An electron microscopic and functional study of very low density lipoproteins in intestinal lymph

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ABSTRACT Previous studies with fasting rats showed that the intestine produces endogenous very low density lipoproteins (VLDL) which resemble those in the plasma. Intestinal VLDL also were found to be important in lipid transport during absorption of saturated but not of unsaturated fat. These findings depended upon separations of a chylomicron-rich fraction (S_f > 400) from VLDL (S_f 20–400) by preparative ultracentrifugation methods based on particle flotation rates. The present studies correlate this method with electron microscopic measurement of lipoprotein particle size.

Almost all intestinal lymph lipoprotein particles from fasting rats were less than 750 A in diameter, and could not be distinguished morphologically from plasma VLDL. Cholestyramine administration or bile diversion led to decreased lymph lipid output, correlating with marked reduction in VLDL. This supports the concept that lymph VLDL contain endogenous lipid which is reabsorbed from the intestinal lumen.

During exogenous fatty acid absorption, lymph lipoprotein particle sizes were significantly smaller after administration of palmitate than after administration of linoleate, a finding consistent with ultracentrifugal evidence of the importance of VLDL in lipid transport during palmitate absorption.

These studies fully confirm and extend earlier observations. Together, they show that the intestine is a source of endogenous VLDL in the fasting animal. In addition, significant quantities of exogenous lipid are transported in VLDL during palmitate absorption, whereas with linoleate absorption nearly all lipid is in chylomicrons. These findings indicate that the small intestine plays a role in lipoprotein metabolism which extends beyond the absorption of dietary fat.

SUPPLEMENTARY KEY WORDS bile diversion · cholesterol · cholestyramine · chylomicrons · intestinal absorption · linoleic acid · palmitic acid · triglyceride

VERY LOW DENSITY ("prebeta") lipoproteins are the major form of endogenous triglyceride transport in plasma (1, 2). It has been conclusively demonstrated that VLDL are produced in the liver, and that free fatty acids, mobilized from adipose tissue, are precursors of VLDL triglyceride (1, 3). There is evidence, however, that synthesis of plasma VLDL may occur in hepatectomized animals, presumably as a result of intestinal lipoprotein production (4, 5).

Recent studies have demonstrated that triglyceride-rich lipoproteins are produced in the rat intestine, and enter plasma in the absence of dietary lipid (6–10). These lipoproteins were found to be similar to plasma VLDL in composition, S_f value (20–400), and electrophoretic mobility (7, 9, 10). In the fasting state, VLDL carried most of the endogenous lipid in intestinal lymph, exceeding in quantitative importance both chylomicrons (S_f > 400) and lipoproteins of d > 1.006.[1] Procedures which impaired absorption of lipid from the intestinal lumen, e.g. bile diversion or administration of cholestyramine, a bile acid sequestrant, led rapidly to a marked

Abbreviations: VLDL, very low density lipoproteins.

[1] The distinction between the two classes of triglyceride-rich lipoproteins, chylomicrons and VLDL, was based operationally on the criteria of Zilversmit (11), in which chylomicrons are defined as alimentary particles of S_f > 400.

decrease in lymph triglyceride and cholesterol, correlating with a nearly complete disappearance of VLDL and chylomicrons as determined by preparative ultracentrifugation. These findings suggested that the endogenous lipids in both VLDL and chylomicrons were derived from bile and from gastrointestinal epithelium which had been shed into the lumen.

VLDL were also found to be of importance in lipid transport during the absorption of exogenous palmitic acid, administered intraduodenally as mixed bile salt–monoolein–fatty acid micelles (7, 12). In marked contrast, however, VLDL played only a minor role during the absorption of linoleic acid, and carried less lipid even than in the fasting state.

In all of these studies, measurement and interpretation of the distribution of lipids among lymph lipoprotein fractions were based on ultracentrifugal separations. It seemed important, therefore, to assess the validity of this approach by methods independent of particle density and flotation rate. In the present study, samples of lymph obtained under the various experimental conditions previously reported (7, 9, 10, 12) were examined by electron microscopy, as well as by preparative ultracentrifugation. Evidence is presented which confirms and extends the earlier interpretations based on ultracentrifugation alone.

METHODS

Materials

Palmitic acid, linoleic acid, glycerol monooleate, and sodium taurocholate were obtained from Calbiochem, Los Angeles, Calif., and tested for purity by thin-layer chromatography (13). Palmitic acid and linoleic acid were more than 95% pure. Monoolein contained small amounts of fatty acid and diglyceride. Sodium taurocholate contained no detectable free cholic acid. Cholestyramine was obtained as Questran from Mead Johnson & Co., Evansville, Ind.

Animals and Experimental Procedure

Male 350–400 g albino Sprague-Dawley rats (Bioscience Animal Laboratories, Oakland, Calif.) were maintained prior to study on a standard laboratory diet (FP Standard Rat and Mouse Food, Feedstuffs Processing Company, San Francisco, Calif.). Operative procedures, including cannulation of the mesenteric lymphatic duct, common bile duct, and duodenum, were carried out as previously described (10). After operation, the animals were placed in restraining cages and received 0.85% NaCl by continuous intraduodenal infusion; they were allowed to stabilize overnight.

