Intracellular apoA-I and apoB distribution in rat intestine is altered by lipid feeding

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Abstract Intracellular forms of chylomicrons, very low density lipoprotein (VLDL) and high density lipoprotein (HDL) have previously been isolated from the rat intestine. These intracellular particles are likely to be nascent precursors of secreted lipoproteins. To study the distribution of intracellular apolipoprotein among nascent lipoproteins, a method to isolate intracellular lipoproteins was developed and validated. The method consists of suspending isolated enterocytes in hypotonic buffer containing a lipase inhibitor, rupturing cell membranes by nitrogen cavitation, and isolating lipoproteins by sequential ultracentrifugation. ApoB and apoA-I mass are determined by radioimmunoassay and newly synthesized apolipoprotein characterized following [^{3}H]leucine intraduodenal infusion. Intracellular chylomicron, VLDL, low density lipoprotein (LDL), and HDL fractions were isolated and found to contain apoB, apoA-IV, and apoA-I. In the fasted animal, less than 10% of total intracellular apoB and apoA-I was bound to lipoproteins and 7% of apoB and 35% of apoA-I was contained in the d 1.21 g/ml infranatant. The remainder of intracellular apolipoprotein was in the pellets of centrifugation. Lipid feeding doubled the percentage of intracellular apoA-I bound to lipoproteins and increased the percentage of intracellular apoB bound to lipoproteins by 65%. Following lipid feeding, the most significant increase was in the chylomicron apoB and HDL apoA-I fractions. These data suggest that in the fasting state, 90% of intracellular apoB and apoA-I is not bound to lipoproteins. Lipid feeding shifts intracellular apolipoprotein onto lipoproteins, but most intracellular apolipoprotein remains non-lipoprotein bound. The constant presence of a large non-lipoprotein-bound pool suggests that apolipoprotein synthesis is not the rate limiting step in lipoprotein assembly or secretion. — **Magun, A. M., B. Mish, and R. M. Glickman.** Intracellular apoA-I and apoB distribution in rat intestine is altered by lipid feeding. *J. Lipid Res.* 1988. **29:** 1107–1116.

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Intracellular chylomicron, VLDL, and HDL lipoproteins have been identified and partially characterized from within rat intestinal enterocytes (1–3). These intracellular particles are thought to be nascent precursors of lymph and plasma lipoproteins (4). Since the intestine synthesizes 50% of apoA-I and apoA-IV in rat plasma (5), the intracellular distribution of apoproteins among nascent lipoproteins and the factors regulating intracellular lipoprotein assembly may be important determinants of plasma lipoprotein levels (6).

Prior studies of intracellular apoprotein distribution have employed immunofluorescent (7, 8) or immunoperoxidase (9, 10) staining methods. These methods do not permit localization of apoprotein to a specific class of intracellular lipoprotein and they are qualitative techniques. A different approach to intracellular apoprotein distribution was taken by Alpers et al. (11), who first described the intracellular distribution of apolipoprotein as isolated from the supernatant of homogenized rat enterocytes. They showed that a large proportion of intracellular apoA-I was non-lipoprotein bound, that most of apoB was lipoprotein bound, and that fat feeding increased apoA-I and apoB in the lipoprotein fractions.

In this study, we report our quantitative findings on intracellular apolipoprotein distribution in rat enterocytes. We describe and validate a method for isolation of intracellular lipoprotein fractions. We describe the composition of intracellular lipoproteins, the distribution of apolipoprotein in the fasted state, and the effect of lipid feeding on intracellular apolipoprotein distribution.

METHODS

Isolation of intracellular lipoproteins

Isolation of enterocytes. Male Sprague-Dawley rats weighing 200–250 g were used in all experiments. Fasted animals were allowed only water for 24–36 hr. Lipid-fed animals were fasted overnight and then administered (by gavage or intraduodenal infusion via a duodenotomy) 4 ml of a 10% solution of Intralipid 1 hr prior to harvesting the

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; RIA, radioimmunoassay; TLC, thin-layer chromatography.

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cells. Intraduodenal infusions were performed as previously described (3). In some experiments, to radiolabel intracellular apolipoprotein, 3 ml of a solution containing 0.75 mCi of [^{3}H]leucine were infused into the duodenum of anesthetized rats for 20 min prior to harvesting the intestines. Four to six rat intestines were used per preparation. The second portion of the duodenum to the distal jejunum was removed for analysis. Rat intestinal epithelial cells were harvested using a modification of the Weiser technique as previously described (3). In one experiment, separate villous and crypt fractions were isolated (12). The villous fraction contained cells isolated during the first 15 min of incubation in phosphate-buffered saline (containing EDTA and dithiothreitol) and the crypt fraction contained cells isolated during the next 20 min of incubation in buffer.

Cellular Homogenization. The isolated cells were suspended in 200 ml of ice-cold hypotonic Veronal buffer (pH 8.3, 37.5 mM sodium diethyl barbiturate and 7.3 mM barbituric acid) which also contained, in some experiments, 2 mM diethyl-*p*-nitrophenylphosphate (E-600), a lipase inhibitor (13). The cellular suspension was kept at 5-10°C throughout the isolation procedure. The cells were dispersed in a glass Dounce homogenizer and passed twice through a nitrogen cavitation apparatus (Parr) at 2000 psi for 15 min, a pressure known to disrupt intracellular organelles (14, 15). The efficacy of the cavitation apparatus as a method to rupture cells was studied by microscopic examination. Slides of the homogenate, supernatant, and pellet revealed no intact epithelial cells.

The homogenate following cavitation (from which the starting concentration of intracellular apoprotein was calculated) was centrifuged at 100,000 *g* for 1 hr in an SW 28 rotor (Beckman). The top of the 100,000 *g* supernatant containing intracellular chylomicrons was removed by pipette. The pellet was resuspended in Veronal buffer, passed through the cavitation apparatus for 15 min at 2000 psi and then added to the remaining solution. The homogenate was then centrifuged at 100,000 *g* for 16 hr in an SW 28 rotor to isolate intracellular VLDL, which were removed by pipette. The resulting infranatant was raised to d 1.070 g/ml with NaBr and centrifuged at 100,000 *g* for 15 hr in a 70 TI rotor (Beckman) to isolate LDL particles. The d 1.070 g/ml infranatant was raised to 1.21 g/ml and centrifuged in a 70 TI rotor at 100,000 *g* for 44 hr, following which HDL lipoproteins were removed by pipette. The LDL and HDL fractions were overlaid with d 1.006 and 1.070 g/ml NaBr solutions and centrifuged for 16 hr in an SW 50.1 rotor (Beckman) to allow any lighter lipoproteins to float. Throughout the preparation, aliquots of the infranatant and pellets were taken for analysis.

