Plasma lipoprotein composition in alcoholic hepatitis: accumulation of apolipoprotein E-rich high density lipoprotein and preferential reappearance of "light"-HDL during partial recovery

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Abstract Abnormal lipoproteins accumulate in the plasma of alcoholic hepatitis patients in association with a deficiency of the cholesterol esterifying enzyme, lecithin:cholesterol acyltransferase. Most of these abnormal lipoproteins are found in the d > 1.006 g/ml density fraction. To investigate the composition and morphology of the lipoproteins at various times during the illness in four patients, we have employed density gradient ultracentrifugation combined with analyses of gradient fractions by polyacrylamide gel electrophoresis, electroimmunoassay, and electron microscopy. At the onset of the illness, plasma cholesteryl esters ranged from 19–34% of total cholesterol; high density lipoprotein (HDL) cholesterol and apoA-I, the major HDL apoprotein, were <10% of normal; and most of the d > 1.006 g/ml triglycerides and phospholipids were found in the LDL density region. A linear correlation (r = 0.964, $P < 0.001$) was found between the d > 1.006 g/ml apoB concentration and the summation of the triglyceride and esterified cholesterol for that fraction, indicating a constant ratio of apoB to the summation of these two "core lipids". ApoA-I was primarily found in the fraction d > 1.18 g/ml (HDL_3 and VHDL) but not at all in the HDL_2 density range of the gradient. No cholesteryl esters were present in the apoA-I containing fractions. In contrast to normal, large amounts of apoE accumulated in lipoproteins isolated at d 1.055–1.114 g/ml. The apoE-rich fractions contained primarily phospholipids and unesterified cholesterol; they appeared by electron microscopy to be mixtures of spherical particles, vesicular particles, and chains of bilamellar discs. Analyses of the density gradient fractions by SDS polyacrylamide gel electrophoresis under reducing conditions indicated that apoA-II levels and distribution paralleled apoA-I, not apoE, providing evidence against appreciable concentrations of apoE–apoA-II complexes. During partial recovery from alcoholic hepatitis in three patients, the d > 1.006 g/ml unesterified cholesterol and triglyceride levels decreased, while esterified cholesterol, HDL-cholesterol, and apoA-I levels increased. The first HDL fractions to reappear were lipoproteins with HDL_2 density characteristics, as evidenced by simultaneous increases of apoA-I, apoA-II, cholesteryl esters and phospholipids. Lipoproteins with HDL_3 density characteristics appeared later. Long-term (6–10 months) follow-up studies indicated a substantial elevation of HDL cholesterol and apoA-I in three of the four patients that appeared to have resulted from further increases in their HDL_2-like subspecies. The above results illustrate the diversity of abnormal lipoproteins in alcoholic hepatitis and the ability of density gradient ultracentrifugation combined with lipid and apolipoprotein quantitation, electron microscopy, and polyacrylamide gel electrophoresis to partially resolve those lipoproteins in the d > 1.006 g/ml plasma fraction.—**Weidman, S. W., J. B. Ragland, and S. M. Sabesin.** Plasma lipoprotein composition in alcoholic hepatitis: accumulation of apolipoprotein E-rich high density lipoprotein and preferential reappearance of "light"-HDL during partial recovery. *J. Lipid Res.* 1982. **23:** 556–569.

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Since the liver is the major source of plasma lipoproteins and of lecithin:cholesterol acyltransferase (LCAT), a key enzyme involved in lipoprotein metabolism, liver injury would be expected to be accompanied by abnormalities of lipoprotein biosynthesis and metabolism (1). Research from this laboratory (2–6) and those of others (7–12) has provided evidence that alcoholic liver injury is associated with the following alterations in lipoprotein composition: *1*) VLDL that is deficient in both apoE and apoC but relatively normal in lipid composition; *2*) LDL that contains mainly apoB but is greatly enriched in TG and deficient in esterified cholesterol (EC) and is also heterogeneous in size; and *3*) HDL that is greatly di-

Abbreviations: apo, apolipoprotein; CH, cholesterol; TG, triglyceride; CM, chylomicrons; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; IDL, intermediate density lipoproteins; HDL_2, high density lipoproteins isolated in the density range d 1.063–1.125 g/ml; HDL_3, high density lipoproteins isolated in the density range d 1.125–1.21 g/ml; DTNB, dithiobisnitrobenzoic acid; LCAT, lecithin:cholesterol acyltransferase (EC 2.3.1.43); EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; UC, unesterified cholesterol; EC, esterified cholesterol; PL, phospholipids; VHDL, very high density lipoproteins.

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TABLE 1. Clinical profiles of alcoholic hepatitis patients at admission

Patient No.	Age/Sex	Weight	Height	Total Bilirubin	Alkaline Phosphatase	SGOT	SGPT	Albumin
		kg	*cm*	*mg/dl*[a]	*mU/ml*[b]	*mU/ml*[c]	*mU/ml*[d]	*gm/dl*[e]
1	M/41	66.8	170	18.4	353	179	30	2.3
2	M/52	95.0	183	15.0	252	116	34	2.8
3	F/47	81.8	196	17.0	302	89	17	1.9
4	M/34	57.3	168	11.6	174	180	N.D.[f]	3.0

[a] Normal range 0.1–1.2 mg/dl.
[b] Normal range 20–115 mU/ml.
[c] Normal range 9–42 mU/ml.
[d] Normal range 5–27 mU/ml.
[e] Normal range 3.0–5.5 g/dl.
[f] Not determined.

minished in concentration and is greatly enriched in apoE and phospholipid (PL), but deficient in apoA-I and EC. Several studies (2, 13–21) have documented low plasma LCAT activity in patients with alcoholic liver disease and have shown that this decrease is associated with impaired cholesterol esterification. Associated with the deficiency of LCAT in these patients is a decreased level of plasma apoA-I, an activator of LCAT. Evidence has been presented that the catabolism of apoA-I is increased in alcoholic hepatitis, providing a rationale for reduced plasma levels (22). Evidence has also been presented for hepatic triglyceride lipase deficiency in liver disease, which may partially account for the accumulation of TG-rich LDL (5). Recently, it was proposed that the apoE-enriched HDL and apoB-enriched VLDL that accumulate in the plasma of patients with alcoholic hepatitis represent nascent HDL and VLDL and may be the principal pathway through which apoE and apoB are secreted by the liver in man (3).

Our previous studies have utilized the classical density cuts to define VLDL, LDL, and HDL fractions. Realizing that the composition of these sequentially isolated lipoprotein fractions probably reflected the sum of the composition of a heterogeneous mixture of lipoproteins, we have now extended these studies, employing a density gradient fractionation procedure of the d > 1.006 g/ml fraction, in order to more clearly define the association of the various apoproteins and lipid classes. A unique feature of our gradient is that it includes a large amount of sucrose mixed with NaBr in order to decrease ionic strength and possibly to decrease dissociation of apoproteins during centrifugation (23).