During the experiments, all animals received intraduodenal infusions of 0.85% NaCl, 5 ml/hr. Where indicated, the infusion also contained mixed micelles (fatty acid–monoolein–taurocholate) or a suspension of cholestyramine. Mixed micelles were prepared in 10 ml of 20 mM sodium taurocholate in 0.85% NaCl, and contained fatty acid (19.2 mM) and monoolein (9.6 mM) (10, 13). 5 ml of this solution was administered in the first 30 min of the experiment; the remaining 5 ml was diluted with an equal volume of 0.85% NaCl, and this material was infused at a constant rate over the final 2 hr. The total amount of lipid administered to these animals was 80–90 mg over a 3 hr period.

Lymph samples were collected at room temperature in tubes previously moistened with a 10% solution of Na_2EDTA. In some experiments, lymph was subjected to preparative ultracentrifugation (10) in order to fractionate it into a chylomicron-rich fraction ($S_f > 400$), VLDL, and $d > 1.006$ lipoproteins. Whole lymph and lymph lipoprotein fractions were analyzed for triglyceride content by the method of Carlson (14), and for cholesterol by the method of Zlatkis, Zak, and Boyle (15), as previously described (10).

Electron Microscopy of Lymph

Samples of lymph were fixed in osmium tetroxide and were examined with a Philips EM-300 electron microscope following shadow-casting with carbon-platinum pellets as described by Jones and Price (16). Particle sizes on photographic negatives (×10,000) were measured with an optical microcomparitor (Gaertner Scientific Corp., Chicago, Ill.).

In most experiments, samples for electron microscopy were taken from the completed collection obtained during the indicated time period. The only exceptions to this were the samples of lymph obtained during mixed micelle infusion; in these experiments, samples were taken at 1 hr. This was the time of maximal rate of appearance of lipid in lymph, as determined by measurement of lymph radioactivity following the intraduodenal infusion of mixed micelles labeled with fatty acid-^{14}C.[2]

RESULTS

Intestinal Lymph Lipoproteins during Intraduodenal Infusion of Isotonic NaCl

Lymph was collected during a 3 hr intraduodenal infusion of isotonic NaCl. Analysis (preparative ultracentrifugation) for distribution of lipids among lipoprotein fractions gave results similar to those reported previously (10): VLDL carried 1.5 mg of triglyceride and 0.18 mg of cholesterol per hr, while chylomicrons carried 1.7 mg and 0.07 mg, respectively.

[2] Ockner, R. K., and A. L. Jones. Unpublished observations.

The importance of VLDL in fasting lymph is confirmed in the electron micrograph of the shadow-casted lymph d < 1.006 lipoproteins (Fig. 1), in which it can be seen that most particles were in the size range of plasma VLDL, that is, 300–1000 A in diameter (16–18). Very few particles with diameters greater than 750 A are seen (Table 1), suggesting that the preparative ultracentrifugation procedure actually overestimated the quantitative significance of chylomicrons and underestimated that of VLDL in intestinal lymph from fasting rats. The inset to Fig. 1 is an electron micrograph of VLDL from the plasma of a fasting, unoperated rat, and is presented for purposes of comparison with the lymph lipoproteins. It is evident that the particles in the two fluids are morphologically indistinguishable.

TABLE 1 SIZE DISTRIBUTION OF PARTICLES (d < 1.006) IN INTESTINAL LYMPH AND PLASMA FROM FASTING RATS

Particle Diameter	Plasma	Lymph
A	*No. of particles*	
150–250		1
250–350	1	1
350–450	16	18
450–550	45	19
550–650	29	27
650–750	9	28
750–850		5
850–950		
950–1050		1
Mean particle diameter	524 A	573 A

The diameters of 100 particles in each group were determined from random areas of the electron microscope negative (see Materials and Methods).

Intestinal Lymph Lipoproteins during Intraduodenal Infusion of Mixed Micelles

Administration of palmitate–monoolein and linoleate–monoolein micelles resulted in similarly increased lymph triglyceride values (20.6 and 16.2 mg/hr, respectively). As before, however (12), preparative ultracentrifugation showed that relatively more lipid was carried in VLDL during palmitate absorption than during linoleate absorption (22% and 6% of total lymph triglyceride, respectively).