Validation experiments

A number of experiments were performed to determine possible effects of the cavitation apparatus on intestinal cells and lipoproteins. Rat plasma (5 ml) was added to 50 ml of Veronal buffer containing E-600. The solution was divided into two halves. One half was passed twice through the cavitation apparatus; the other half was not. Apolipoprotein mass of both halves was measured and then the density of both solutions was raised to d 1.21 g/ml and the solutions were centrifuged for 72 hr at 35,000 rpm in an SW 50.1 rotor. The d 1.21 g/ml top and bottom fractions of each solution were removed by pipette and the apolipoprotein distribution was determined.

Further studies of possible effects of the cavitation apparatus on lipoproteins were performed by injecting 1.5 mCi of [^{3}H]oleate into the peritoneum of a rat and then removing blood after 60 min, from which plasma VLDL were isolated. Lipid analysis by thin-layer chromatography and quantitation of the radioactivity in each lipid class revealed that 95% of the total radioactivity was in triglyceride. The radiolabeled VLDL were then added to a typical homogenate from an intracellular preparation and the solution was repassed through the nitrogen cavitation apparatus. Lipoproteins were isolated from the homogenate and all fractions were analyzed for distribution of the radiolabel. In another experiment, the [^{3}H]oleate-labeled VLDL were added to Veronal buffer free of any cellular material and the solution was passed through the cavitation apparatus and centrifuged, and radioactivity was determined in the isolated fractions.

The effect of centrifugation on intracellular particles was studied by preparing a cellular homogenate, raising the density of the homogenate to 1.21 g/ml, and then layering a density gradient of NaBr over the homogenate. The gradient was left undisturbed for 48 hr, following which the chylomicron/VLDL fraction which floated to the top was removed by pipette. The chylomicron/VLDL fraction (which had not been subject to centrifugation) was loaded over a d 1.21 g/ml NaBr bottom, centrifuged in an SW 28 rotor for 16 hr at 100,000 *g*, and the mass distribution of apoA-I was determined. In an additional experiment examining the effect of centrifugation on intracellular particles, [^{3}H]leucine-labeled intracellular chylomicron and VLDL particles were isolated (by centrifugation) following intraduodenal administration of [^{3}H]leucine. These radiolabeled particles were layered onto the top of a d 1.21 g/ml NaBr buffer and then centrifuged for the same number of *g*-min used to isolate lipoproteins. [^{3}H]Leucine-labeled apoA-I distribution was determined by quantitative immunoprecipitation in the resultant fractions.

Analysis of intracellular fractions

ApoA-I and apoB mass were measured by specific radioimmunoassay as described previously (3). Though the pellet fractions can be assayed for mass directly, conclusions concerning the total mass in the pellets are based

on subtracting the lipoprotein-bound fraction and the d 1.21 g/ml infranatant from the starting homogenate. This was done because of the large dilution factor inherent in resuspending the pellet fraction for mass measurements by RIA. Intracellular epitope expression was compared to plasma epitope expression by using the starting intracellular homogenate as the standard instead of the plasma standard to construct an RIA displacement curve. **Fig. 1** compares the displacement curves of plasma and the homogenate fraction in apoA-I and apoB RIA assays. The displacement curve slopes in each assay are almost identical.

Lipids were analyzed by quantitative thin-layer chromatography following chloroform–methanol extraction as previously described (3). To determine radioactivity after [^{3}H]oleate administration, the lipids were scraped from the TLC plate and radioactivity was measured by scintillation counting.

Isotope incorporation into individual apoproteins was determined by SDS gel polyacrylamide electrophoresis followed by gel slicing, overnight incubation in liquid scintillation solution, and determination of radioactivity, as previously described (3).

Total protein was measured by the method of Lowry et al. (16).

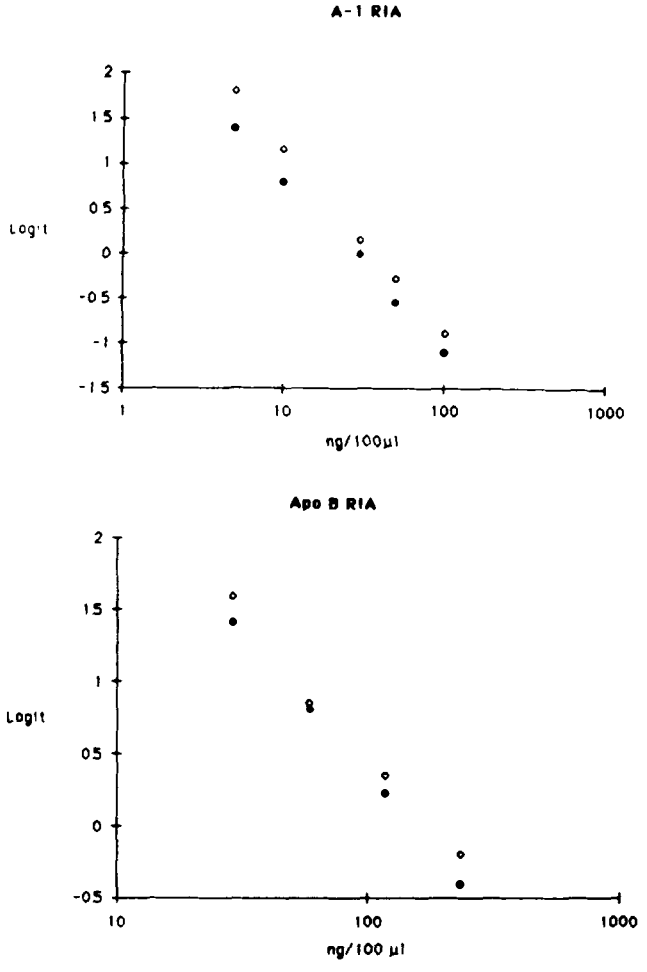

Fig. 1. Comparison of plasma (◆) and intracellular homogenate (◇) displacement curves in apoA-I and apoB radioimmunoassays. Dilutions of the starting intracellular homogenate (immediately post-nitrogen cavitation) and of plasma standard were analyzed by radioimmunoassay. The abscissa is the concentration and the ordinate is the logit function.