We have applied the above techniques to four alcoholic hepatitis patients, three of whom were sampled at intervals during their partial recovery. The results have provided new insights into the HDL subspecies distribution early in the recovery period as well as the changes occurring in HDL_2 and HDL_3 density ranges with partial recovery. After discharge from the Clinical Research Center, the patients were studied again 6–10 months later. Three of the patients had striking elevations in their HDL levels, which appear to have resulted from selective increases in the HDL_2 density range.

METHODS

Patients

Three patients with the typical clinical and biochemical features of acute hepatitis were admitted to the Clinical Research Center for investigation (**Table 1**). Informed consent was obtained from each patient and the research protocol was approved by the Patient Participation Committee, University of Tennessee Center for the Health Sciences. Patient No. 4 was sampled twice while in the Prison Ward. The patients entered the hospital with complaints of weakness, anorexia, nausea, abdominal discomfort, and jaundice. None of the patients complained of pruritis. All patients admitted to excessive alcohol consumption for several years and indicated that their intake of alcohol was greatly increased for several weeks before hospitalization. Percutaneous liver biopsies obtained from each patient revealed massive hepatocellular lipid accumulation, alcoholic hyaline, mild hepatocellular necrosis, inflammation, and cholestasis. In two patients (Nos. 1 and 3) there was evidence of early micronodular cirrhosis.

Collection of samples and sample handling

All blood samples for lipoprotein studies were obtained after a 12–14 hr fast and were collected in EDTA (1.5 mg/ml) tubes to which DTNB (1 mM) was added to inhibit LCAT activity. The plasma lipoproteins were separated as follows: *a*) chylomicrons or $S_f > 400$ lipoproteins were isolated and washed by ultracentrifugation in the Beckman SW 50.1 rotor for 10^6 *g*-min at d 1.006 g/ml; *b*) VLDL was recovered from a 17-hr spin in the SW 50.1 rotor (2×10^6 *g*-min) at d 1.006 g/ml; and *c*)

TABLE 2. Density ranges covered by individual density gradient fractions

Fraction No.	Density Range
	g/ml
1	1.017–1.021
2	1.021–1.026
3	1.026–1.031
4	1.031–1.036
5	1.036–1.042
6	1.042–1.049
7	1.049–1.055
8	1.055–1.064
9	1.064–1.074
10	1.074–1.085
11	1.085–1.098
12	1.098–1.114
13	1.114–1.134
14	1.134–1.158
15	1.158–1.182
16	1.182–1.205
17	1.205–1.224
18	1.224–1.244
19	1.244–1.300
20	1.300–1.403

[a] Fraction densities obtained on both blank (no plasma) and patient gradients. Density ranges were obtained from a plot of fraction number vs. fraction density.

the d > 1.006 g/ml fraction (IDL + LDL + HDL) was separated in a 10-ml exponential salt plus sucrose gradient extending from d 1.01 to d 1.21 g/ml. Two ml of the plasma remaining after VLDL isolation was adjusted to density of 1.2406 g/ml with 762 mg of sucrose and 391 mg of NaBr and overlaid with the gradient using an Isco model 570 gradient former and a Buchler Densiflow. The solutions mixed to form the gradient were: heavy solution, 2.133 m NaBr containing 323.8 g/l sucrose (final density 1.2295 g/ml) and 0.1 g/l EDTA Na_2; and light solution, 0.195 m NaCl plus 0.1 g/l EDTA Na_2. After spinning at 40,000 rpm for 24 hr in a Beckman SW 41 Ti rotor, the tube was punctured and 20 separate 0.6-ml fractions were collected from the top by pumping 7.95 m NaBr (d = 1.49 g/ml) to the bottom of the tube using an Isco Model 640 gradient fractionator. Plasma proteins were located in fractions 15–20 of the gradient (d 1.169–1.403 g/ml). Gradient fraction densities were measured at 25°C with a Newtec high precision densimeter (Newtec Inc., Birmingham, AL) (**Table 2**). TG, PL, CH (total and unesterified), apoB, and apoA-I were determined in whole plasma, CM, VLDL, and the d > 1.006 g/ml lipoprotein fraction. All density gradient fractions were analyzed for PL and total and unesterified CH. Fractions 1–9 (top of the gradient) were analyzed for apoB and fractions 10–20 were analyzed for apoA-I. Sequential ultracentrifugal preparation of IDL (d 1.006–1.019 g/ml), LDL (d 1.019–1.063 g/ml), and HDL (d 1.063–1.21 g/ml) and subsequent density gradient fractionation revealed that these lipoproteins floated in fractions Nos. 1, 2–8, and 9–17, respectively, in agreement with the density regions covered by these fractions as measured in the blank (no protein) gradients.

The densities of the fractions from blank gradients containing no plasma and of those with the patient samples were comparable and highly reproducible. When duplicate blank gradients were spun and the density of each fraction was compared with that of the duplicate, the two densities agreed within 1%. Duplicate gradients of the d > 1.006 g/ml fraction from normolipidemic subjects, when analyzed for total CH and PL and each fraction compared with its duplicate, agreed within 5 and 7%, respectively. Similar agreement was also noted in three out of four of the alcoholic hepatitis patients when samples were spun in duplicate at each time point. The remaining patient's sample could not be spun in duplicate due to the limited quantity of plasma available.

Analytical techniques

CH and CH esters were determined by gas–liquid chromatographic analysis of extracts of saponified and non-saponified samples containing a known amount of β-sitosterol as an internal reference. TG was determined by the colorimetric method of Biggs, Erickson, and Moorehead (24). Plasma and lipoprotein phospholipids were determined by extraction of samples by sequential addition of 50 vol of methanol and 50 vol of chloroform. Extracts were dried under nitrogen, digested with 70% perchloric acid and phosphorus was determined by the method of Rouser, Fleischer, and Yamamoto (25). Phosphorus values were converted to phospholipid values using a factor of 25. HDL-CH was determined by heparin-Mn^{2+} precipitation of apoB-containing lipoproteins (26). A turbid supernate from a hypertriglyceridemic sample was cleared by centrifugation at 12,000 *g* for 10 min.

Apolipoproteins A-I and B were determined in samples by the electroimmunoassay (rocket) method of Laurell (27) using antibodies prepared in rabbits (28, 29). The apoB assay was linear over the range of 1/200 to 1/50 dilution of the reference plasma (standardized with LDL) in 0.024 M barbital buffer (pH 8.6, Bio Rad Laboratories, Richmond, CA) or 28–280 ng applied to the plate, while the apoA-I assay was linear over the range of 1/200 to 1/30 dilution of the reference plasma (standardized with pure apoA-I) in the same buffer or 32–325 ng applied. The range of apoA-I and apoB concentrations obtained for normolipidemic subjects in our laboratory are 130 to 190 mg/dl and 75 to 110 mg/dl, respectively. Samples of whole plasma and sequential ultracentrifugal lipoprotein fractions from all times for a given patient were always run on the same immunoassay plate.