In Figs. 2 and 3 are shown typical electron micrographs of the palmitate and linoleate lymph preparations, respectively. The following several differences between the two samples are evident. Particles in the lymph of the linoleate-infused animal show significantly greater electron density, reflecting the greater osmiophilia of the unsaturated fatty acid (compare also Figs. 4 and 5). The particles from the animal given linoleate show a greater tendency to clump (see also Fig. 6 *a–c*). The individual particles in the linoleate lymph are generally of larger diameter than in the palmitate lymph (also, Figs. 4 and 5). The particles in the palmitate lymph are relatively poorly fixed, as evidenced by the lesser degree of electron density and the apparent "collapse" of many of the larger particles on the thin Formvar film of the electron microscope grid. This apparent lack of particle stabilization is in all likelihood due to the lesser degree of osmiophilia of the saturated fatty acid. This has the additional effect of permitting the particles to flatten somewhat, instead of remaining spherical. Thus, it can be seen that the shadows of the larger particles of the palmitate preparation are shorter than expected, indicating a lower particle "height" or vertical diameter. Although precise quantitative interpretations of this phenomenon cannot be made, the flattening has the effect of falsely increasing apparent particle diameters when "viewed" from above microscopically. This not only precludes any accurate assessment of particle size, but also strongly suggests that the difference in particle sizes between the palmitate and linoleate lymph is even greater than is immediately evident from inspection of the electron micrographs.

DISCUSSION

In these studies we have shown that intestinal lymph from fasting animals contains abundant lipoprotein particles similar in size (300–1000 A) to the VLDL in plasma of both man and rat, and in the perfusate from the isolated perfused liver preparation (17, 18). Moreover very few particles larger than 1000 A (chylomicrons) were seen. These observations confirm and extend earlier evidence that in the fasting state most of the endogenous lipid in intestinal lymph is transported in VLDL which resemble plasma VLDL in composition, S_f value, and electrophoretic mobility (7, 9, 10). In addition, the relative scarcity in the electron micrograph of particles in the size range of chylomicrons suggests that fractionation of lymph lipoproteins from fasting animals by preparative ultracentrifugation, both in the present study and as previously described (10), leads to an underestimation of the quantitative importance of VLDL.

This underestimation of VLDL resulting from ultracentrifugation appears to be caused by two factors. First, there is overlap between chylomicrons and VLDL with regard to particle density and flotation rate, and, therefore, the dividing line between the two classes is necessarily arbitrary. Although endogenous VLDL are generally regarded as having an S_f of 20–400 (corresponding to diameters of up to 750 A), there is evidence that this class may include particles up to 1000 A and perhaps slightly over (18, 19), corresponding to S_f of approximately 800 (20) and within the chylomicron range (11). Second, electron microscopic examination of the

