extracted specimens (Figs. 1 to 4). Label incorporated into goblet cells, however, remains stable throughout the extraction procedure (Figs. 2 and 4). In electron micrographs of extracted specimens, sites previously occupied by fat droplets are now visible as clear, unoccupied spaces: circular profiles of endoplasmic reticulum, vacuolar clusters in the Golgi zone, and enlarged lateral intercellular spaces (Fig. 13). The comparatively small amount of label which resists extraction in the absorptive cells is represented by a few scattered grains over areas of endoplasmic reticulum, the Golgi apparatus, mitochondria, and in the vicinity of the lateral plasma membrane (Fig. 13). Infrequent grains are also found over the microvilli, terminal web, and nuclei in distribution and number similar to those seen in these areas of unextracted specimens (compare Figs. 8 and 13). Radioautographs prepared from intestinal segments injected with glucose-H^3, but not fat, demonstrate a grain distribution and frequency similar to those described for fat-extracted specimens (Fig. 14). Comparative grain counts and distributions are summarized in Table I.

The results obtained from scintillation counts on intestinal homogenate fractions are given in Table II. These data show the highest activity to be in the fat fraction (chloroform phase) with a fivefold increase in the incorporation of glucose-derived label when fatty acids are injected along with glucose into the intestinal segments. Following saponification of the fats in the chloroform phase, the radioactivity is shown to reside primarily in the aqueous phase. It is assumed that glycerol, released by saponification and present in this phase, is responsible for the radioactivity here. Very little labeling of the fatty acids occurs.

DISCUSSION

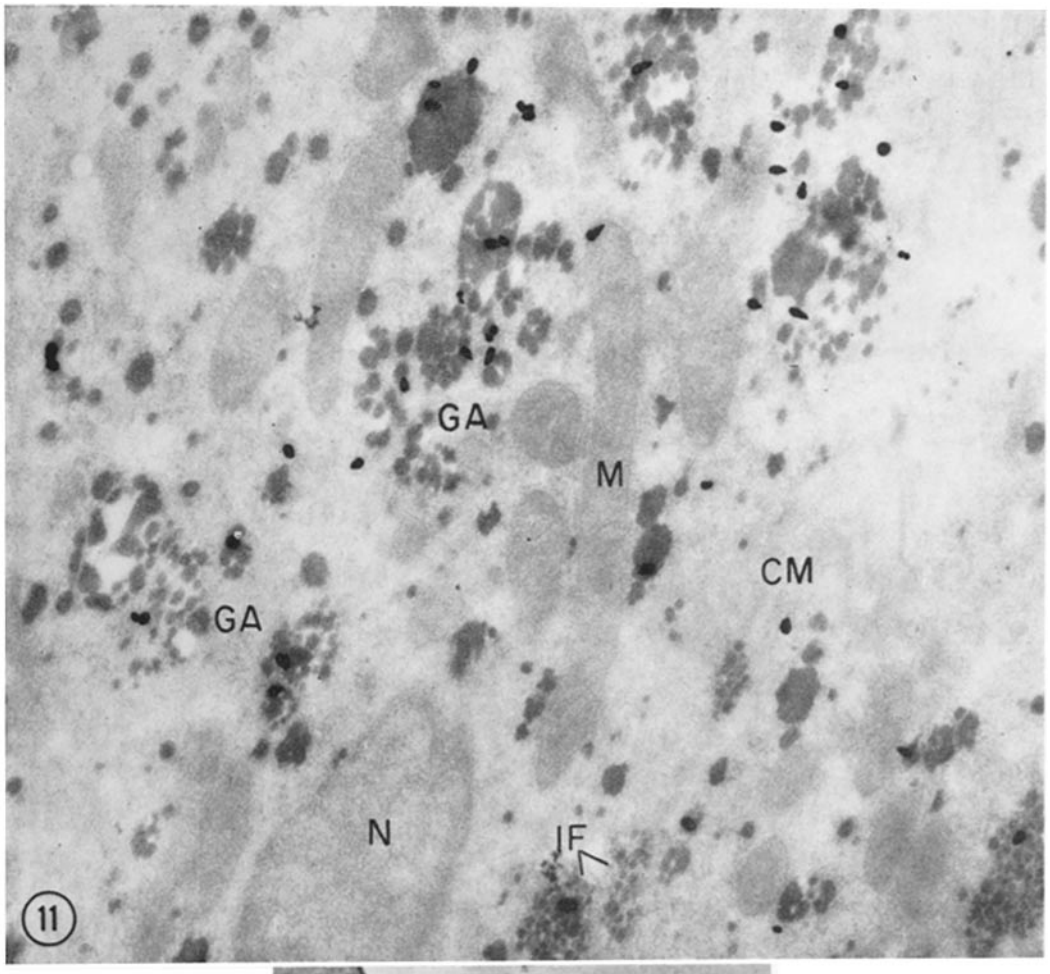
In studying intestinal triglyceride synthesis, one of two alternative metabolic pathways can be followed, the monoglyceride or the glycerophosphate –phosphatidic acid pathways (12, 18, 33). Although there is evidence to suggest that the monoglyceride pathway is the more active under physiological conditions (24), the glycerophosphate pathway was chosen for this study. For radioautographic demonstration of synthetic processes it is essential that any remaining free, radioactive precursor be selectively removed from the tissues. The use of labeled monosaccharides which are soluble in conventional aqueous fixatives seems a proper choice.

