papers and notes on methodology

Isolation of hepatocellular lipid droplets: the separation of distinct subpopulations

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Abstract A new method for the isolation of lipid droplets from rat liver has been devised. The procedure involves tissue homogenization and discontinuous density gradient centrifugation. Six discrete bands of lipid particles, rich in triglyceride and cholesterol, are visible following 30 min of centrifugation at 25,770 *g* (max) in a swinging bucket rotor. An entire rat liver can be processed in 1–2 hr. Differences in the density of these liver lipid particles correlate with their triglyceride, sterol, phospholipid, and protein contents. The separation of these distinct populations of intracellular particulate neutral lipids provides an approach to the study of their origin and metabolism. This procedure may also be useful in the isolation of various populations of lipid-rich particles from other tissues. — **Ontko, J. A., L. W. Perrin, and L. S. Horne.** Isolation of hepatocellular lipid droplets: the separation of distinct subpopulations. *J. Lipid Res.* 1986. **27:** 1097–1103.

Supplementary key words cholesterol • cholesteryl esters • density gradient centrifugation • lipid-rich particles • liver • metabolism • method • microsomes • triglyceride • very low density lipoproteins

Lipid droplets are present in numerous cell types in animal tissues and in cell cultures (1–10). In studying the composition and metabolism of lipid droplets, the high-yield isolation of these particles in pure and native states is of fundamental importance.

The isolation of lipid droplets from animal tissues presents a number of problems including the lengthy procedures required, alterations produced in droplet structure, droplet destruction via coalescence, poor yields, the presence of impurities in the final droplet suspension, and the lack of separation of droplet subfractions. In the course of studies on the metabolism of hepatocellular lipid droplets, these problems were addressed and an improved procedure for the isolation of these particles was developed.

EXPERIMENTAL PROCEDURES

Animals

Male Holtzman rats were obtained from Charles River Breeding Laboratories. These animals (290–420 g) were maintained on water and Purina Laboratory Chow ad libitum, and were housed at constant 22°C, with lights on at 6:00 AM and off at 6:00 PM.

Preparation of sucrose solutions

The sucrose was placed in a graduated beaker and a volume of boiling water equal to 80% of the desired final volume was added; the sucrose was dissolved with constant stirring on a hot plate. The cooled solution was transferred to a volumetric flask. Redistilled water was used for preparation of all reagents. A 100% sucrose solution was also prepared to adjust individual bands, removed from the gradient, to 65% sucrose for recentrifugation when washing was required. The 100% sucrose solution was prepared by dissolving the sucrose in hot water in a graduated beaker in a slowly boiling water bath with constant stirring, with subsequent transfer to a volumetric container. All sucrose solutions contained 10 mM Tris-HCl at pH 7.2. They were stored at 0–5°C and freshly prepared at intervals not exceeding 3 weeks.

Tissue homogenization

Rats were lightly anesthetized with diethyl ether (ether for anesthesia, Mallinckrodt, Paris, KY) and decapitated.

Abbreviations: VLDL, very low density lipoproteins.

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Livers were rapidly excised and plunged into ice-cold 0.15 M NaCl. Nonhepatic tissue was removed from the livers. All subsequent procedures were at 0–5°C. The livers were blotted dry, weighed, and placed in 20 ml of 65% sucrose (Baker Analyzed Reagent, J. T. Baker Chemical Co., Phillipsburg, NJ). The liver was cut into small pieces with scissors and transferred with an additional 10 ml of 65% sucrose to a 55-ml homogenizer of the Potter-Elvehjem type, size C (#3431-E25, Thomas Scientific, Philadelphia, PA). The tissue was homogenized with three complete strokes of the serrated Teflon pestle (0.15–0.23 mm clearance) with a TRI-R STIR-R motor at speed 2 (150 rpm). The remaining volume of 65% sucrose required for a 20% liver homogenate (for example, 10 ml for a 10-g liver) was then mixed into the homogenate.

Preparation of the discontinuous sucrose gradient

Gradients were prepared as follows. Ten ml of the 20% liver homogenate in 65% sucrose was pipetted with a wide-bore pipet into the bottom of the Sorvall 50-ml polycarbonate centrifuge bottle (tube) Oak Ridge style, 28 × 103 mm (#03934, Sorvall Instruments, DuPont Co. Bioresearch Systems, Wilmington, DE). The centrifuge tubes were kept in an ice bath. The 52% sucrose solution (4 ml) was slowly layered over the homogenate with a volumetric pipet. Four ml of 44% sucrose, 4 ml of 35% sucrose, 3 ml of 25% sucrose, 3 ml of 10% sucrose, and 6 ml of redistilled water were overlayered consecutively (**Fig. 1A**). The tubes were then sealed by hand with a three-part sealing assembly (Sorvall #03277) consisting of an O-ring, a plug, and a screw cap.