The recoveries of total CH, unesterified CH, TG, PL, apoB, and apoA-I from the sequential ultracentrifugal separation of CM, VLDL, and the d > 1.006 g/ml fraction were 96, 91, 92, 106, 102, and 96%, respectively. Recoveries of total CH, unesterified CH, PL, apoB, and apoA-I from the density gradient separation were 94, 90, 92, 63, and 91, respectively. The reason for the lower recovery of apoB from the gradient samples is not presently known.

Proteins were determined by a modified Lowry technique (30) using crystalline bovine albumin standards. VLDL apoproteins and density gradient apoproteins were analyzed by the SDS-PAGE method of Laemmli (31); separating gels were a 10–20% gradient; gels were stained with 0.025% Coomassie Brilliant Blue R-250 and destained in the solutions of Weber and Osborn (32), and scanned with an Isco Model 1310 gel scanner attachment to the model UA-5 absorbance monitor.

Electron microscopy

Samples for electron microscopy were dialyzed against 1 mM EDTA, 0.02% sodium azide, pH 7.6. Lipoproteins were negatively stained for electron microscopy with 2% phosphotungstate solutions (pH, 5.9). To insure uniformity in morphological analysis of density gradient fractions, lipoproteins were floated on Formvar-coated grids from equal fraction volumes for 3 sec. Negative staining was then carried out for 2 min in an identical manner for all gradient fractions. Particle size was determined with a magnifying micrometer. In each preparation the diameters of 50–100 free-standing spherical particles were measured. In each preparation containing discoidal particles, the diameter and periodicity of 100 particles was measured.

RESULTS

Changes in plasma and lipoprotein lipid and apoprotein levels during patient recovery

Three of the four patients (Nos. 1–3) were studied in the Clinical Research Center until clinical evaluation and laboratory studies indicated recovery from their alcoholic hepatitis. The study period varied from 18–38 days. The fourth patient was sampled twice in the prison ward and could not be transferred to the Clinical Research Center. He was sampled again during our follow-up studies and is therefore included as a subject. However, his lipid parameters are not included in Figs. 1 and 2, since he was not serially sampled during his recovery period.

None of the four patients was severely hypertriglyceridemic when sampled at day 0 of the study (plasma TG range 157–243 mg/dl). During recovery, plasma TG levels fell to <100 mg/dl as a result of decreases in the d > 1.006 g/ml plasma fraction (IDL + LDL + HDL). Most of the plasma TG was carried in this plasma fraction. VLDL-TG levels ranged from 4.3–9.1 mg/dl and did not change appreciably with recovery. CM-TG levels were <3.8 mg/dl and also did not change greatly with recovery.

Plasma CH levels initially ranged from 108–166 mg/dl with practically all of the CH carried in the d > 1.006 g/ml plasma fraction (VLDL-CH < 3.4 mg/dl). Most of this cholesterol was unesterified, presumably reflecting the low levels of plasma LCAT activity that have been reported previously in alcoholic hepatitis (2, 13–21).

Plasma phospholipids (PL) at day 0 were in the normal range (patients, 184–285 mg/dl; normals, 125–350 mg/dl) with most of the phospholipids carried in the d > 1.006 g/ml fraction. In two of the three patients the d > 1.006 g/ml PL levels decreased initially during recovery, but increased towards basal levels near the end of this period. The third patient's plasma PL levels were essentially invariant during recovery.

The percentage of CH esterified in plasma was low (19–34%) initially and increased rapidly with recovery (**Fig. 1**) to near normal levels (61–67%). Similarly, plasma apoA-I and HDL-CH levels were very low initially (<10% of normals) and increased during the recovery period. However, the levels attained at the end of the study were still subnormal. There appeared to be a lag in the response of apoA-I and HDL-CH levels compared to the increase in percent plasma CH esters (Fig. 1). Essentially, all of the plasma apoA-I was carried in the d > 1.006 g/ml fraction. No apoA-I could be detected in the VLDL fraction by our immunoassay.

Initial plasma apoB levels were in the normal range for patients Nos. 1 and 3, but were elevated in patient No. 2 (**Fig. 2**). Essentially all of the plasma apoB was carried in the d > 1.006 g/ml fraction, since VLDL-apoB levels were <2.1 mg/dl. The response during recovery of the patients' d > 1.006 g/ml apoB was varied: in patient No. 1, levels were essentially constant, patient No. 2's level decreased dramatically by one-third, and patient No. 3's levels were constant during the first half and decreased by 50% in the latter half of her recovery. These responses were not well-correlated with changes in the summation of UC and EC in the d > 1.006 g/ml fraction. However, the apoB levels in the d > 1.006 g/ml fraction were highly correlated ($r = 0.964$, $P < 0.001$) with the summation of EC and TG in those fractions. Increases in VLDL apoB were also noted in the patients during the latter stage of the study (Fig. 2).

VLDL apoprotein composition

Analyses of the VLDL from these patients by SDS PAGE revealed a deficiency of apoE and the C apopro-

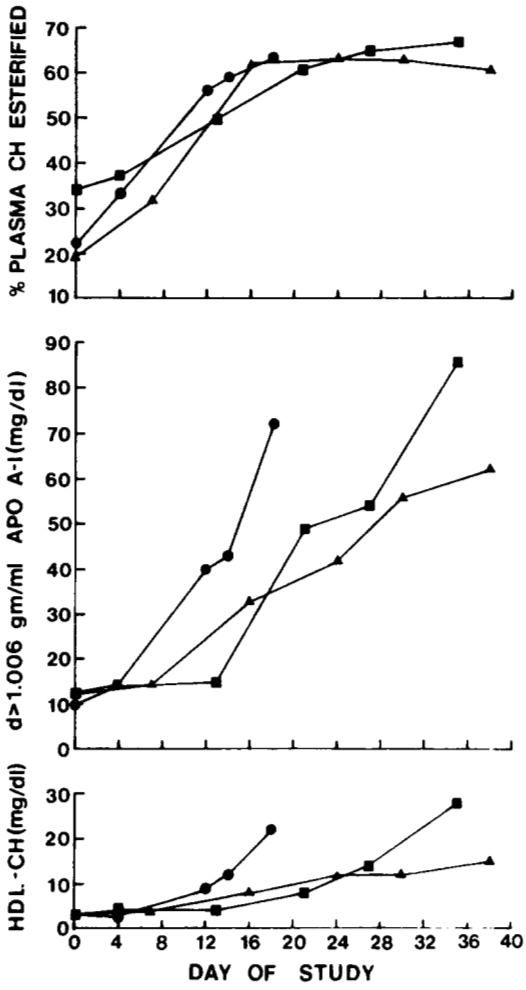

Fig. 1. Changes in percent plasma cholesterol esterified, d > 1.006 g/ml fraction apoA-I, and HDL cholesterol levels during recovery of alcoholic hepatitis patients. Symbols: ●, patient No. 1; ■, patient No. 2, ▲, patient No. 3.

teins. As recovery progressed, VLDL apoE content increased to near normal levels. However, even when plasma EC improved to near normal levels, VLDL was still considerably deficient in the C apoproteins. Since similar results have been previously reported (4), the data are not shown.