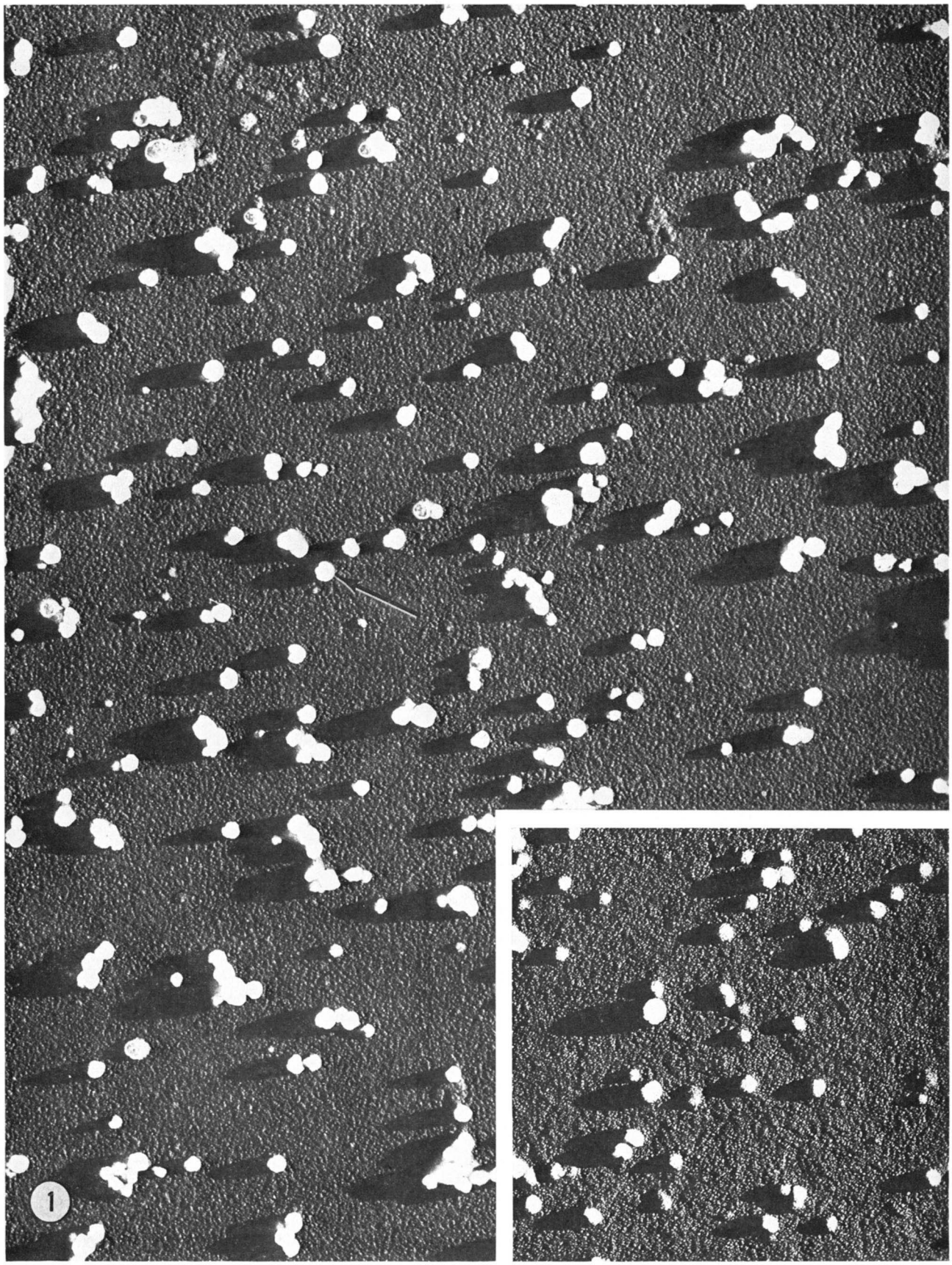
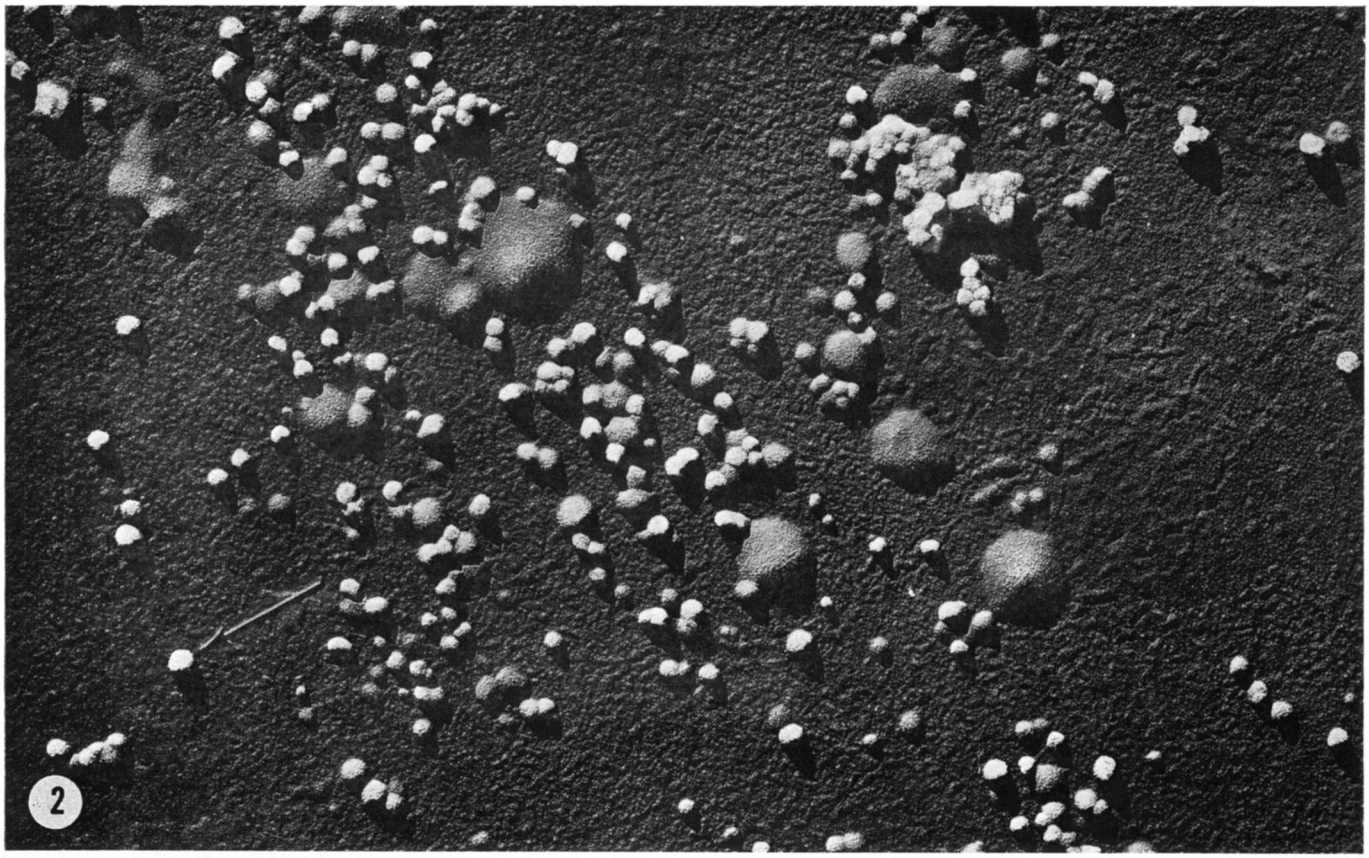