Centrifugation

The tubes were then centrifuged in a precooled Sorvall HB-4 swinging bucket rotor in a Sorvall RC5C superspeed refrigerated centrifuge at 12,500 rpm (25,770 *g*-max) at 4°C for 30 min. Fig. 1B illustrates the observed banding of lipid-rich particles within the gradient. The locations of these bands were always measured and found to be highly reproducible, as were the volumes of these bands. The Sorvall RC5C centrifuge was equipped with an automatic rate control for slow acceleration and deceleration. Similar results were obtained with or without activation of this feature. Similar results were also obtained using 1″ × 3½″ (38.5 ml) ultraclear centrifuge tubes (#344058, Beckman Instruments, Palo Alto, CA) in a Beckman SW 28 swinging bucket rotor and a Beckman L5-75 ultracentrifuge, with ultracentrifugation at 11,750 rpm (25,700 *g*-max) for 30 min.

Collection of individual bands from the sucrose gradient

The distinct bands resolved within the sucrose gradient were readily harvested in separate vessels by careful suction with Pasteur pipets. These pipets, which were bent at an angle of approximately 135°, were connected via flexible tubing to collecting flasks fitted with vacuum adapters leading to a mild vacuum, available as a routine laboratory utility outlet. A water aspirator vacuum pump is also suitable for this purpose.

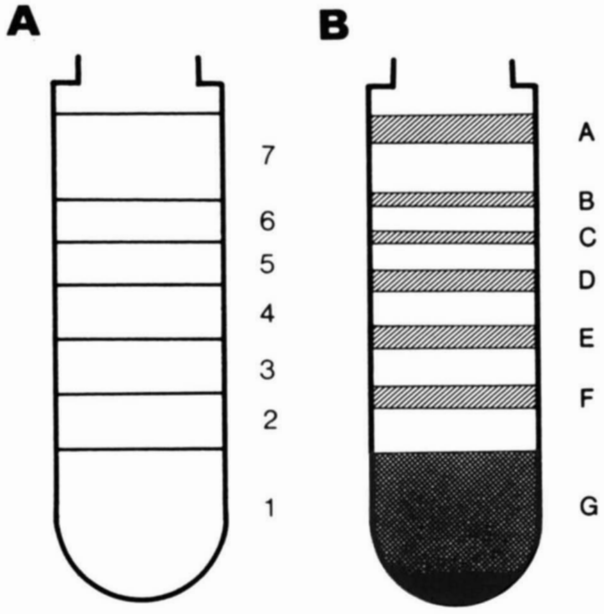

Fig. 1. Panel A: the discontinuous sucrose gradient layered above the 20% liver homogenate in 65% sucrose. The layers 2–7 contain 52% sucrose (4 ml), 44% sucrose (4 ml), 35% sucrose (4 ml), 25% sucrose (3 ml), 10% sucrose (3 ml), and water (6 ml), respectively. All layers contained 10 mM Tris-HCl at pH 7.2. Panel B: location of bands following migration of lipid-rich particles during 30 min of centrifugation at 12,500 rpm (25,770 *g*-max). Bands are designated A–F. Zone G represents the 10 ml of residual homogenate in 65% sucrose and contains a sediment at the bottom.

Lipid extraction

One volume of liver homogenate, or subfraction from the gradient, was diluted with 7 volumes of methanol, mixed, and 14 volumes of chloroform were then admixed. After heating at 40°C for 30 min in a water bath, the samples were filtered through coarse sintered glass. The filtrates were washed once with water and twice with blank upper phase according to Folch, Lees, and Sloane Stanley (11).