Separation of the d > 1.006 g/ml lipoproteins by density gradient ultracentrifugation

The separation of IDL, LDL, and HDL was achieved by density gradient ultracentrifugation in an exponential salt plus sucrose gradient. The EC, UC, apoB, PL, and apoA-I profiles of such a gradient for a normolipidemic subject is shown in **Fig. 3A** (concentrations shown are corrected to that in plasma). It is seen that apoB-containing lipoproteins are found in fractions 1–8 which contain IDL (fraction 1, d 1.017–1.021 g/ml) and LDL (fractions 2–8, d 1.021–1.064 g/ml) (see Experimental Section for verification), while apoA-I containing lipoproteins (HDL and VHDL) are found in fractions 10–20 (d 1.074–1.40 g/ml). Further validation of this gradient with HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.21 g/ml) prepared by sequential ultracentrifugation indicated that these subspecies would be found in fractions 9–13 and 14–17, respectively. However, since HDL_3/HDL_2 ratios are frequently >5:1, and the technique does not resolve a distinct shoulder or peak in the HDL_2 density region, except in subjects with high HDL_2 levels, fractions 9–13 in the gradient would be contaminated with HDL_3 (33). Therefore, we have denoted the summation of total CH or apoA-I in fractions 9–13 and 14–20 as "light" and "heavy" HDL, respectively, or as material isolated in the specified density range.

The apoprotein compositions of equal volume aliquots of the first 15 gradient fractions of Fig. 3A as revealed by SDS-PAGE are shown in Fig. 3B (fractions 16–20 contain too high a concentration of other plasma proteins). Fractions 1–9 contain mainly apoB and traces of

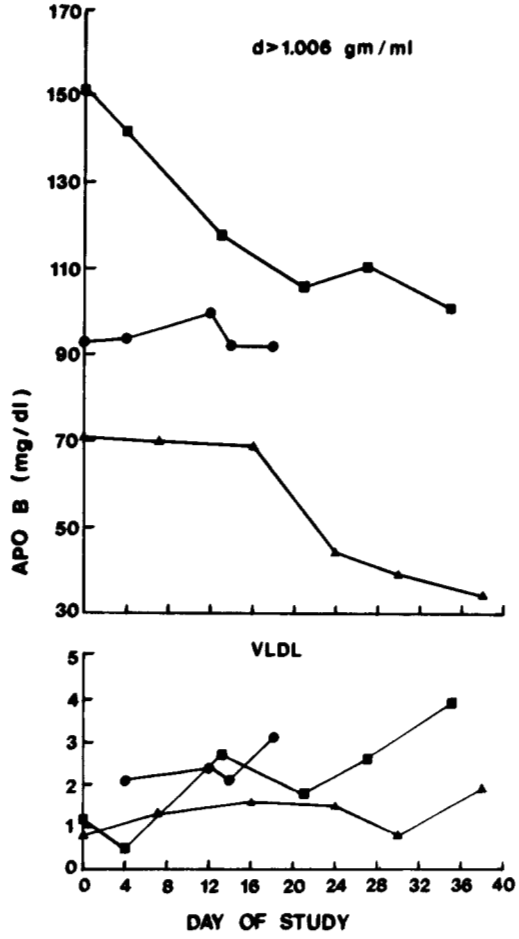

Fig. 2. Changes in d > 1.006 g/ml fraction and VLDL apoB levels during recovery of alcoholic hepatitis patients. Symbols same as Fig. 1.

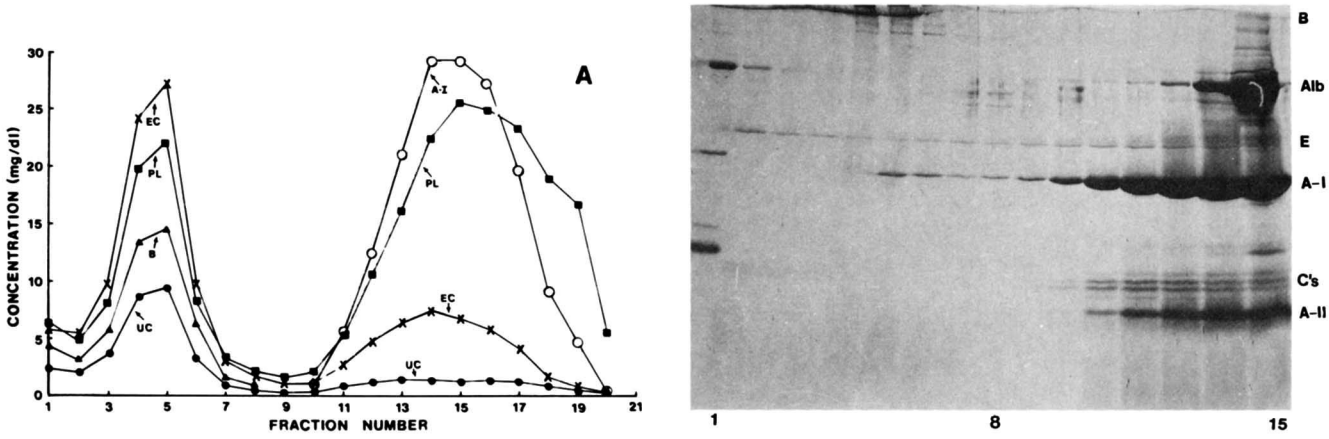

Fig. 3. Distribution of lipids and apoproteins in the density gradient fractionation of the d > 1.006 g/ml plasma fraction of a normolipidemic subject. A, Distribution of esterified CH (EC), unesterified CH (UC), phospholipids (PL), apoB, and apoA-I. B, SDS gradient slab gel electrophoresis of the proteins. Equal volumes (35 μl) of density gradient fractions were solubilized in 1% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol and electrophoresed in the presence of 0.1% SDS. Gels are a linear gradient in polyacrylamide which ranges from 10% (top) to 20% (bottom). Molecular weight standards were run in the far left lane. Only a portion of the apoB of the sample runs into the gel. The remainder is in the stacking gel (not shown).

albumin and apoA-I, while fractions 10–15 contain mainly apoA-I with appreciable concentration of C apoproteins and apoA-II. ApoE appears in minor concentrations in fractions 10–15, but appears to be increasing in higher number fractions.