Buell and Reiser (6) concluded from their study that *a*-glycerophosphate is the immediate precursor of glyceride-glycerol. They indicated that glycolysis is involved since both unlabeled glycerophosphate and dihydroxyacetone reduce the amount of labeled fructose diphosphate incorporated into glycerides. This has been further substantiated with the demonstration that glucose can serve as a source of the glyceride-glycerol (5, 13). Because glucose is a common natural sugar of many diets, it was chosen as the labeled precursor for the present study.

Radioautographs of absorptive cells following absorption of glucose-H^3 and fatty acids clearly

FIGURE 11 Apical cytoplasm of an absorptive cell following 20 min absorption. This radioautograph was treated with a physical developer which produces small spherical, rod- and teardrop-shaped photographic grains. Unstained, the organelles are viewed in reduced contrast: Golgi apparatus (*GA*), mitochondria (*M*), cell membranes (*CM*), and nucleus (*N*). Most grains show a close relationship to the dense fat droplets. Intercellular fat clusters (*IF*). × 22,000.

FIGURE 12 Light microscope radioautograph of a villus following 20 min absorption of safflower oil chyme + glucose-H^3. Considerable labeling is present throughout the cells. Concentrations of grains over the Golgi zone are discernible only in places (arrow). The intensity of the labeling is somewhat less, however, than with linoleic acid (cf. Fig. 3). 7 days' exposure. Phase contrast. × 600.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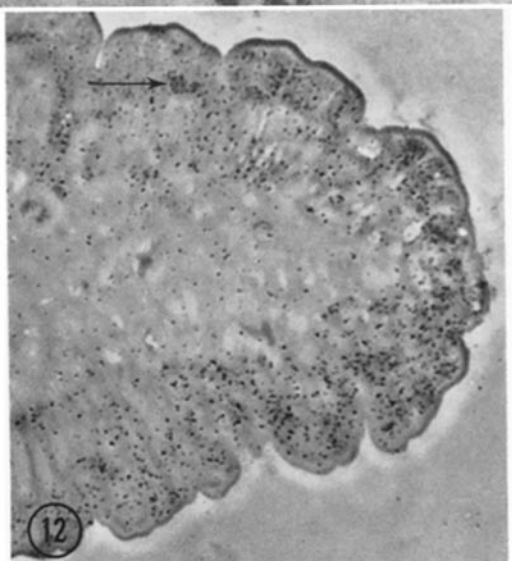
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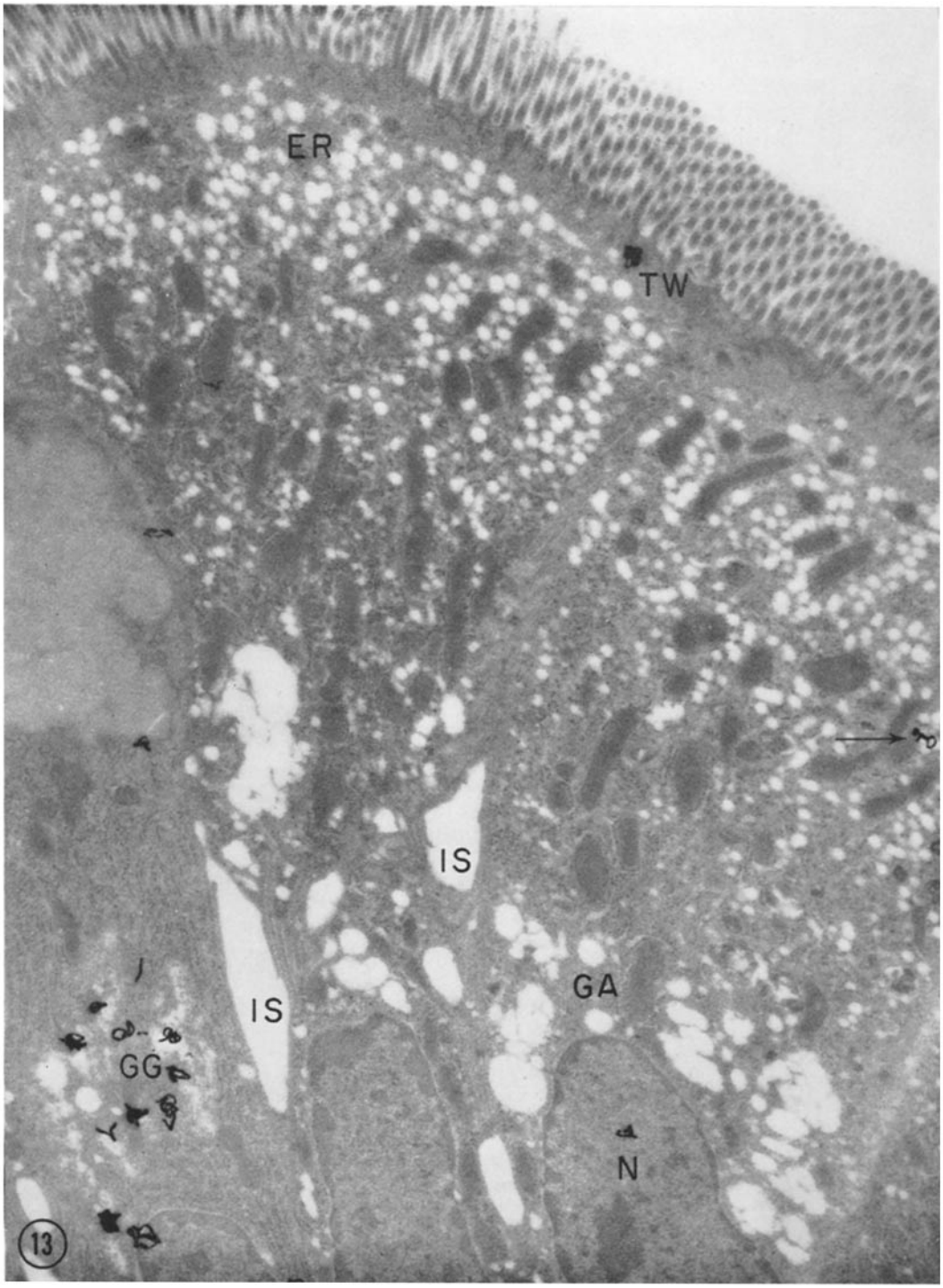