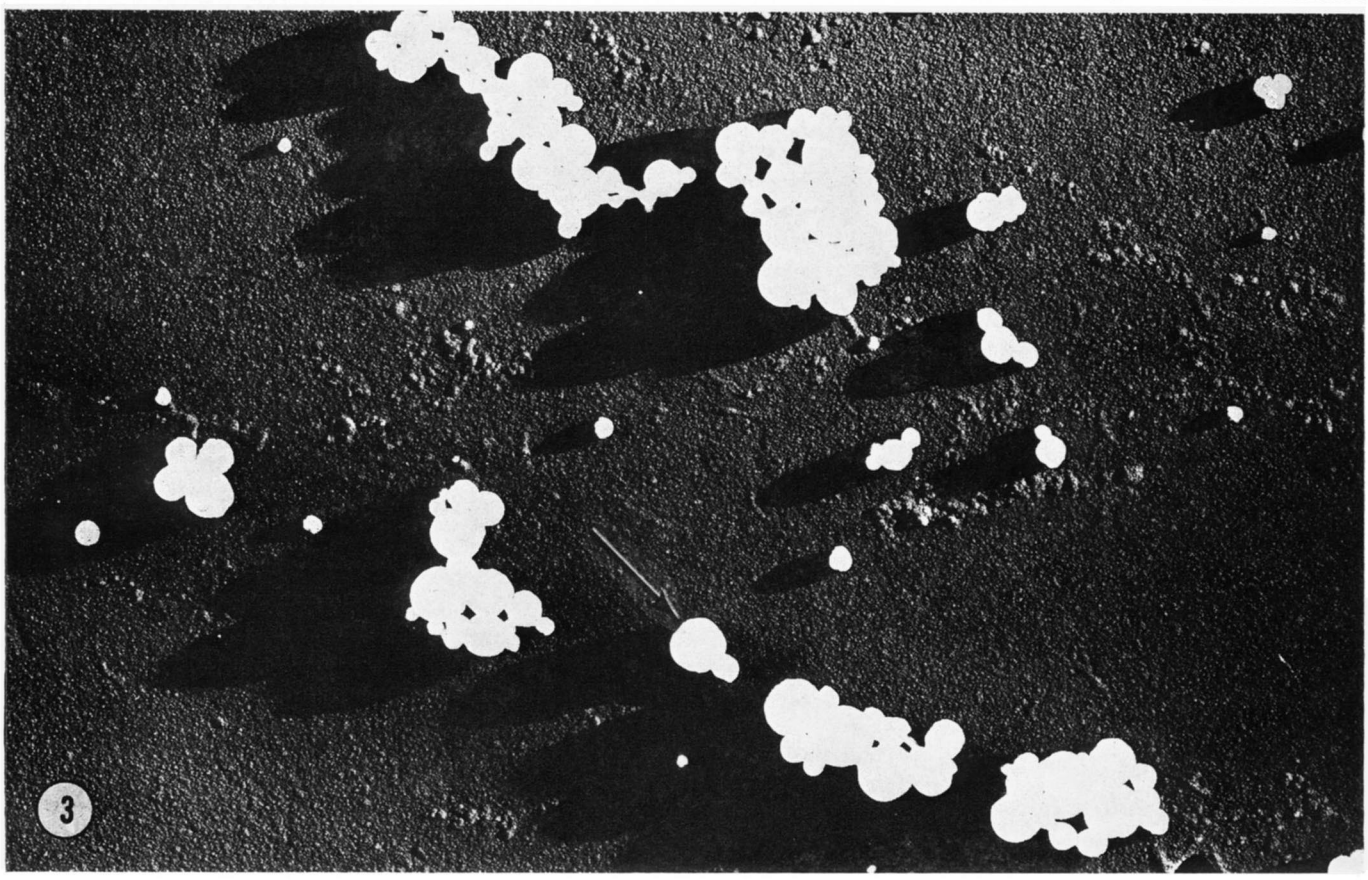
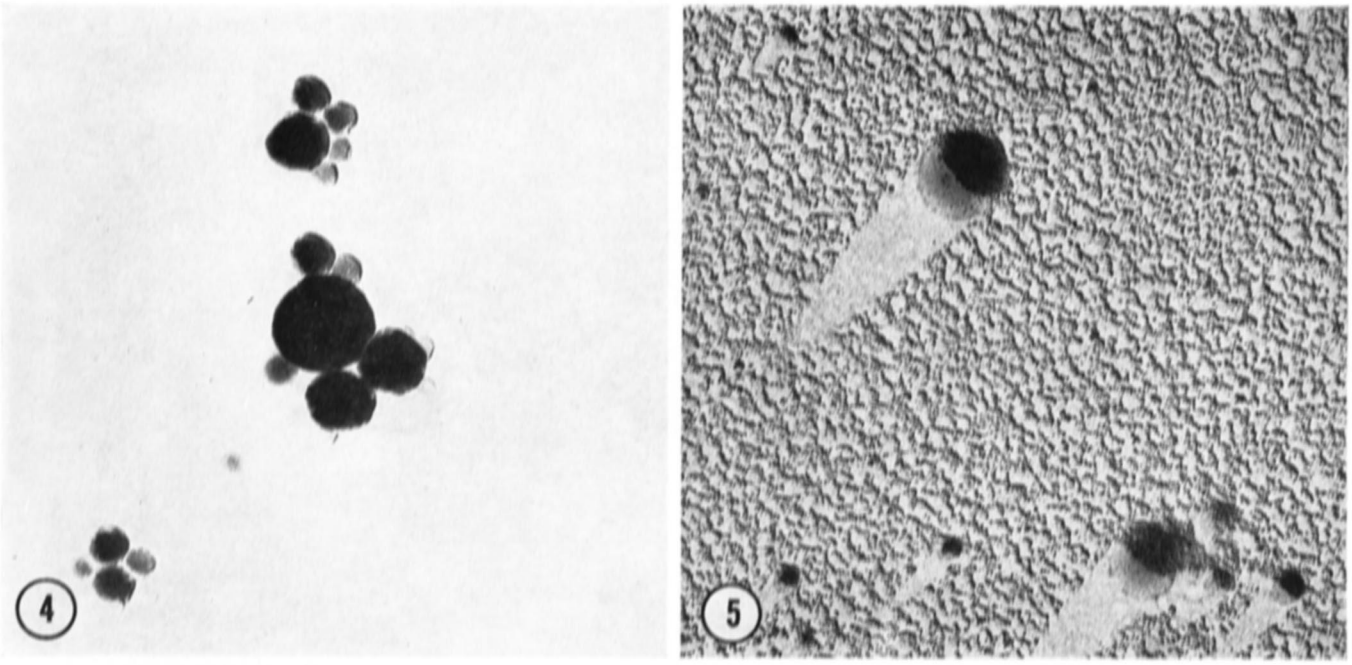