Density gradient profiles from the fractionation of the d > 1.006 g/ml fraction for the four patients at or near the initial day of study are shown in **Fig. 4A–D**. The density gradient profiles of the alcoholic hepatitis patients differ from that of a normal subject (Fig. 3A) in the following respects: *1*) UC is the predominant form of cholesterol throughout the gradient; *2*) the apoB maxima occur at a slightly lower density than that of normal LDL and are enriched in UC; *3*) in three of the four patients, second and third shoulders or peaks of UC and PL occur in the density region 1.055–1.098 g/ml (fractions 8–11) where there is very little apoB or apoA-I; *4*) in patient No. 3 this second peak or shoulder occurs at a density of 1.042–1.049 g/ml (fraction 6); *5*) most of the PL is found in the density range of 1.017–1.064 g/ml (fractions 1–8); *6*) there is very little cholesterol in the HDL density region of the gradient (fractions 9–20); and *7*) a small amount of apoA-I is detected in the HDL_3-VHDL region of the gradient (fractions 14–20, d = 1.134–1.40 g/ml) along with significant amounts of PL. Very little apoA-I is detected by immunoassay in the HDL_2 density region of the gradient (fractions 9–13).

The apoprotein composition of the first 15 basal gradient fractions as determined by SDS-PAGE is shown for patient No. 1 in **Fig. 5A**. It can be seen that the LDL density range contains appreciable quantities of apoE and trace amounts of apoA-I and C apoproteins. The apoE peak is not coincident with the apoB peak. There are second peaks of apoE and apoA-I at fractions 9 and 10 and 11 (d 1.064–1.098 g/ml), respectively, where the PL/UC weight ratio is approximately 2:1. The apoA-I concentration in these fractions was below the limit of detection of our immunoassay. It should be noted that apoE here is not co-distributed in the gradient with apoA-II. The small amounts of apoA-I and apoA-II present appear to co-distribute just as they do in the normal gradient. Comparing Fig. 3B with Fig. 5A, it can also be seen that the apoprotein composition of fraction 8, corresponding to the second PL + UC peak from the top of the gradient, consists mainly of apoE, some apoB, and trace amounts of albumin. Although not shown here, SDS-PAGE analyses of the first 15 basal gradient fractions of the other patients indicated a similar apoE and apoA-I profile. There were qualitative differences in the apoE and apoA-I contained in the LDL fractions, but we consistently observed an apoE peak at fraction 10 which contained trace amounts of apoA-I and was enriched in PL and UC.

Portions of the gradient fractions of the normolipidemic subject and of patient No. 1 (at day 4) were dialyzed, negatively stained, and examined in the electron microscope (**Figs. 6 and 7**). Fractions 1–3 (d 1.017–1.031 g/ml) of the gradient of patient No. 1 contain predominantly spherical lipoproteins ranging from a diameter of 27.8 ± 3.9 to 22.4 ± 2.1 nm (mean ± S.D.). Fractions 4–10 (d 1.031–1.085 g/ml) contain mixtures of normal appearing lipoproteins (mean diameters ranging from 26.7 ± 1.9 to 24.6 to 5.0 nm) plus larger vesicular structures (mean diameters ranging from 63.7 ± 18.4 to 71.0 ± 21.0 nm) with discoidal structures becoming more prevalent in the latter fractions. Fraction 11 (d 1.085–1.098 g/ml) contains predominantly discoidal particles

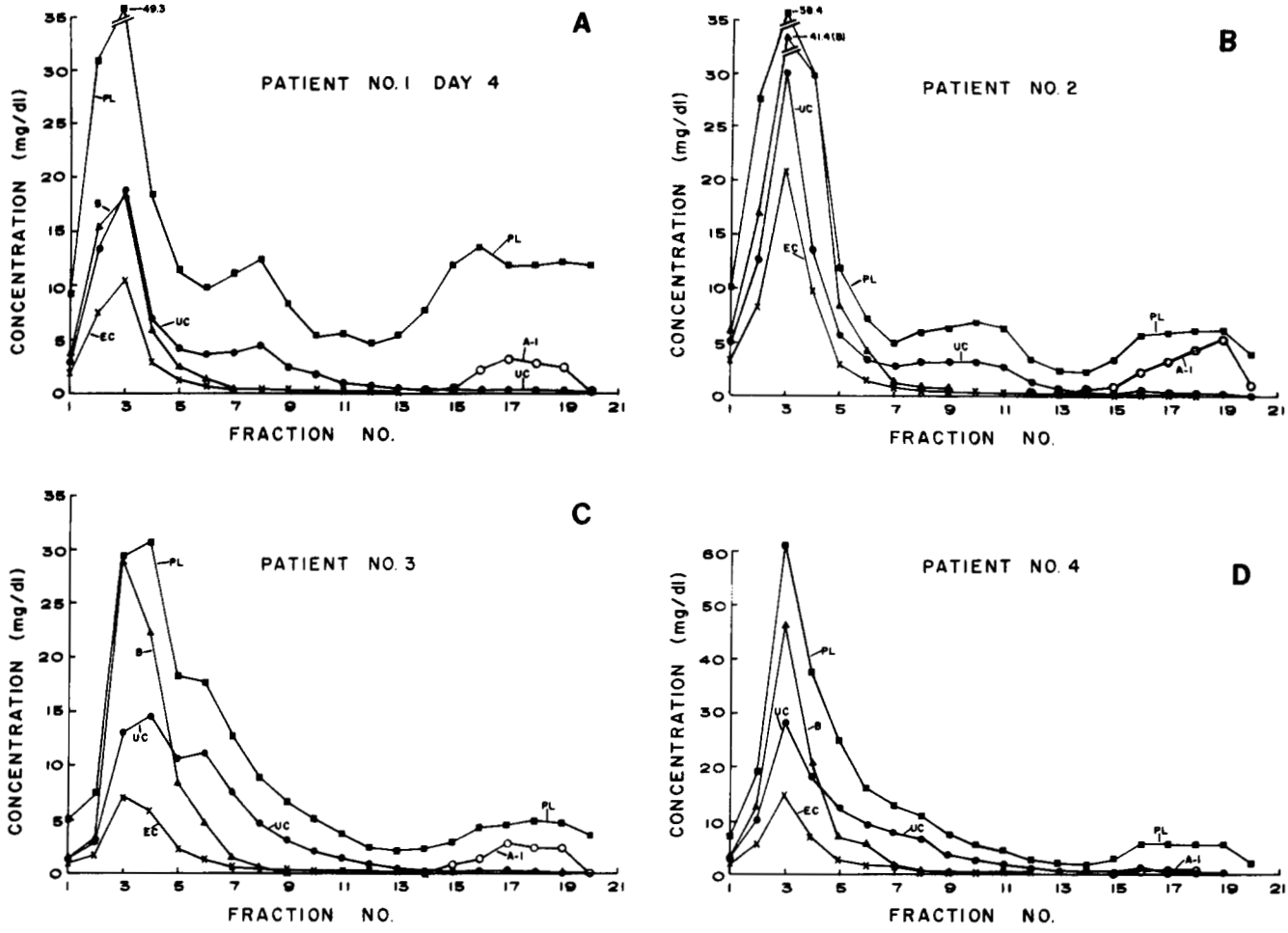

Fig. 4. Distribution of lipids and apoproteins in the density gradient fractionation of the d > 1.006 g/ml plasma fraction of alcoholic hepatitis patients near the onset of illness. A, Patient No. 1; B, patient No. 2; C, patient No. 3; D, patient No. 4. Symbols, ■, PL; ▲, apo B; ●, UC; X, EC; O, apoA-I.