FIGURE 13 Glutaraldehyde-fixed, fat-extracted epithelium following 20 min absorption of linoleic acid chyme + glucose-H^3. Clear areas represent sites previously occupied by fat droplets. In the endoplasmic reticulum (*ER*), the profiles relate to separate individual droplets; in the Golgi apparatus (*GA*) and intercellular spaces (*IS*), to droplet clusters. Single grains are scattered over the terminal web (*TW*), *ER* (arrow), and nucleus (*N*). Grains are particularly numerous over the Golgi apparatus (*GG*) of the goblet cell at the left. 4 months' exposure. × 12,000.

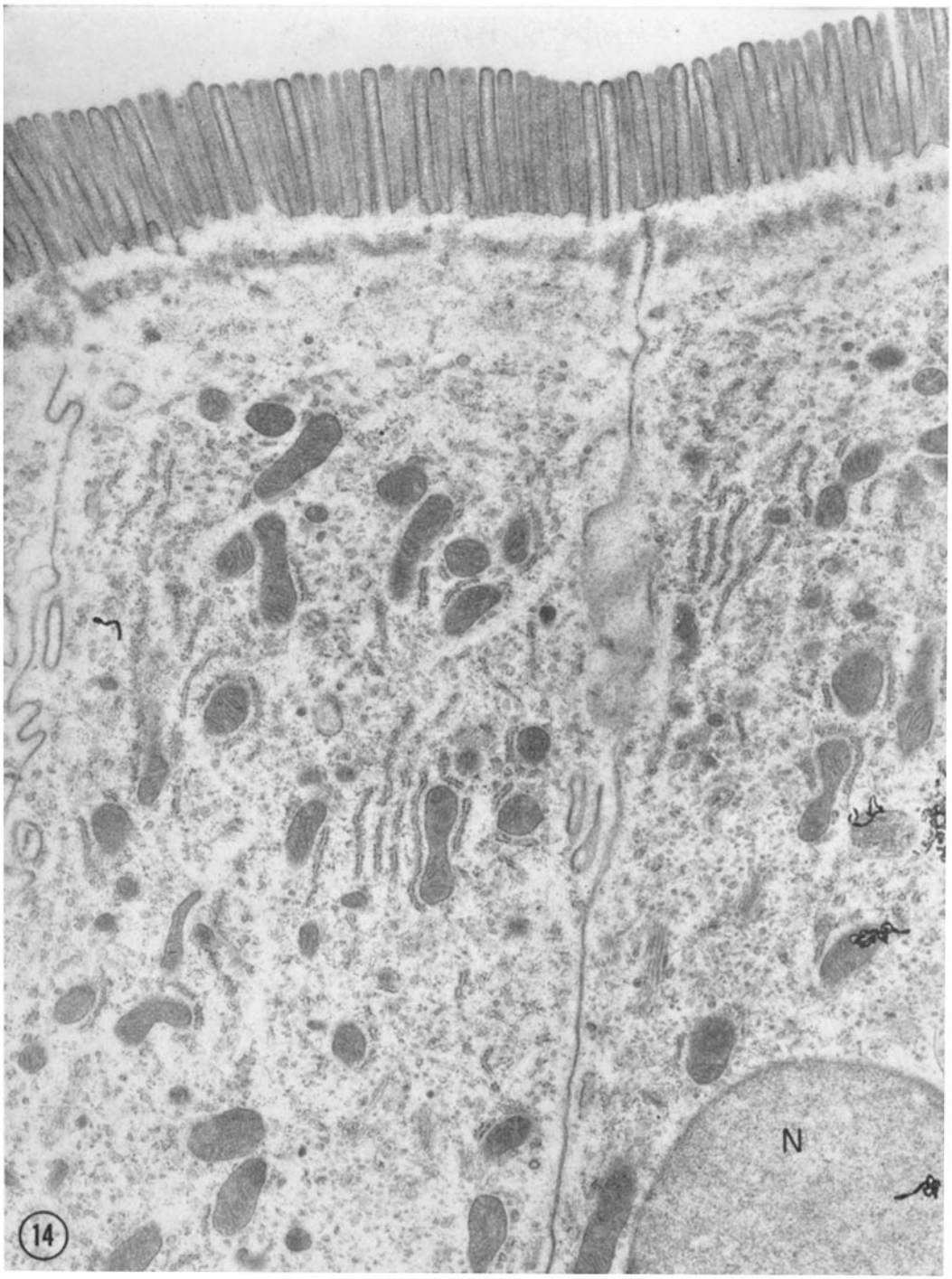

FIGURE 14 Apical cytoplasm of absorptive cells following 5 min absorption of aqueous glucose-H^3. Chyme was omitted. Only 3 grains are present over cytoplasmic elements in comparison to concomitant fat and glucose absorption as shown in Fig. 7. 1 grain is over a nucleus (*N*). 2½ month exposure. × 15,000.

TABLE I

Distribution of Grain Counts over Various Cell Components

Grain counts were made over 20 fields from each condition. Grains over apparently fat-free areas of the cytoplasm include those over the endoplasmic reticulum, mitochondria, Golgi apparatus, and matrix. Percentages are given in parentheses.

Absorption Conditions	Fat droplets	Cytoplasm	Microvilli terminal web	Nuclei	Total count
Linoleic acid + glucose, 5 min	398 (77)	83 (16)	29 (6)	4 (1)	520
Glucose 5, min		79	6	9	94
Linoleic acid + glucose, 20 min	600 (82)	106 (15)	5 (1)	19 (3)	730
Linoleic acid + glucose, 20 min, fat-extracted		53	8	1	62

TABLE II

Glucose-6-H³ Incorporation into Rat Intestinal Segments

Scintillation counts of various fractions following the Folch extraction procedure and saponification of homogenates. CPM × 100.

	100 μc Glucose-H³ in	Seg. wt.	Aqueous phase	Residue	Chloroform phase	Non-sapon. material	Free fatty acids	Aqueous residue (by subtraction)