FIG. 1. Lipoproteins (d < 1.006) in intestinal lymph of the fasted rat. Lymph lipoproteins (d < 1.006) were fixed in osmium tetroxide and shadowed with carbon–platinum pellets. The particles range in size from 300 to 1000 A. The particle at the arrow is 700 A in diameter. The inset at the lower right shows a similar preparation of plasma VLDL from a normal fasting rat at the same magnification for comparison. Note the striking similarity in particle size distributions in the two preparations. ×51,000.





FIGS. 2 and 3. Lymph lipoproteins during absorption of mixed micelles. These figures at the same magnification show the relative size distribution of intestinal lymph particles following intraduodenal infusion of palmitate–monoolein and linoleate–monoolein micelles, respectively.



FIGS. 4 and 5. Positive image of lymph lipoprotein particles. Samples of lymph from linoleate- and palmitate-infused animals, respectively Note the electron opacity of the linoleate-rich particles in Fig. 4. Although in Fig. 5 the outlines of the particles are clearly shown, it is readily noted that the particles are less electron opaque. The "cap" observed around the leading edge of the palmitate chylomicron is due to the accumulation of platinum which otherwise (in the absence of the particle) would have distributed itself within the shadow area. Both of these figures represent examples of the largest chylomicrons obtained from either animals. Characteristically, the particles of the linoleate-infused animal are larger than those which are obtained after palmitate infusion. The largest particle observed in Fig. 4 is 3000 A in diameter, while the largest particle observed in Fig. 5 is 2500 A in diameter. ×44,000.

chylomicron-rich fraction of lymph from fasting rats showed that virtually all particles were in the VLDL size range, indicating that the conditions employed for preparative ultracentrifugation of lymph led to the inclusion of some VLDL in the chylomicron rich fraction.[2] Therefore, separation of these two classes by the preparative procedure unavoidably results in the inclusion, in the chylomicron fraction, of some lymph particles which actually are similar in size to endogenous VLDL.

The infusion of cholestyramine or the diversion of bile caused a rapid and significant decrease in both the abundance and size of the VLDL in intestinal lymph as studied by electron microscopy, correlating with a decrease in lymph triglyceride and cholesterol.[2] This finding confirms previous evidence, obtained by preparative ultracentrifugation, that the fall in lymph lipid output in these two experimental situations is due primarily to a decrease in VLDL and chylomicrons (10). These observations strongly support the concept that the lipids in lymph VLDL are derived from the intestinal lumen during the fasting state, thereby confirming that these endogenous particles originate in the intestine rather than in the liver. Direct evidence for this conclusion has come from recent electron microscopic studies (21), in which we demonstrated that VLDL are present in the smooth endoplasmic reticulum and Golgi apparatus of the intestinal absorptive cells of fasting rats, as well as in the intercellular spaces and lacteals. It is interesting that these intracellular locations correspond precisely to those of VLDL in the hepatocyte during active

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In Fig. 2, following palmitate–monoolein infusion, the great majority of the particles shown are within the size range of very low density lipoproteins (300–1000 A). The particle at the arrow is 700 A in diameter. The larger particles appear to have flattened out on the Formvar film. Their shadow lengths, which reflect particle height, should be proportionately longer than those of the smaller particles, but in fact they are actually shorter. This type of distortion makes estimates of particle size difficult if not impossible. By carefully scanning the electron microscope grid, occasionally one can find large palmitate-rich particles which have retained their spherical configuration (see Fig. 5). These then could be used for size evaluation.