with a mean diameter of 19.6 ± 2.9 nm and a periodicity of 6.7 ± 0.7 nm with some spherical particles of mean diameter 18.7 ± 3.0 nm. Fractions 12–15 (d 1.098–1.182 g/ml) of the patient contained relatively few lipoprotein structures having mean diameters ranging from 11.4 ± 1.5 nm (small particle population) to 28.3 ± 7.2 nm (larger particles). Fractions 16–20 (d 1.182–1.40 g/ml) could not be examined due to the presence of other plasma proteins. The basal density gradient fractions of patients No. 2–4 were also examined by this technique with similar conclusions (data not shown).

Density gradient changes with recovery

Patients No. 1–3 were sampled during their recovery period. **Fig. 8** shows the density gradient profile at recovery (day 18) for patient No. 1. Similar profiles were obtained for the other two patients. At recovery, the LDL portion of the gradient d 1.021–1.064 g/ml (fractions 2–8) had become EC-rich, the band of PL and UC at fraction 8 (Fig. 4A) had disappeared, and the HDL_2 d 1.064–1.125 g/ml (fractions 9–13) region of the gradient had increased in EC, PL, and apoA-I. Notice that the restoration of HDL involved mainly the d 1.064–1.125 g/ml density (light HDL) region with smaller increases in the HDL_3 + VHDL (d > 1.125 g/ml, heavy HDL) region (fractions 14–20). The relatively large amounts of apoE seen in fractions 8–11 (d 1.055–1.098 g/ml) of the SDS-gels (Fig. 5A) at day 4 diminished appreciably (Fig. 5B). The gel picture clearly confirms the results of the immunoassay of apoA-I, i.e., apoA-I first returns chiefly in the light HDL region of the gradient. Electron micrographs of negatively stained samples of the density gradient shown above for patient No. 1 at recovery are shown in **Fig. 9.** The LDL region now contained particles that were much more normal in appearance and more homogeneous in size. Discoidal particles were much less prevalent in fractions 10 and 11; and the HDL particles, though few in number compared to normal, ap-

peared normal in shape and size. Even though the patient had medically recovered from alcoholic hepatitis, his plasma lipoprotein levels were still not normal.

Follow-up studies

The four patients were sampled again 6–10 months after their initial samples as alcoholic hepatitis patients. The results are given in **Table 3** along with laboratory results indicative of liver function. Except for patient No. 3, liver function parameters were still elevated, although the serum bilirubin levels were much improved. Patients No. 1–3 had normalized their plasma CH and TG levels along with their percentage of cholesterol esterified. Patient No. 4's percent esters were subnormal, probably as a result of another hospitalization with alcoholic hepatitis about 3.5 months after his first sample date in this study. Patients No. 2, 3, and 4 had HDL-CH and plasma apoA-I levels that were elevated above normal. Conversations with these three patients revealed they were still drinking heavily. Patient No. 1, who had HDL-CH and plasma apoA-I levels in the normal range, indicated he had reduced his alcohol consumption considerably. The density gradient profile obtained by fractionation of the d > 1.006 g/ml fraction from patient No. 3 is presented in **Fig. 10**. Summation of CH and apoA-I in fractions 9–13 (d 1.064–1.125 g/ml) and comparison with that in fractions 9–20 indicated more light HDL than heavy HDL in the gradient. Similar density gradient profiles were obtained for patients No. 2 and 4. Patient No. 1, on the other hand, had an HDL-CH and apoA-I density gradient distribution similar to that of a normal subject (Fig. 3A). Thus, the three patients who continued to drink heavily after hospitalization continued to elevate their light HDL subspecies, which were increasing when the patients were discharged.

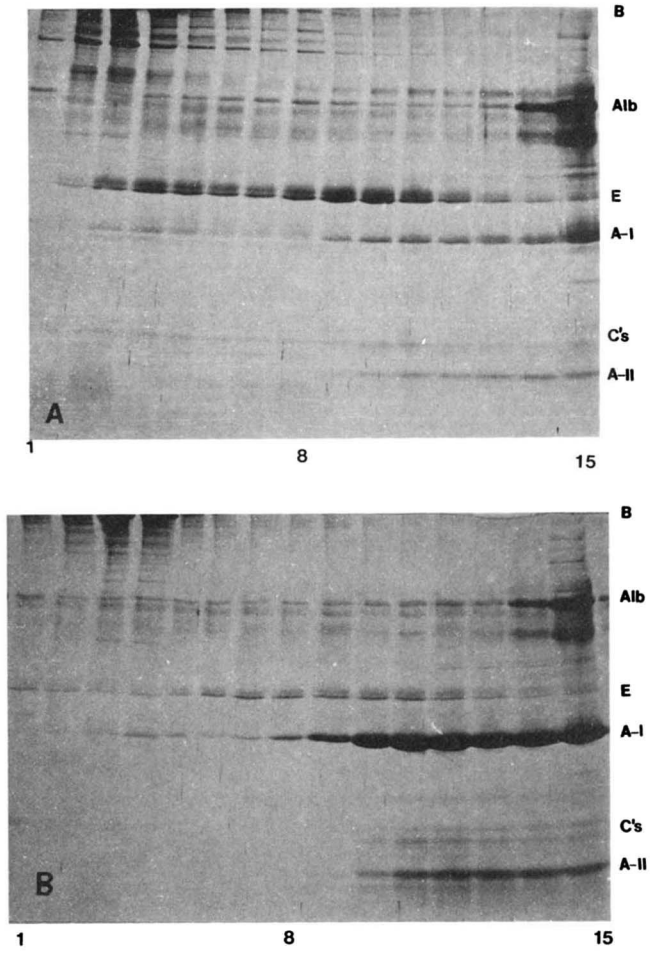

Fig. 5. SDS–gradient slab gel electrophoresis of proteins in density gradient fractions from patient No. 1. A (top), day 4 of illness; B (bottom), day 18 (recovered). Fractions were electrophoresed as in Fig. 3B, using equal volumes (35 μl) of density gradient fraction in each lane.