RESULTS

Distribution of intracellular apolipoprotein in fasting rats

Table 1 shows the distribution of intracellular apoA-I and apoB in the lipoprotein-bound fraction, expressed as a percentage of the starting intracellular apoprotein mass (i.e., chylomicrons + VLDL + LDL + HDL apoprotein mass/total intracellular apoprotein mass determined prior to ultracentrifugation). One series of experiments (five preparations of four to six rats) was done without use of E-600, a lipase inhibitor (13), in the buffers and another series of experiments (six preparations) included E-600 in the isolation buffers. Use of E-600 significantly reduced the recovery of apoA-I-containing lipoproteins, but did not affect apoB recovery. All further experiments were performed using E-600 in the isolation buffers. As is seen, greater than 90% of intracellular apoB and apoA-I is not bound to intracellular lipoproteins.

The non-lipoprotein-bound fractions consisted of the d 1.21 g/ml infranatant and the pellets of centrifugation. The d 1.21 g/ml infranatant contained 6.8 ± 0.5% of intracellular apoB (n = 4) and 35 ± 11% of intracellular apoA-I (n = 3). Approximately 60% of intracellular apoA-I and 84% of apoB are, therefore, contained in the pellets of centrifugation.

Effect of nitrogen cavitation on lipoproteins

We conducted experiments to determine whether nitrogen cavitation is associated with lipoprotein fragmentation or redistribution of apolipoproteins. Plasma from a rat was added to Veronal buffer (with E-600) and the buffer was then divided in half. One half was passed through the cavitation apparatus and the other half was not. ApoA-I and apoB levels were determined on the two halves and they were identical. The density of the two halves was then raised to 1.21 g/ml and the solutions were centrifuged for 72 hr at 35,000 rpm in a SW 50.1 rotor, which is approximately equivalent to the centrifugation time used in the isolation protocol. The distribution of apoA-I and apoB in the d 1.21 g/ml top fractions and infranatants from the two solutions is seen in **Table 2.** The distributions are almost identical. This proves that

TABLE 1. Percent of total intracellular apolipoprotein bound to intracellular lipoproteins (mean ± SD)

	Without E-600 (n = 5)	With E-600 (n = 6)
	%	
ApoA-I	7.1 ± 3.3[a]	3.6 ± 0.85[a]
ApoB	7.11 ± 3.0	8.9 ± 5.2

[a]Significantly different means by unpaired t test, $P < 0.025$.

the nitrogen cavitation apparatus does not affect the determination of apolipoprotein mass nor does it dislodge apolipoproteins bound to plasma lipoproteins.

We examined whether nitrogen cavitation could fragment lipoprotein particles during the isolation method by adding plasma VLDL labeled with [^{3}H]oleate (95% of the label was in triglyceride) to the buffer solution. The suspension was passed through the nitrogen cavitation apparatus two times. Lipoproteins were re-isolated by sequential ultracentrifugation as in a typical intracellular preparation, except that membranes and cytoplasm were absent. The d 1.21 g/ml infranatant contained only 9% of the added counts, which were almost all in triglyceride. Thus, nitrogen cavitation could account for, at most, a 9% loss of triglyceride-rich lipoproteins.

Effect of centrifugation on intracellular lipoproteins

The possibility that intracellular apolipoproteins are dislodged from the surface of lipoproteins during centrifugation was examined. In one experiment, an intracellular chylomicron/VLDL fraction was isolated by allowing these triglyceride-rich particles to rise freely to the top of a density gradient without centrifugation. This fraction was then loaded onto a density gradient, centrifuged for 16 hr, and the mass distribution of apoA-I was determined. The d 1.21 g/ml infranatant contained 19% of the apoA-I in the fraction. In a second experiment, intracellular [^{3}H]leucine-labeled chylomicrons and VLDL were isolated from a typical cell homogenate and then layered onto a d 1.21 g/ml NaBr buffer. The lipoproteins were centrifuged for the total number of g-min used to isolate intracellular lipoproteins and the distribution of labeled apoA-I was determined by immunoprecipitation. Only 14% of the added apoA-I was in the d 1.21 g/ml infranatant.

Evidence against lipolysis or fragmentation of intracellular lipoproteins

Enzymatic lipolysis of intracellular chylomicrons could decrease the recovery of lipoprotein-bound intracellular apolipoprotein. Addition of a lipase inhibitor to the medium would increase the recovery of lipoprotein-bound apolipoprotein if lipolysis were occurring. As described above (Table 1), addition of E-600 did not increase the recovery.

We further examined the question of lipolysis by adding plasma VLDL containing [^{3}H]oleate to a cellular homogenate following the first pass through the nitrogen cavitation apparatus. The mixture was then repassed through the cavitation apparatus and intracellular lipoproteins were isolated. The lipid content of each fraction was analyzed by thin-layer chromatography and the radioactivity was determined. If lipolysis were occurring, then the total amount of triglyceride radioactivity in the various fractions should have diminished and free fatty acid radioactivity should increase. All of the triglyceride radiolabel was recovered without any loss; all of the radioactivity in the fatty acid fraction was recovered without any gain in activity. Thus, there is no evidence that any significant lipolysis of triglyceride-rich lipoproteins occurs during the isolation methodology.

The distribution of the radiolabel among the lipoprotein fractions was examined following addition of the [^{3}H]oleate-labeled VLDL. The intracellular chylomicron and VLDL fraction contained 96% of the total lipoprotein counts, 3% of the counts were in the LDL fraction, and under 1% were in the HDL fraction. Thus, there is no significant fragmentation or lipolysis of lipoproteins leading to artifactual production of HDL from chylomicrons.