Fig. 3 shows intestinal lymph particles after linoleate–monoolein infusion. In general, the particles are of greater size and show a tendency to clump. Note the relatively long shadow length of the 1800 A particle at the arrow. (These micrographs are printed as the negative to enhance depth perception. As a result, the increased whiteness seen in the linoleate-rich particles actually indicates greater electron density, due to the relative osmiophilia of the unsaturated fatty acids.) ×41,000.

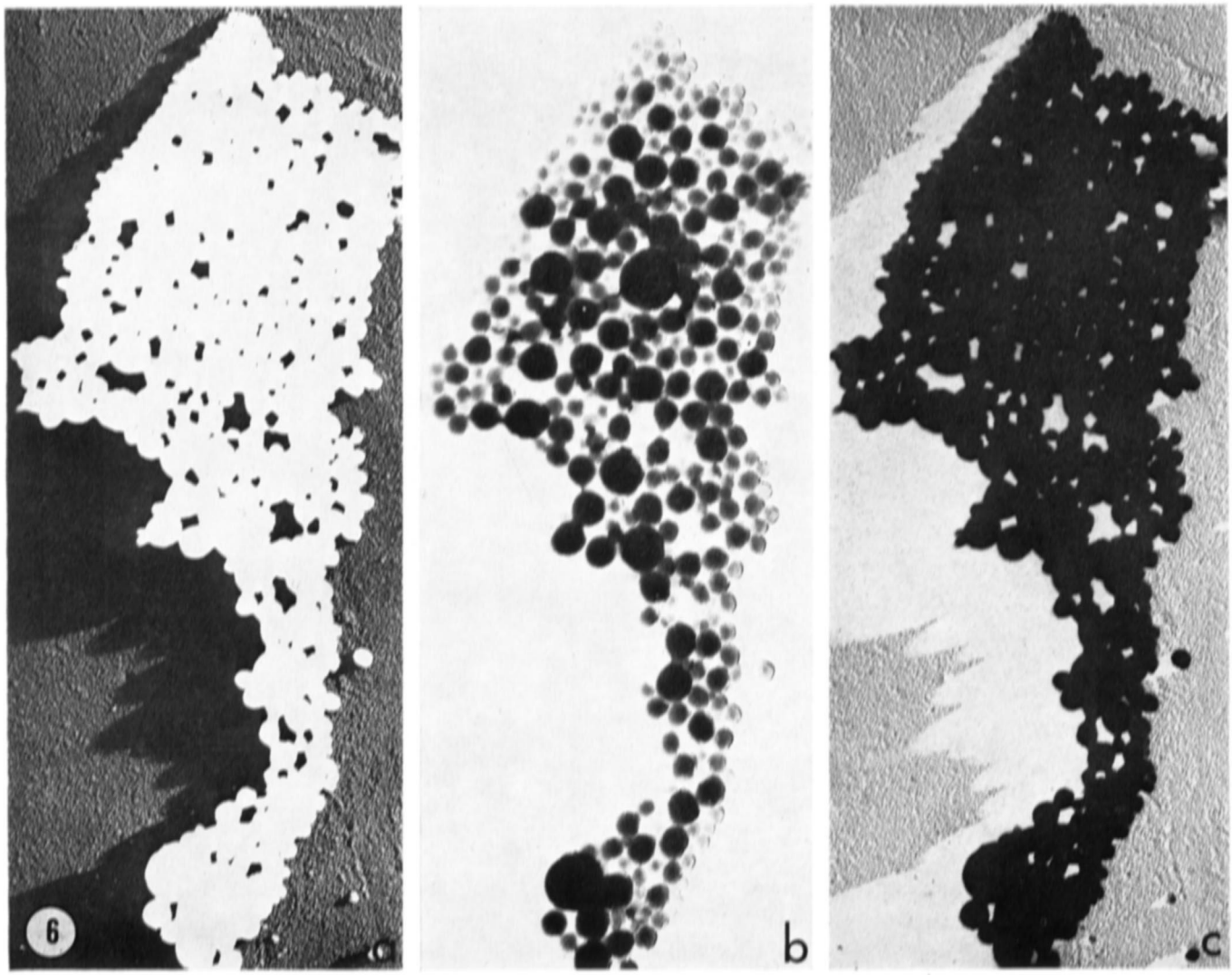

FIG. 6*a–c*. Effect of photographic technique on particle resolution. These micrographs of the same clump of linoleate-rich lymph particles have been processed by different techniques. When the electron image of shadowed clumped lipoproteins is printed conventionally as the negative as in Fig. 6*a*, or as the positive in Fig. 6*c*, it is difficult to estimate particle size. However, if the particles are electron opaque, the micrograph can be printed on very high contrast photographic paper and the background reduced, resulting in the image seen in Fig. 6*b* (also Fig. 4). With the background absent, it is possible to delineate clearly the boundaries of the clumped particles, and, thereby, to measure the size of the individual particles. ×31,000.

lipoprotein secretion (17). These particles are virtually absent from the intestinal mucosa following bile diversion, and are markedly reduced in the mucosa of animals receiving intraduodenal infusions of cholestyramine.