DISCUSSION

Previous studies from this laboratory, which have documented lipoprotein compositional alterations in human alcoholic hepatitis, have utilized primarily sequential ultracentrifugation procedures for isolation of lipoproteins. In these studies we utilized the classical density cuts to define CM, VLDL, LDL, and HDL fractions. Based on these studies, we have demonstrated the presence in plasma of abnormal lipoproteins which we postulate to represent the accumulation of secretory forms. In the present study we have utilized an exponential salt plus sucrose density gradient in order to more completely resolve the d > 1.006 g/ml fraction into its constituent lipoproteins, IDL, LDL, HDL_2, HDL_3, and VHDL. In addition, we have combined this technique with SDS-PAGE and electron microscopic studies of the individual density gradient fractions to define lipoprotein apoprotein composition and morphology. We reasoned that, although we may not be able to completely resolve all of the different types of lipoprotein particles found in the d > 1.006 g/ml fraction, we should be able to draw conclusions as to their composition based on the gradient profiles of the various apoprotein and lipid constituents and correlate these with electron micrographs. Thus, if two or more constituents demonstrate an identical gradient profile, we would assume that they are associated in a single type of lipoprotein particle. On the other hand, a difference in gradient profile of two constituents might indicate that they exist in different particles. An example of the latter type of finding is the difference in the distribution of apoE and apoA-II in the gradient, which suggests that these apoproteins do not exist as a complex in a single lipoprotein particle.

Heterogeneity in the LDL region of the density gra-

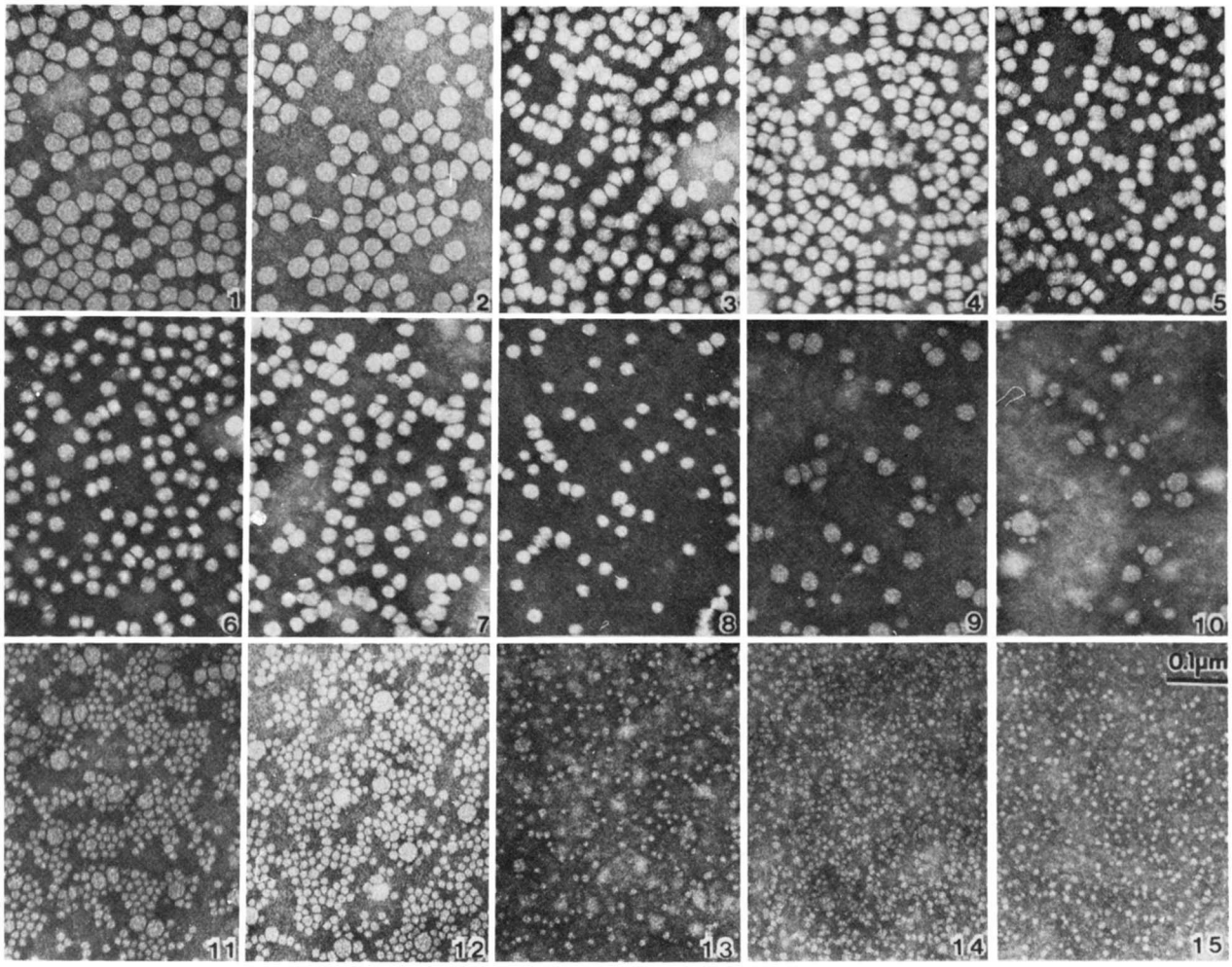

Fig. 6. Electron micrographs of negatively stained samples of the density gradient fractions No. 1–15 shown in Fig. 3A. (normolipidemic subject). Final magnification 100,000×. IDL, LDL, and HDL are found in fractions 1, 2–8, and 9–17, respectively. The mean diameter of the particles in fraction 1 was 31.0 ± 7.7 nm (S.D.). The mean diameters of particles in fractions 2–8 (LDL) ranged from 28.9 ± 2.9 nm (fraction 2) to 18.7 ± 2.8 nm (fraction 8). The mean particle diameter in fraction 5 (peak in lipid and apoB concentrations) was 23.4 ± 2.3 nm. Fractions 9–13 (light HDL) contained two populations of spherical particles. The smaller particles had a mean diameter ranging from 16.6 ± 2.6 nm (fraction 9) to 9.1 ± 1.8 nm (fraction 13). Larger particles ranged from 31.3 ± 4.2 nm to 26.3 ± 2.9 nm. The smaller particles are probably HDL while the larger ones could be Lp(a). Fractions 14 and 15 contained only HDL-sized particles with a mean diameter of 7.8 ± 1.2 nm and 7.2 ± 1.5 nm, respectively.

dient can be seen from both the SDS-PAGE analyses of the apoproteins and electron microscopic studies of each fraction (Figs. 4A and 5A). ApoB, PL, EC, and UC peak together in fraction 3 (d 1.026–1.031 g/ml). An apoE peak can be seen in fraction 4 (d 1.031–1.036 g/ml) and an albumin peak occurs in fraction 8 (d 1.055–1.064 g/ml) together with peaks in PL and UC. Thus, the LDL region of the gradient appears to consist of lipoproteins varying in lipid and protein composition. Electron micrographs of negatively stained samples of the density gradient fractions 3 and 4 (Fig. 7) indicated the presence of larger vesicular structures in fraction 4 in addition to small, spherical particles common to both fractions. Fraction 8 contained fewer stained particles, but appeared to consist of spherical particles similar to those in fractions 3 and 4; in addition, it contained smaller vesicular structures than present in fraction 4 along with some stacked discs. Since a normal sequential ultracentrifugal density cut (d 1.019–1.063 g/ml) for LDL would have contained all three types of particles, it can be seen that the density gradient ultracentrifugation technique can provide a partial resolution of these particles. Kostner et al. (34) have also reported heterogeneity in the lipoproteins in the 1.019–1.063 g/ml density region in the plasma of patients with secondary LCAT deficiency resulting from extrahepatic cholestasis.