TABLE 2. Effect of nitrogen cavitation on plasma lipoproteins

	ApoB		ApoA-I	
	Cavitation	No Cavitation	Cavitation	No Cavitation
		%		
1.21 g/ml Top	97	93	56	60
1.21 g/ml Infranatant	4	7	44	40
Total	101	100	100	100

A solution of plasma lipoproteins in Veronal buffer was divided and half the sample was subjected to nitrogen cavitation. Apoproteins A-I and B mass distributions were determined after centrifugation for 72 hr at d 1.21 g/ml.

Evidence against lipoprotein sequestration

Intracellular lipid distribution and composition were also examined. Triglyceride and cholesteryl ester intracellular distributions from ad libitum-fed animals were determined by quantitative thin-layer chromatography analysis of all the isolated fractions. As seen in **Table 3,** 29% of intracellular triglyceride is in the lipoprotein fraction and 71% is in the pellets. The lipid composition of the pellets is shown in **Table 4.** Though a large amount of total intracellular triglyceride is found in the pellets of centrifugation, triglyceride comprises less than 4% of the lipid mass of the pellet.

The finding that a large percentage of intracellular triglyceride and the finding that 60% of apoA-I and 84% of apoB are contained in the pellets of centrifugation led us to consider whether the pellets contained lipoproteins. We examined this issue by immediately resuspending the pellets from an ad libitum-fed group of animals in hypotonic Veronal buffer and twice repassing the suspended pellets through the cavitation apparatus. This solution was then centrifuged, as if it were the starting homogenate, in an attempt to liberate and isolate any trapped lipoproteins. The triglyceride and apolipoprotein yields from this experiment are shown in **Table 5.** The reprocessing of the pellet of centrifugation released only an additional 3% of triglyceride and less than 1% of apolipoprotein into the lipoprotein-bound pool. Multiple attempts to isolate lipoproteins from the pellets of centrifugation in many different preparations met with similar minimal increases in lipid and apolipoprotein yield. In one experiment, the pellet of centrifugation was resuspended in a solution so dilute that no pellets were formed upon further centrifugation following repassage through the cavitation apparatus. This was done to eliminate the possibility that membranes were mechanically preventing lipoproteins from floating during isolation centrifugation. The results of this experiment were similar; only 8% more triglyceride bound to lipoproteins could be isolated from the resuspended pellet even though no new pellets were being formed. Thus, though the pellets of centrifugation contain a large percentage of the total intracellular apolipoprotein mass and a variable percentage of intracellular lipid, there is no evidence that there are significant amounts of sequestered lipoproteins in the pellets.

TABLE 3. Intracellular distribution of triglyceride and cholesteryl ester from animals fed ad libitum (one preparation of four rats)

	Triglyceride	Cholesteryl Ester
	%	
d < 1.21 g/ml (Lipoproteins)	29	30
d > 1.21 g/ml (Infranatant)	1	8
Pellet	71	61
Total	101	99

Following isolation of the lipoprotein fractions from the cellular homogenate, triglyceride and cholesteryl ester mass were determined by quantitative TLC.

TABLE 4. Lipid composition of the pellets of centrifugation from preparations from animals fasted or fed ad libitum

	Fasted		Fed	
	Prep. 1	Prep. 2	Prep. 1	Prep. 2
	%			
Phospholipid	68	64	62	67
Fatty acid	21	20	24	21
Cholesterol	7	13	9	8
Triglyceride	4	2	4	3
Cholesteryl ester	0	0	0	1
Total	100	99	99	100

The pellets of centrifugation from two preparations from fasted animals and two preparations from animals fed ad libitum were extracted and the lipid composition was determined by quantitative TLC.

Composition of intracellular lipoproteins

The newly synthesized apolipoprotein composition of intracellular lipoproteins was determined by infusing [^{3}H]leucine intraduodenally and isolating intracellular lipoproteins from epithelial cells. The lipoproteins were delipidated, the protein was chromatographed on 5.6% SDS gels, and the gels were sliced and counted. **Fig. 2** illustrates that newly synthesized apoB, apoA-IV, and apoA-I are present in each of the lipoprotein classes. Coomassie blue-stained gels of the lipoprotein fractions resembled the radiolabeled gel profiles in that the chylomicron and VLDL fractions contained apoproteins B, A-IV, A-I, and other intracellular proteins (data not shown). Extensive centrifugation of the chylomicron and VLDL fractions did not reduce the amount of nonspecific intracellular proteins composing the background of the chylomicron and VLDL fractions. ApoC (R_f 0.9), which has been reported to be synthesized in the intestine (17), may be present in the radiolabeled chylomicron and VLDL fractions.

The lipid composition of each intracellular lipoprotein class was analyzed by thin-layer chromatography as seen in **Table 6.** The total protein and lipid mass were determined for each of the fractions in one preparation. Chylomicrons contained 25.3% protein and 74.7% lipid; VLDL contained 56.9% protein and 43.1% lipid; HDL contained 77% protein and 22% lipid.

Effect of lipid feeding on intracellular lipoprotein distribution

Table 7 summarizes the distribution of apoA-I and apoB among the intracellular lipoprotein fractions as a percentage of total intracellular apolipoprotein mass and (in parentheses) as a percentage of the total lipoprotein-

TABLE 5. Yield of lipoprotein-bound triglyceride and apolipoprotein reisolated from the centrifugation pellet

	Triglyceride	ApoA-I	ApoB
		μg	
Starting homogenate[a]	12,800	288	550
Chylomicrons[b]	231	1.3	0.7
VLDL[b]	82	0.4	2.0
LDL[b]	68	0	
HDL[b]	18	0.8	2.0
Total	399	2.5	4.7
Percent yield[c]	3.1%[b]	0.9%[b]	0.8%[b]

[a]The total intracellular starting mass.

[b]These fractions represent intracellular lipoproteins isolated from the pellet of centrifugation. The pellet was resuspended in hypotonic Veronal buffer, twice repassed through the cavitation apparatus, and lipoprotein fractions were then isolated by sequential ultracentrifugation.