VLDL-size particles also are produced by the intestine during fat absorption, as shown by electron microscopy of intestinal lymph during the intraduodenal infusion of mixed micelles. The quantitative significance of this fraction, however, was influenced by the major fatty acid being absorbed. Thus, VLDL were significantly more abundant with palmitate absorption than with linoleate absorption. Conversely, the lymph during linoleate absorption contained a larger number of particles having diameters greater than 1000 A. Furthermore, these differences in particle size are almost certainly even greater than apparent because of the partial collapse, and the resulting falsely increased transverse diameter of the palmitate-rich particles. Poor fixation by osmium of particles rich in saturated fatty acids has also been observed by others (22, 23). A further difference was that linoleate-rich particles had a greater tendency to clump than did the palmitate-rich particles. While this could have been due to an artifact of the fixation process, it suggests the possibility that clumping of linoleate-rich particles in vivo may lead to their coalescing, and thus contribute to the formation of the larger particles seen in lymph during linoleate absorption.

The morphological evidence that lymph lipoprotein particle sizes differ during the absorption of these two fatty acids is consistent with the findings obtained when lipoprotein fractions were separated by preparative ultracentrifugation and analyzed for distribution of lipid. In the lymph preparations examined by electron microscopy in the present study, and in the larger groups previously reported (12), more triglyceride and cholesterol were carried in VLDL during the absorption of palmitate than during the absorption of linoleate.

The present studies, combining functional and morphologic approaches, fully confirm and extend earlier observations concerning two important aspects of the role of the small intestine in mammalian lipoprotein metabolism. First, it is evident that the intestine produces VLDL which are similar to those in plasma when compared by a variety of analytical, physical and morphologic techniques. Even in the absence of dietary lipid, these lipoproteins enter the intestinal lymph and the plasma continuously, and are produced in the intestine from the lipids of bile and shed gastrointestinal epithelium. The VLDL of intestinal origin are almost certainly included with other plasma VLDL in the lipoprotein fractionation techniques generally employed, such as ultracentrifugation and electrophoresis. In addition to the liver, therefore, the intestine is a source of endogenous plasma VLDL. The quantitative significance of this source has not as yet been determined. In the rat, however, endogenous intestinal lymph triglyceride enters the plasma at a rate of approximately 5–6 mg/hr (6, 10), while total plasma triglyceride turnover is of the order of 15 mg/hr (24). These observations suggest that the intestine may contribute up to 40% of plasma triglyceride in the fasting rat (10), although others have made lower estimates (8). Because the secretion of bile and the turnover of gastrointestinal epithelium are functions common to all mammals, the formation of intestinal VLDL would be expected to occur in the absence of dietary lipid in other species, including man. Indeed, we have recently demonstrated that the intestinal mucosa of normal human volunteer subjects, following a 40 hr fast, contains particles similar in size and location to the VLDL in rat intestine referred to above (21, 25). This observation further supports the concept that in man as in the rat, the small intestine is a source of endogenous plasma VLDL.

A second aspect of intestinal lipoprotein production confirmed in these experiments is that the absorption of various long-chain fatty acids may lead to differences in distribution of lipids among lymph lipoproteins. For example, it was shown (7, 12) that during the absorption of unsaturated fatty acids (oleic or linoleic), lymph cholesterol is transported to a far greater extent in chylomicrons than in other fractions, whereas during absorption of saturated fatty acid (palmitic), cholesterol was transported equally in VLDL and chylomicrons. Such differences in the lipoprotein form in which lymph lipids enter plasma may be of metabolic significance, and it has been shown that chylomicron cholesterol has a half-time of survival in plasma only one-half that of VLDL cholesterol (7, 12). These findings suggest that the cholesterol-lowering effect of dietary unsaturated fatty acids may be mediated in part through their effect on lipoprotein formation at the intestinal level.

The authors wish to acknowledge the helpful comments of Dr. Richard Havel and Dr. Rudi Schmid during the preparation of this manuscript, and the technical assistance of Mr. Edward A. Miller, Miss Gabrielle Rouiller, and Mrs. Janice Yager.

The research was supported in part by Grants AM-13328 and AM-05598 from the National Institutes of Health, and by the Veterans Administration Hospital, San Francisco, Calif.

Manuscript received 26 August 1969 and in revised form 1 December 1969; accepted 13 February 1970.

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