Analytical methods

Triglyceride was assayed as previously described (12). In some cases the triglyceride content of individual lipid bands from the gradient was also analyzed with a kit assay procedure based on the reaction of glycerol produced by lipase-catalyzed hydrolysis (#65103/93, EM Diagnostic Systems, Gibbstown, NJ, procured from Scientific Products, McGaw Park, IL). In this procedure, H_2O_2 generated by glycerol phosphate oxidase reacts with 4-amino-

antipyrene and *p*-chlorophenol to produce a visible chromophore. Protein was measured with the dye-binding procedure of Bradford (13) with the Bio-Rad protein assay dye reagent concentrate (#500-0001, Bio-Rad Laboratories, Richmond, CA). A microassay procedure was employed with a standard curve from 1–20 μg of protein. Sucrose did not interfere. The measurement of the protein content of gradient band A required correction for the turbidity of lipid droplets. Free cholesterol and cholesteryl esters were measured by gas–liquid chromatography (14). Glucose-6-phosphatase activity was assayed by the procedure of Baginski, Foa, and Zak (15). N-Acetyl-β-glucosaminidase activity was measured according to Beaufay et al. (16). After 20 min of incubation, the reaction was stopped with trichloroacetic acid (17). Following centrifugation, the supernate was adjusted to pH 10.0 as described by Sellinger et al. (17). The substrate was *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma Chemical Co., St. Louis, MO), and *p*-nitrophenol (Sigma Chemical Co.) was employed for standardization. Total phospholipids were analyzed by Bligh and Dyer extraction (18) with lipid phosphorus determination according to Bartlett (19). In this procedure, 1.0 ml of the lipid-rich fraction from the sucrose gradient and, in the case of the parent homogenate and zone G at the bottom of the gradient, 0.1 ml of sample plus 0.9 ml of water was extracted with 3.75 ml of chloroform–methanol 1:2 (v/v). Thirty min later, 1.25 ml of chloroform and 1.25 ml of 2 M KCl were admixed. Following centrifugation and removal of the upper phase, aliquots of the lower phase were analyzed for phosphorus (19).

RESULTS AND DISCUSSION

Following centrifugation for 30 min at 12,500 rpm (25,770 *g*-max), six distinct bands were clearly and reproducibly observed in the sucrose gradient above the liver homogenate. These bands are designated A–F (Fig. 1B). Zone G in Fig. 1B represents the liver homogenate with a pellet shown as the darkest region. **Fig. 2A** shows a photograph of bands as observed with side lighting. Here the individual bands are displayed in relation to their turbidity. Band A was fluffy white with a highly concentrated thin zone at the upper surface. Bands B–D were cloudy white with fine, uniformly dispersed particles. Band D had a slightly grey case. Band E was two-tone; the upper portion was faintly grey and the lower region slightly tan. Band F had a tan cast with a greyish central zone. In Fig. 2A, band F does not appear to be clearly resolved because the zone between band F and the surface of the homogenate is cloudy. In actual appearance, however, band F is distinct. Fig. 2B shows the bands with a light background and band F is more clearly demonstrated. The intensities of the various bands are closely proportional to their lipid contents, described below.

The triglyceride and cholesterol composition of these bands are provided in **Table 1.** The lipid constituents are expressed in units per gram of liver, fresh weight. The sucrose gradient in each tube was constructed upon 10 ml of a 20% liver homogenate. Therefore, the actual amounts present in each band of a single centrifuge tube equal the quantity shown in Table 1 multiplied by 2. Band A at the top of the gradient contains the greatest amounts of both triglyceride and cholesteryl ester (Table 1). These particles of lowest density also contain the least quantity of free cholesterol on a percentage basis. These properties indicate that the band A particles are large lipid droplets. The relative abundance of free cholesterol in bands C–F is not consistent with the core composition of lipid droplets (20, 21). Much of the free cholesterol in these fractions is presumably associated with surface constituents. The lipid composition of bands B, C, and D, and their respective positions in the density gradient, suggest that these bands contain progressively smaller lipid droplets. The increasing ratio of free cholesterol to cholesteryl esters in bands B, C, and D is consistent with a gradual increase in the surface area of these particles in relation to their core volume. These successive alterations in lipid composition extend to bands E and F. However, these bands are considerably