[c]The percent of total intracellular mass contained in the lipoproteins reisolated from the pellet of centrifugation.

bound apolipoprotein (chylomicrons + VLDL + LDL + HDL = 100%). Six preparations using four to six rats per experiment were analyzed. The distribution data reveal that almost all lipoprotein-bound apoA-I in fasting animals is either on triglyceride-rich particles of density < 1.006 g/ml or on HDL particles. In contrast, lipoprotein-bound apoB in fasting animals distributes diffusely among all the lipoprotein classes. The effect of lipid feeding was examined after intraduodenal administration of 10% Intralipid for 60 min and then isolation of intracellular lipoproteins. The data reveal that lipid feeding increases the percent of total intracellular apoA-I and apoB bound to lipoproteins. The percent of intracellular apoA-I bound to lipoproteins more than doubled ($P < 0.01$) and the percent of intracellular apoB increased by 65%. Lipoprotein-bound apoA-I as a percent of total apoA-I increased in each lipoprotein class, with the largest increase occurring in the HDL fraction (threefold increase, $P < 0.05$). Lipoprotein-bound apoB, as a percent of total apoB, increased in all but the LDL fraction, with the largest significant increase (also threefold) occurring in the chylomicron fraction. During lipid feeding, the percent of total intracellular apoA-I or apoB bound to lipoproteins remained under 15%.

The possibility that the distribution of apolipoprotein in the villous cells was different than in the crypt cells was examined by separately analyzing villous and crypt cell fractions from one preparation post-lipid feeding. Total intracellular apoA-I bound to lipoproteins was 7.4% in the villous cells and 8.4% in the crypt cells. Total intracellular apoB bound to lipoproteins was 8% in the villous cells and 13% in the crypt cells. These results are not significantly different from the data derived from the combined villous and crypt fractions (Table 7).

DISCUSSION

The aim of this investigation was to examine intracellular intestinal apolipoprotein distribution in fasting and lipid-fed animals. We found greater than 90% of total intracellular apoA-I and apoB to be non-lipoprotein-bound in fasting animals. Though our initial interest was in the lipoprotein-bound fraction, the conceptual importance of a large non-lipoprotein-bound pool of intracellular apolipoprotein necessitated a detailed examination of the methodology used to rupture cells and isolate lipoproteins.

Nitrogen cavitation was used to disrupt cells; this method has been shown to reproducibly rupture large (or small) volumes of cells (18, 19). Comparison with techniques such as Potter-Elvehjem homogenization have shown that nitrogen cavitation reliably yields cell-free homogenates while mechanical homogenization can leave clumps of unruptured cells (20). Cavitation is also felt to be a gentler technique (21). It does not generate heat and is performed in an inert environment. In this study, we showed that in a hypotonic buffer, which induces cellular swelling, all cell membranes are ruptured after passage through the cavitation apparatus. Previous studies have shown that Golgi and other intracellular organelles reliably rupture with pressure greater than 1500 psi (14, 15).

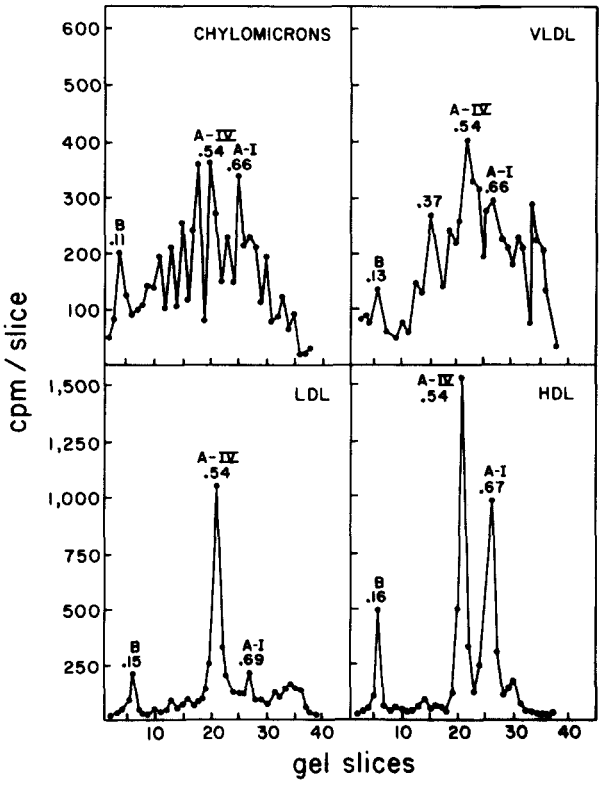

Fig. 2. [^{3}H]Leucine incorporation into apolipoproteins of nascent intracellular lipoproteins isolated following [^{3}H]leucine intraduodenal infusion.

TABLE 6. Lipid composition of intracellular lipoproteins

	Chylomicrons	VLDL	LDL	HDL
Triglyceride	64 ± 5	48 ± 9	31 ± 4	13 ± 7
Cholesteryl ester	7 ± 3	8 ± 3	7 ± 5	9 ± 6
Fatty acid	9 ± 2	22 ± 13	11 ± 5	17 ± 7
Cholesterol	5 ± 1	6 ± 1	12 ± 4	11 ± 5
Phospholipid	15 ± 5	16 ± 10	39 ± 15	50 ± 9
Total	100	100	100	100

All solutions contained E-600. Data are means ± SD; n = 5 preparations.

Although the cavitation apparatus does not generate shearing force, it was important to prove that there was no artifactual alteration of lipoproteins during the isolation methodology. Dislodging of surface apolipoprotein or fragmentation of lipoproteins was ruled out by suspending rat plasma lipoproteins in the isolation buffer, passing them through the cavitation apparatus, and then isolating lipoprotein fractions by centrifugation. The distribution of apoA-I and apoB was identical to plasma lipoproteins similarly suspended in the buffer and centrifuged, but not passed through the cavitation apparatus.