		mg						
Rat 1	0.1 cc Fatty chyme	190	217	9	2752	45	30	2677
	0.1 cc Water	170	87	4	529	22	26	481
Rat 2	0.1 cc Fatty chyme	140	491	6	2310	28	22	2261
	0.1 cc Water	170	63	10	454	31	31	392

Saponification

demonstrate that a considerable amount of label is incorporated into the cells. The results obtained in this study give convincing evidence that a high percentage of this incorporated label is in the glycerol moiety of newly synthesized glycerides. It can be seen from Table I that about 80% of the grains was found over areas containing droplets of fat. The use of physical developer with attending smaller grain size highly suggests that the source of emissions is from within the droplets. The resolution obtainable by the methods employed has been shown to depend on a number of parameters (2, 9), among which is the size of the silver halide crystals. With Ilford L-4 emulsion, this size (120 mμ) is within the size range of many of the fat droplets dispersed throughout the endoplasmic reticulum. In that the resolution can be no better than the size of the silver halide crystal (27), it cannot be stated with certainty that the source is derived from within these fat droplets. However, droplets within the dilations of the Golgi cisternae and in the intercellular spaces are frequently numerous and closely packed. The concomitant concentration of grains over such aggregations is more indicative of localization of the label within the fat droplets. Furthermore, the localization and distribution of grains are very similar to those observed in radioautographs of intestinal absorptive cells exposed to oleic acid-H³ chyme over the same range of absorptive periods (20). It is of course possible that not all glycerides are in droplet form, but are also present in molecular dispersion, such as indicated in a radioautographic study by Strauss (37).