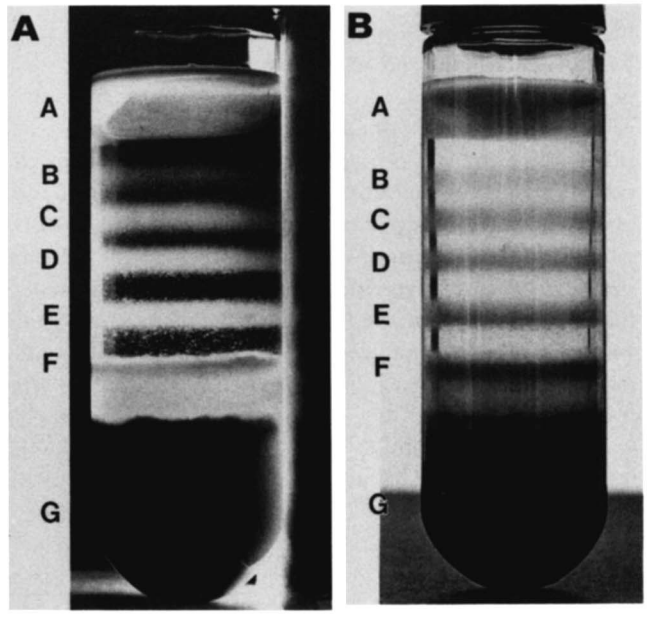

Fig. 2. Photographs of centrifuge tubes following 30 min of discontinuous sucrose gradient centrifugation of liver homogenates. Panel A: bands viewed with side lighting which accents the appearance of creamy lipid particles which equilibrate in layers within the gradient. Panel B: bands are photographed with ordinary lighting and a light background. Band F is more visible under these conditions.

TABLE 1. Triglyceride and cholesterol contents of individual bands isolated from sucrose gradients following centrifugation of liver homogenates

Band	Triglyceride	Cholesterol			Free Cholesterol[c]		
		Free	Esterified[a]	Total[b]	Triglyceride	Free Cholesterol	Cholesteryl Esters
		μg/g liver			*% of total*[d]	*% of total*[e]	
A	1867	8	119	1994	93.6	0.4	0.1
B	80	2	5	87	92.0	2.3	0.4
C	132	7	8	147	89.8	4.8	0.9
D	159	15	11	185	85.9	8.1	1.4
E	319	46	21	386	82.6	11.9	2.2
F	637	190	43	870	73.2	21.8	4.4
G	1425	2029	72	3526	40.4	57.5	28.2
Total	4619	2297	279	7195	64.2	31.9	8.2

These data were obtained in a representative experiment in which the individual bands from four centrifuge tubes, each containing 10 ml of the same 20% liver homogenate, were combined for analysis.

[a] Combined mass of cholesteryl esters containing 16-, 18-, and 20-carbon fatty acids.

[b] Triglyceride + free cholesterol + cholesteryl esters.

[c] Mass ratio of free cholesterol to cholesteryl esters containing 16-, 18-, and 20-carbon fatty acids.

[d] [Triglyceride ÷ (triglyceride + free cholesterol + cholesteryl esters)] × 100.

[e] [Free cholesterol ÷ (triglyceride + free cholesterol + cholesteryl esters)] × 100.

richer in phospholipid and protein, as described below. Therefore, these particles appear to be more complex and their identification remains the subject of further investigation.

The reproducibility of the separation of different populations of lipid-rich particles from rat liver by this density gradient centrifugation procedure is shown in **Table 2.** The quantities of triglyceride isolated in the six gradient bands from seven different livers from fed rats were compared. The standard errors of the mean were less than 20% of the mean values in each band. The amounts of triglyceride found in each of bands A, B, E, and F differed significantly from the triglyceride contents of each of the other bands. The amounts of triglyceride found in bands C and D were not significantly different from each other when compared as groups. However, in individual livers, the quantity of triglyceride in band D was always greater than that observed in band C. When the triglyceride values were statistically analyzed as paired observations, the triglyceride contents of all bands were found to differ significantly (Table 2). The average total liver triglyceride was 6009 μg/g (Table 2). It is evident that 95% of the total, specifically 5696 μg/g, was recovered in gradient bands A-F plus zone G. These combined data document the reproducibility of the gradient centrifugation procedure.

The total phospholipid contents of the populations of lipid particles in bands A-F are shown in **Table 3.** The phospholipid:triglyceride ratios are also provided. Phospholipids have greater densities than triglycerides. The sequential increase in density of the lipid particles in bands A-F is clearly related to their phospholipid content. The phospholipid content of band A particles is similar to that found in the hepatic lipid droplets isolated by DiAugustine, Schaefer, and Fouts (6). These investigators reported a phospholipid:triglyceride ratio of 0.018-0.021. Kondrup (9) found the somewhat higher ratio of 0.04-0.05, which coincides with the calculated ratio in combined bands A-C (Table 3), namely 70 μg of phospholipid and 1629 μg of triglyceride with a ratio of 0.043. The average ratio of phospholipid to triglyceride in the liver was 6.5 (Table 3). Bands A-G all exhibited higher ratios consistent with the enrichment of all bands with triglyceride. Bands E and F possess more phospholipid than triglyceride.