As we and others have shown, centrifugation of plasma can cause a 35–40% loss of apoA-I from lipoproteins into the d 1.21 g/ml infranatant (22). This raises the possibility that some fraction of the 35% of intracellular apoA-I found in the d 1.21 g/ml infranatant may be due to centrifugation losses. We showed that 14–19% of the starting apoA-I mass on triglyceride-rich intracellular lipoproteins may be lost during centrifugation. Since the amount of mass in the chylomicron and VLDL fractions represents less than 2% of the total intracellular mass, the amount of apoA-I which might be lost during centrifugation is about 0.5% of total intracellular apoA-I. Thus, centrifugation losses represent a minor fraction of the apoA-I in the d 1.21 g/ml infranatant. Possible fragmentation or lipolysis of intracellular lipoproteins was examined by adding triglyceride radiolabeled plasma VLDL to a cellular homogenate or to Veronal buffer and then proceeding with the usual isolation techniques. These experiments showed that the isolation method does not disrupt, fragment, or induce lipolysis of lipoproteins.

The possibility that lipoproteins were sequestered in the pellets of centrifugation by fragments of cells was carefully examined. Resuspension and repassage of the pellets of centrifugation through the cavitation apparatus in very dilute solutions resulted in minimal increases in triglyceride or apolipoprotein in the lipoprotein-bound fraction. This was examined multiple times in different preparations in fasted and fed animals. Lipid analysis of the pellets revealed that less than 4% of the lipid mass was triglyceride, similar to previous analyses of the lipid composition of enterocyte membranes (23, 24).

The extensive control experiments seeking evidence for possible significant effects of the isolation methodology on alterations of surface apolipoproteins, fragmentation, lipolysis, or sequestration of particles were unrevealing. Certain possible effects of rupturing cells can never be totally excluded. The cavitation apparatus may affect plasma lipoproteins differently than intracellular lipoproteins. Proteolysis may be occurring when cells are ruptured, though the serine protease inhibitor E-600 was added to diminish this possibility (25). If proteolysis were occurring, however, both the lipoprotein and nonlipoprotein fractions should be equally affected. Aggregation of lipid and protein during cell fractionation is a theoretical concern. This issue was examined in detail in our

TABLE 7. Effect of lipid feeding on apolipoprotein distribution[a]

	ApoA-I				ApoB			
	Fasted (n = 6)		Fed (n = 6)		Fasted (n = 6)		Fed (n = 6)	
	%				%			
Chylomicrons	0.54 ± 0.28	(15)	1.1 ± 0.9	(17) NS	0.98 ± 0.43***	(11)	3.1 ± 1.5***	(21)
VLDL	1.4 ± 1.1	(39)	1.6 ± 0.89	(21) NS	2.2 ± 1.0*	(25)	5.1 ± 2.5*	(34)
LDL	0.16 ± 0.21	(4)	0.35 ± 0.38	(5) NS	3.2 ± 3.5	(36)	2.6 ± 2.3	(11) NS
HDL	1.5 ± 1.3*	(41)	4.7 ± 3.3*	(61)	2.5 ± 1.5	(28)	3.9 ± 2.3	(26) NS
Total	3.6 ± 0.85**	(100)	7.75 ± 3.7**	(100)	8.9 ± 5.2	(100)	14.7 ± 7.1	(100) NS

[a]The distributions are calculated as a percent of total intracellular apolipoprotein. The figures in parentheses represent the distribution of apolipoprotein as a percent of lipoprotein-bound apolipoprotein. All experiments were performed with 2 mM E-600 in the solutions.

Difference between fasted and fed values: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; NS, not significant.

previous work on the isolation of HDL (3). Furthermore, recombinant studies of apolipoprotein and lipid have shown no evidence of lipoprotein formation at temperatures below 20°C (26). Therefore, we are confident that significant artifactual alterations of intracellular lipoproteins do not occur.

The composition of the isolated intracellular lipoprotein fractions revealed that there was newly synthesized apoB, A-IV, and A-I in each fraction. The radiolabeled gel slices showed that in the chylomicron and VLDL fractions there were other intracellular proteins. The relatively high protein content of these fractions similarly suggested that the fractions contained adherent intracellular proteins. Repeated centrifugations of these fractions did not significantly alter the presence of the intracellular proteins. Of note was the finding that the LDL fraction contained 36% of the apoB and 4% of the apoA-I bound to lipoproteins, suggesting that there are apoB-rich particles in the LDL density range. Previous intestinal intracellular lipoprotein compositional data are limited to two studies. Redgrave (27) described intracellular prechylomicrons as being 92% triglyceride and 4% protein. Swift et al. (2) characterized VLDL from Golgi organelles and found them to be 72% triglyceride, 13% protein, 8% phospholipid, and 5% cholesterol.

It must be emphasized that the lipoprotein fractions in this study are derived from cellular homogenates. These fractions should thus contain nascent lipoproteins in various stages of assembly, as would be found in rough and smooth endoplasmic reticulum, Golgi, and secretory vesicle organelles. A number of previous studies support the concept that intracellular lipoproteins undergo both protein and lipid modification during assembly. Glaumann, Bergstrand, and Ericsson (28) have shown that nascent lipoproteins from hepatic rough endoplasmic reticulum have markedly increased protein content and decreased triglyceride as compared to lipoproteins derived from Golgi organelles. Higgins and Fieldsend (29) have recently shown that transfer of newly synthesized phospholipids onto lipoproteins occurs within hepatic Golgi organelles. It was, therefore, not unexpected that the composition of our lipoprotein fractions derived from cellular homogenates differs somewhat from secreted lipoproteins.

In fasting animals, lipoprotein-bound apoA-I distributed into two fractions, triglyceride-rich particles and HDL particles, while lipoprotein-bound apoB distributed more broadly into each lipoprotein density range. Lipid feeding had two clear effects on intracellular apoprotein distribution. One effect was that a greater percentage of intracellular apolipoproteins was associated with lipoproteins. In the lipoprotein fraction, apoA-I more than doubled and apoB increased by 65%. The other effect of lipid feeding was a change in the distribution of apoA-I and apoB among the lipoproteins. Following lipid feeding, the percentage of HDL-associated apoA-I tripled and the percentage of intracellular apoB on chylomicrons tripled. The HDL fraction became the predominant intracellular apoA-I lipoprotein fraction, containing 61% of lipoprotein-bound apoA-I, as compared to 41% in the fasting state. The chylomicron and VLDL fractions together became the predominant intracellular apoB lipoprotein fractions, containing 55% of lipoprotein-bound apoB as compared to 36% in the fasting state. Even after lipid feeding, however, the percent of total intracellular apoA-I bound to lipoproteins remained less than 10% and the percent of lipoprotein-bound apoB remained less than 15% of total intracellular apolipoprotein. The possibility that these data averaged large variations in intracellular apolipoprotein distribution in villous and crypt cells was examined by separately isolating and characterizing villous and crypt fractions. There were no significant differences in apolipoprotein distribution in the fractions.