It is additionally significant that, following the absorption of aqueous glucose, or when the fat is extracted from the tissue following glucose–fatty chyme absorption, the grain counts are consider-

ably lower. It has previously been shown that labeled glucose is incorporated into complex carbohydrates in intestinal epithelial cells (30, 31) and into mucopolysaccharides which concentrate on the surface coat of microvilli after 1 hr (19). It is assumed that most of the "residual" labeling observed in the present study, as well as that observed in the goblet cells, is of this nature. The significant increase in glucose incorporation during fat absorption as observed in the radioautographs and in the tissue homogenates is in agreement with the findings of Dawson and Isselbacher (13) and Holt et al. (17) in their in vitro studies. It was clearly demonstrated by the former and again in this study that incorporation of glucose carbon into glyceride-glycerol is greatly enhanced when fatty acids are added to the absorption mixture.

Therefore it is suggested that following the absorption of fatty acids and glucose-H^3 most of the grains observed relate to the synthesis of glycerol-labeled glycerides. However the use of labeled glyceride-glycerol precursors does not establish the degree to which synthesis is completed. Intermediates in the synthetic pathway, the phosphatidic acids (21), as well as triglycerides may be present.

It is of interest that a similar pattern of grains can be seen following absorption from safflower oil–prepared chyme. This suggests that the *a*-glycerophosphate pathway is utilized under conditions when monoglycerides are presumably absorbed. The relative importance of this finding to the total synthetic mechanism cannot be inferred from this study since the monoglyceride pathway was not examined and the degree of lipid hydrolysis in the lumen was not known. The results obtained in a recent study by Mattson and Volpenhein (25), however, suggest that approximately 28% of dietary fats is completely hydrolyzed and that a corresponding amount of *de novo* synthesis of triglycerides is involved. Although no attempt was made at quantification in this study, it is tempting to speculate that after safflower oil absorption the failure of labeling to approach the over-all concentration, which was observed following linoleic acid absorption, may reflect a more rapid absorption of free fatty acids than glycerides, such as described by Borgström (3) and Burr et al. (7). This could account for the similarity in the radioautographs of linoleic acid and safflower oil absorption during the initial phases and the apparent disparity in more prolonged stages when monoglyceride uptake might prevail in the latter.

Glyceride synthesis in the region immediately subjacent to the terminal web zone is strongly implied by the fact that grains are readily visible over this area at all time intervals studied (from 2 to 20 min). This synthesis most likely involves the abundant endoplasmic reticulum in the area. Indeed, a good deal of evidence is available which indicates that the enzymes involved have the highest activities in the microsome fraction of absorptive cells (4, 18, 34). Evidence for lipid uptake by the cells from micellar solutions (16) is quite convincing (8, 22, 35, 36). It would follow that glyceride synthesis begins when incoming lipid molecules in the cell matrix contact the most superficial elements of the endoplasmic reticulum.

The appearance of labeled glycerides across the entire epithelium is very rapid, grains being observed over the length of the cells within 2 min after the initiation of absorption. Fat droplets likewise are distributed throughout the cells following brief exposures to chyme. The use of radioautography has shown, in fact, that oleic acid-H^3 traverses the epithelium within 1 to 2 min (20). A rapid transport of glycerides or their precursors across the cell is certainly indicated. It is difficult, therefore, to determine whether glyceride synthesis is limited to the most apical portions of endoplasmic reticulum and the triglycerides rapidly transported to deeper parts of the cell, or whether all portions of this organelle throughout the cell are involved.

The absorptive cells frequently display a proportionately greater concentration of grains over the Golgi apparatus following absorption periods of 10 to 20 min. It is possible to interpret this observation as evidence for considerable glyceride synthesis within this organelle. The Golgi apparatus is known, however, to retain large numbers of fat droplets (28) beginning very early in the absorptive process (20). The abundance of label in this area may be best explained, therefore, as resulting from an accumulation of glycerides synthesized in more apical portions of the cell. These considerations regarding the site of glyceride synthesis are currently under additional study.

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