TABLE 2. Triglyceride contents of sucrose gradient bands isolated from seven rat livers

Band	Triglyceride Content[a]	Significance[b]	
		I[c]	II[d]
	μg/g liver		
A	1800 ± 216[e]	1[f]	1[f]
B	61 ± 12	2[g]	2[h]
C	107 ± 17	3	3[g]
D	153 ± 25	3	4[g]
E	410 ± 73	4[h]	5[f]
F	917 ± 84	5[f]	6[f]

[a] The parent homogenates of these seven livers from fed rats contained 6009 ± 322 μg of triglyceride/g liver. Gradient zone G contained 2248 ± 145 μg of triglyceride/g liver.

[b] Values designated by different numbers are significantly different, as described in footnotes below.

[c] Column I is a two-tailed comparison of groups by Student's *t*-test with 12 degrees of freedom.

[d] Column II is a two-tailed comparison of paired observations by Student's *t*-test with 6 degrees of freedom.

[e] Mean ± SE, n = 7.

[f] $P < 0.005$.

[g] $P < 0.05$.

[h] $P < 0.01$.

TABLE 3. Phospholipid contents of lipid-rich fractions isolated from the sucrose gradients

Band	Phospholipid[a,b]	Phospholipid[a,c] / Triglyceride
	μg/g liver	
A	28	0.019
B	9	0.149
C	33	0.347
D	111	0.668
E	599	1.216
F	1702	2.169
G	30647	14.058
H[d]	35474	6.505

[a] Mean values of three rat livers.
[b] Total phospholipid based on an average molecular weight of 775.
[c] Mass ratios.
[d] Parent homogenates.

The protein contents of the bands A through F are shown in **Table 4.** Bands E and F were each washed once in order to remove proteins that may have diffused into these regions from the underlying liver homogenate. In this procedure, bands E and F were isolated following initial centrifugation. These turbid fractions were then individually adjusted to 65% sucrose with a 100% sucrose solution (see Experimental Procedures). Then, another gradient of identical composition (Fig. 1A) was constructed upon 10 ml of each fraction. Following the second centrifugation, again at 12,500 rpm, the vast majority of the lipids in bands E and F migrated again to their original locations. This behavior demonstrates that the particles in these bands possess and retain different and specific densities. The lipid particles in bands A and D exhibited similar behavior, namely repeated migration to the same location within the gradient. This has not as yet been extensively studied with bands B and C. The gradual increase in the protein content of bands A through F (Table 4) suggests this component as a possible contributing determinant of their respective differences in density.

To examine the possibility that constituent particles of certain bands were artificially produced by the tissue homogenization procedure, alternative procedures of tissue disruption were investigated. These included freezing by two methods, namely slow cooling in the freezer to −10°C and instant freezing at −20°C with the freeze-clamping technique. In the latter procedure, aluminum blocks of the Wollenberger type (22) were cooled to −20°C in a Dewar flask containing ethanol adjusted to −20°C with dry ice. Following freezing, the tissues were ground to a uniform homogenate with a porcelain mortar and pestle. Other procedures that did not utilize freezing were also tested. The tissue was passed through a screw-type stainless-steel tissue press fitted with a fine sieve (#141 Harvard Apparatus, Millis, MA). In this device the tissue was extruded through 1-mm diameter round holes. The minced tissue was then ground in 65% sucrose by hand in a Ten Broeck homogenizer. In other methods, the freeze-clamped liver tissues were thawed and separate portions were homogenized in Ten Broeck and Dounce homogenizers. Finally, the simple procedure of passage of the fresh liver through the tissue press, as described above, followed by grinding to a uniform consistency with a mortar and pestle was also accomplished. The liver homogenates prepared by these six different procedures all developed bands A–F in similar yields following centrifugation in the discontinuous sucrose gradient. These results provide strong evidence for the natural occurrence of the different populations of lipid-rich particles resolved within the gradient.