The only previous published data on quantitative intestinal intracellular apolipoprotein distribution is that of Alpers et al. (11). We, too, found that most intracellular apoA-I (>90%) is non-lipoprotein-bound and that apoA-I shifts onto lipoproteins (mostly HDL) after lipid feeding. Alpers et al. (11) suggested that intracellular chylomicrons might not contain apoA-I. We found that there is definitely apoA-I bound to intracellular chylomicrons in the fasted and lipid-fed states, albeit a small percentage of the total intracellular apoA-I. We both found that apoB is present in all intracellular lipoprotein-bound fractions, including HDL, confirming our previous report that intracellular HDL contain apoB (3). We, however, found that lipoprotein-bound apoB represented only 9–15% of total intracellular apoB with the remainder of intracellular apoB being membrane-associated in the pellets of centrifugation. Recent work by Wong and Pino (30) on the intracellular distribution of apoB in the liver has shown that the microsomal fraction contains more than 50% of apoB, 90% of which is membrane-associated, similar to our findings on intracellular apoB distribution.

Our data suggest the following model of intracellular intestinal lipoprotein assembly. In the fasting state, there is a preformed pool of apoA-I and apoB, as has been reported in a number of studies (31, 32). Apolipoprotein in this pool is largely non-lipoprotein-bound, presumably in the rough endoplasmic reticulum, as suggested by Christensen et al. (9). Almost all of the non-lipoprotein-bound apoB is in the pellets of centrifugation, suggesting that apoB is tightly membrane associated. The pellets of centrifugation contain 60% of intracellular apoA-I while 35% of intracellular apoA-I is in the d 1.21 g/ml infranatant, perhaps bound to small amounts of lipid. Less than 10% of intracellular apoA-I or apoB in the fasted state is bound to lipoproteins. Lipid feeding increases the percent of intracellular apoprotein bound to intracellular lipoproteins, but 85–90% of intracellular apoprotein remains non-lipoprotein-bound, suggesting that only a

small fraction of intracellular apolipoprotein is mobilized during lipid feeding. The distribution of apolipoprotein among lipoproteins also changes with lipid feeding in that HDL become the predominant apoA-I lipoprotein while chylomicrons and VLDL become the predominant apoB lipoproteins.

Our finding that most intracellular apolipoprotein in fasting and lipid feeding is non-lipoprotein-bound suggests that the basal constitutive rate of apolipoprotein synthesis ensures an abundance of intracellular apolipoprotein available for lipoprotein formation. The constant presence of a non-lipoprotein-bound pool furthermore suggests that apolipoprotein synthesis is not the rate-limiting step in lipoprotein assembly or secretion. Previous work from this laboratory showing that apoA-I concentrations in lymph were unaffected by bile diversion is consistent with there being a pool of apolipoprotein available for lipoprotein formation even when lipid flux is at a nadir (33). Variations in apolipoprotein synthesis and content might have little effect on intestinal apolipoprotein secretion if the non-lipoprotein-bound intracellular pool is large. Apparent increases in apolipoprotein secretion following lipid feeding (32, 34) may reflect mobilization of apolipoprotein from the intracellular pool rather than alterations in apolipoprotein synthetic rates. In agreement with this concept is the finding of Davidson et al. (35, 36) that jejunal apoB and apoA-I synthesis is not regulated by triglyceride under physiological conditions.

The presence of a large non-lipoprotein-bound pool of apoA-I is consistent with the hypothesis that apoA-I may be secreted into the lamina propria free of significant lipid (11). However, the presence of apoB, A-IV, and A-I on intracellular chylomicrons and VLDL and the existence of intracellular forms of LDL and HDL suggest that intestinal apoprotein is secreted bound to lipoproteins. The finding that, with lipid feeding, HDL becomes the predominant intracellular apoA-I lipoprotein raises the possibility that some HDL particles may be precursor forms of nascent chylomicrons while other particles in the fraction may be secreted as nascent HDL. The nature of these intracellular precursor-product relationships is the subject of current investigation.

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REFERENCES

1. Mahley, R. W., B. D. Bennett, D. J. Morre, M. E. Gray, W. Thistlethwaite, and V. S. LeQuire. 1971. Lipoproteins associated with the Golgi apparatus isolated from epithelial cells of rat small intestine. *J. Lab. Invest.* **25:** 435–444.
2. Swift, L. L., P. D. Soulé, M. E. Gray, and V. S. LeQuire. 1984. Intestinal lipoprotein synthesis. Comparison of nascent Golgi lipoproteins from chow-fed and hypercholesterolemic rats. *J. Lipid Res.* **25:** 1–12.
3. Magun, A. M., T. A. Brasitus, and R. M. Glickman. 1985. Isolation of high density lipoproteins from rat intestinal epithelial cells. *J. Clin. Invest.* **75:** 209–218.
4. Tytgat, G. N., C. E. Rubin, and D. R. Saunders. 1971. Synthesis and transport of lipoprotein particles by intestinal absorptive cells in man. *J. Clin. Invest.* **50:** 2065–2078.
5. Windmueller, H. G., and A. L. Wu. 1981. Biosynthesis of plasma apolipoproteins by rat small intestine without dietary or biliary fat. *J. Biol. Chem.* **256:** 3012–3016.
6. Green, P. H. R., and R. M. Glickman. 1981. Intestinal lipoprotein metabolism. *J. Lipid Res.* **22:** 1153–1173.
7. Glickman, R. M., A. Kilgore, and J. Khorana. 1978. Chylomicron apoprotein localization within rat intestinal epithelium: studies of normal and impaired lipid absorption. *J. Lipid Res.* **19:** 260–268.
8. Schonfeld, G., E. Bell, and D. H. Alpers. 1978. Intestinal apoproteins during fat absorption. *J. Clin. Invest.* **61:** 1539–1550.
9. Christensen, N. J., C. E. Rubin, M. C. Cheung, and J. J. Albers. 1983. Ultrastructural immunolocalization of apolipoprotein B within human jejunal absorptive cells. *J. Lipid Res.* **24:** 1229–1242.
10. Green, P. H. R., J. H. Lefkowitch, R. M. Glickman, J. W. Riley, E. Quinet, and C. B. Blum. 1982. Apolipoprotein localization and quantitation in the human intestine. *Gastroenterology.* **83:** 1223–1230.
11. Alpers, D. H., D. R. Lock, N. Lancaster, K. Poksay, and G. Schonfeld. 1985. Distribution of apolipoproteins A-I and B among intestinal lipoproteins. *J. Lipid Res.* **26:** 1–10.
12. Weiser, M. M. 1973. Intestinal epithelial cell surface membrane glycoprotein synthesis. *J. Biol. Chem.* **248:** 2536–2541.
13. Quinn, D. M. 1985. Diethyl-*p*-nitrophenyl phosphate: an active site titrant for lipoprotein lipase. *Biochim. Biophys. Acta.* **834:** 267–271.
14. Fleischer, B. 1974. Isolation and characterization of Golgi apparatus and membranes from rat liver. *Methods Enzymol.* **31:** 180–191.
15. Fleischer, S., G. Meissner, M. Smigel, and R. Wood. 1974. Preparation of submitochondrial vesicles using nitrogen decompression. *Methods Enzymol.* **31:** 292–299.
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
17. Schonfeld, G., N. Grimme, and D. Alpers. 1980. Detection of apolipoprotein C in human and rat enterocytes. *J. Cell Biol.* **86:** 562–567.
18. Mircheff, A. K., J. D. Hanna, M. W. Wolling, and E. M. Wright. 1979. Large scale analytical method for isolating basal lateral plasma membranes from rat duodenum. *Prep. Biochem.* **9:** 133–148.
19. Hunter, M. J., and J. L. Commerford. 1961. Pressure homogenization of mammalian tissues. *Biochem. Biophys. Acta.* **47:** 580–586.
20. Short, C. R., M. H. Maines, and L. E. Davis. 1972. Preparation of hepatic microsomal fraction for drug metabolism studies by rapid decompression homogenization. *Proc. Soc. Exp. Biol. Med.* **140:** 58–65.
21. Dowben, R. M., T. A. Gaffey, and R. M. Lynch. 1968. Iso-

lation of liver and muscle polyribosomes in high yield after cell disruption by nitrogen cavitation. *FEBS Lett.* **2:** 1-3.
22. Kunitake, S. T., and J. P. Kane. 1982. Factors affecting the integrity of high density lipoproteins in the ultracentrifuge. *J. Lipid Res.* **23:** 936-940.
23. Brasitus, T. A., K. Yeh, P. R. Holt, and D. Schachter. 1984. Lipid fluidity and composition of intestinal microvillus membranes isolated from rats of different ages. *Biochim. Biophys. Acta.* **778:** 341-348.
24. Glaumann, H., and G. Dallner. 1968. Lipid composition and turnover of rough and smooth microsomal membranes in rat liver. *J. Lipid Res.* **9:** 720-729.
25. Henson, P. M., D. Gould, and E. L. Becker. 1976. Activation of stimulus specific serine esterases (proteases) in the initiation of platelet secretion. *J. Exp. Med.* **144:** 1657-1673.
26. Hirz, R., and A. M. Scanu. 1970. Reassembly in vitro of a serum high density lipoprotein. *Biochim. Biophys. Acta.* **207:** 364-367.
27. Redgrave, T. G. 1971. Association of Golgi membranes with lipid droplets (pre-chylomicrons) from within intestinal epithelial cells during absorption of fat. *Aust. J. Exp. Biol. Med. Sci.* **49:** 209-224.
28. Glaumann, H., A. Bergstrand, and J. L. E. Ericsson. 1975. Studies on the synthesis and intracellular transport of lipoprotein particles in rat liver. *J. Cell. Biol.* **64:** 356-377.
29. Higgins, J. A., and J. K. Fieldsend. 1987. Phosphatidylcholine synthesis for incorporation into membranes or for secretion as plasma lipoproteins by Golgi membranes of rat liver. *J. Lipid Res.* **28:** 268-278.
30. Wong, L., and R. M. Pino. 1987. Biogenesis of very low density lipoproteins in rat liver. *Eur. J. Biochem.* **164:** 357-367.
31. Glickman, R. M., J. Khorana, and A. Kilgore. 1976. Localization of apolipoprotein B in intestinal epithelial cells. *Science.* **193:** 1254-1255.
32. Glickman, R. M., and P. H. R. Green. 1977. The intestine as a source of apolipoprotein A-I. *Proc. Natl. Acad. Sci. USA.* **74:** 2569-2573.
33. Bearnot, H. R., R. M. Glickman, L. Weinberg, P. H. R. Green, and A. R. Tall. 1982. Effect of biliary diversion on rat mesenteric lymph apolipoprotein A-I and high density lipoprotein. *J. Clin. Invest.* **69:** 210-217.
34. Imaizumi, K., R. J. Havel, M. Fainaru, and J. L. Vigne. 1978. Origin and transport of the A-I and arginine-rich apolipoproteins in mesenteric lymph of rats. *J. Lipid Res.* **19:** 1038-1046.
35. Davidson, N. O., and R. M. Glickman. 1985. Apolipoprotein A-I synthesis in rat small intestine: regulation by dietary triglyceride and biliary lipid. *J. Lipid Res.* **26:** 368-379.
36. Davidson, N. O., M. E. Kollmer, and R. M. Glickman. 1986. Apolipoprotein B synthesis in rat small intestine: regulation by dietary triglyceride and biliary lipid. *J. Lipid Res.* **27:** 30-39.