The preparation of tissue homogenates and the isolation of subcellular fractions by centrifugation techniques are commonly conducted at 0–4°C. This temperature range was routinely employed in the present study. Since temperatures in this region are known to cause alterations in the structure of lipoproteins in rats fed saturated fatty acids (23), the possibility that cooling to 0–4°C induced changes in hepatic lipid particles, and thereby artificially produced populations that migrated into certain bands, was considered. Rat livers were therefore homogenized at room temperature. Equivalent portions were *a*) subjected to gradient centrifugation at the same temperature (20–25°C) and *b*) cooled in an ice bath and processed as usual at 0–4°C. The same banding pattern of bands A–F developed in all gradients. Triglyceride assays revealed no significant differences in the triglyceride contents of individual bands. Accordingly, cooling per se did not promote the formation of specific bands nor did it appreciably alter the distribution of triglyceride-rich particles within the gradient. Although small alterations in the density of triglyceride, caused by crystallization, could have escaped detection under the conditions employed, it

TABLE 4. Protein contents of individual bands isolated from the sucrose gradients

Band	Protein	Protein / Triglyceride
	mg/g liver	
A	0.015[a]	0.01
B	0.012	0.18
C	0.048	0.44
D	0.139	0.88
E	0.316[b]	1.04[b]
F	2.396[b]	3.78[b]

[a] Average of three livers from fed rats.
[b] These fractions were washed by recentrifugation under the same conditions.

is unlikely that cooling caused crystallization. Thus, the liver triglycerides of chow-fed rats contain an abundance of unsaturated fatty acids (24) with an anticipated freezing point considerably below 0°C.

It was also considered that certain bands could significantly consist of lipoproteins derived from blood present in the liver when it was removed from the animal. This possibility was eliminated when it was observed that removal of blood by in situ perfusion of the liver with saline via the portal vein prior to excision of the organ did not alter the development of bands A–F in the gradient.

Since microsomes contain nascent very low density lipoprotein (VLDL) particles and since these secretory lipoproteins represent a significant fraction of liver cellular triglyceride (3, 8, 9, 25–27), the bands of the gradient were examined for the presence of the endoplasmic reticulum marker enzyme, glucose-6-phosphatase. Bands A–D were devoid of activity (**Table 5**). Band E contained a trace of activity. Band F contained a small amount of glucose-6-phosphatase activity, approximately 3% of that present in the parent homogenate, while 96% of the homogenate enzyme activity was recovered in zone G (Fig. 1B, Table 5). The lysosomal enzyme N-acetyl-β-glucosaminidase was also measured. No activity was detected in bands A–D (Table 5). Band E contained detectable activity but it was only 0.2% of that present in the total homogenate. Band F contained about 2% of the total and 98% was recovered in zone G. These findings indicate that liver microsomes and lysosomes migrate significantly only into band F.

The presence of several populations of neutral lipid-rich particles in rat liver, which are rapidly separable based on differences in their density, suggest differences in their origin, metabolism, and function. It is possible that distinct populations of particles are derived from different cell types in the liver, notably parenchymal and various nonparenchymal cells. Further, even within the same cell type, the architecture of the microcirculation produces heterogeneity. Thus, hepatocytes in the portal region and those in the venous zone of the sinusoids exhibit histochemical and metabolic differences (28–30). Accordingly, cells in different anatomical zones may produce lipid particles of somewhat different composition and density, and thereby contribute to the multiplicity of populations observed (Fig. 2). Differences in the density of lipid droplets may also be related to their size and age spectra within individual cells. In addition, subpopulations may reflect involvement in different phases of intracellular lipid sorting (31). Compositional analyses (Tables 3–5) suggest that the bands of greatest density contain membranous elements, possibly VLDL particles in Golgi and secretory vesicles (3, 26, 27, 32–34), chylomicron and VLDL remnants in endocytic vesicles (35, 36) and multivesicular bodies (37), and triglyceride-containing secondary lysosomes (35–37). This density gradient centrifugation procedure may therefore also provide a method for the rapid concentration of these triglyceride-rich organelles for further purification and study.

The present studies reveal the presence of several different populations of neutral lipid particles in the liver. Although differences in the density of these populations are related in part to their sterol and protein contents, further investigation may identify other physical and chemical properties that characterize their heterogeneity. The resolution and compositional analysis of these lipid particles provides an experimental approach to definition of the molecular mechanisms involved in their formation and mobilization in both normal and pathological conditions.

TABLE 5. Microsomal and lysosomal enzyme activities in lipid-rich bands isolated from the sucrose gradients

Band	Glucose-6-phosphatase[a,b]	N-acetyl-β-glucosaminidase[a,c]
	% of total	*% of total*
A	0	0
B	0	0
C	0	0
D	0	0
E	0.3	0.2
F	3.2	1.7
G	92.0	94.5
H[d]	100	100

[a] Three different rat livers were analyzed; mean values are shown.
[b] The recovery of glucose-6-phosphatase in the gradient fractions was 96%.
[c] The recovery of N-acetyl-β-glucosaminidase in the gradient fractions was 96%.
[d] Enzyme activities of the parent homogenates were set at 100%.

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