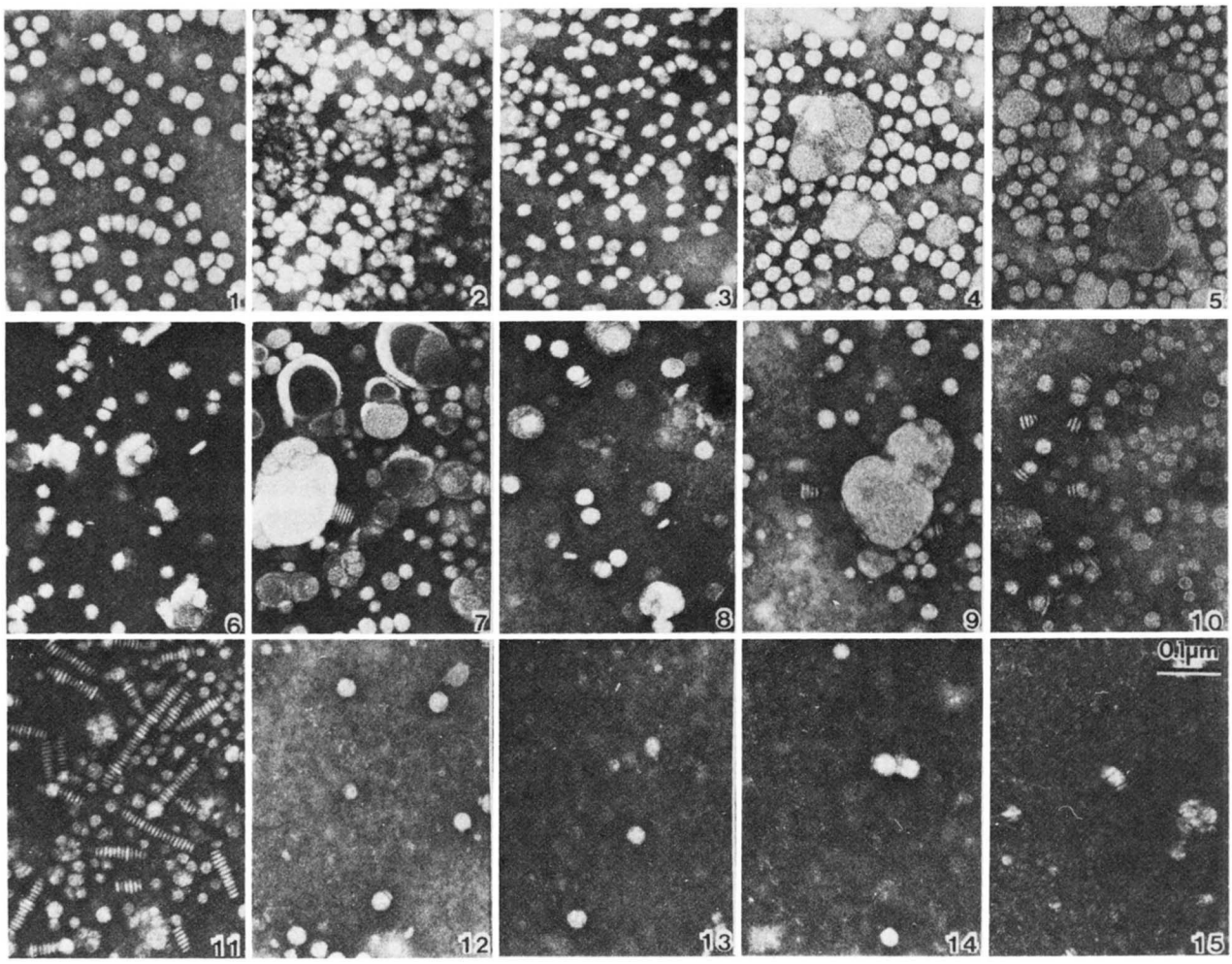

Fig. 7. Electron micrographs of negatively stained samples of the density gradient fractions No. 1–15 shown in Figure 4A. Alcoholic hepatitis patient No. 1. Final magnification 100,000×.

The further characterization of these lipoproteins contained in our density gradient fractions is being pursued in our laboratory.

Since the majority of the plasma lipids and apoB are carried in the d > 1.006 g/ml fraction in alcoholic hepatitis, and more specifically in the LDL density region, we were prompted to examine the EC + TG (core lipids) content of this fraction. We found a strong, significant linear correlation to exist between these lipids and the apoB concentration of the d > 1.006 g/ml fraction. We speculate that this finding may indicate that TG and EC bind equally to apoB-containing particles in the LDL density range, since the binding ratio of apoB to TG + EC is constant. Since many of these "LDL-like" particles are similar to normal LDL in both size and density, we suggest that they may arise from nascent VLDL that has been partially depleted of TG by the action of peripheral lipoprotein lipase. In the absence of normal

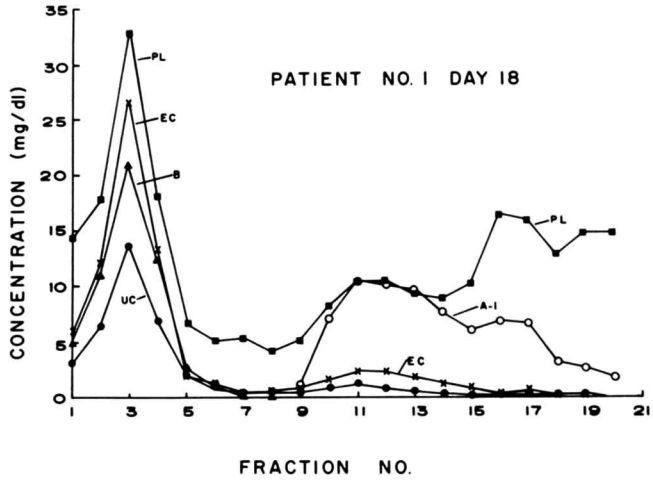

Fig. 8. Distribution of lipids and apoproteins in the density gradient fractionation of the d > 1.006 g/ml plasma fraction of alcoholic hepatitis patient No. 1 at day 18 (recovered). Symbols same as Fig. 4.

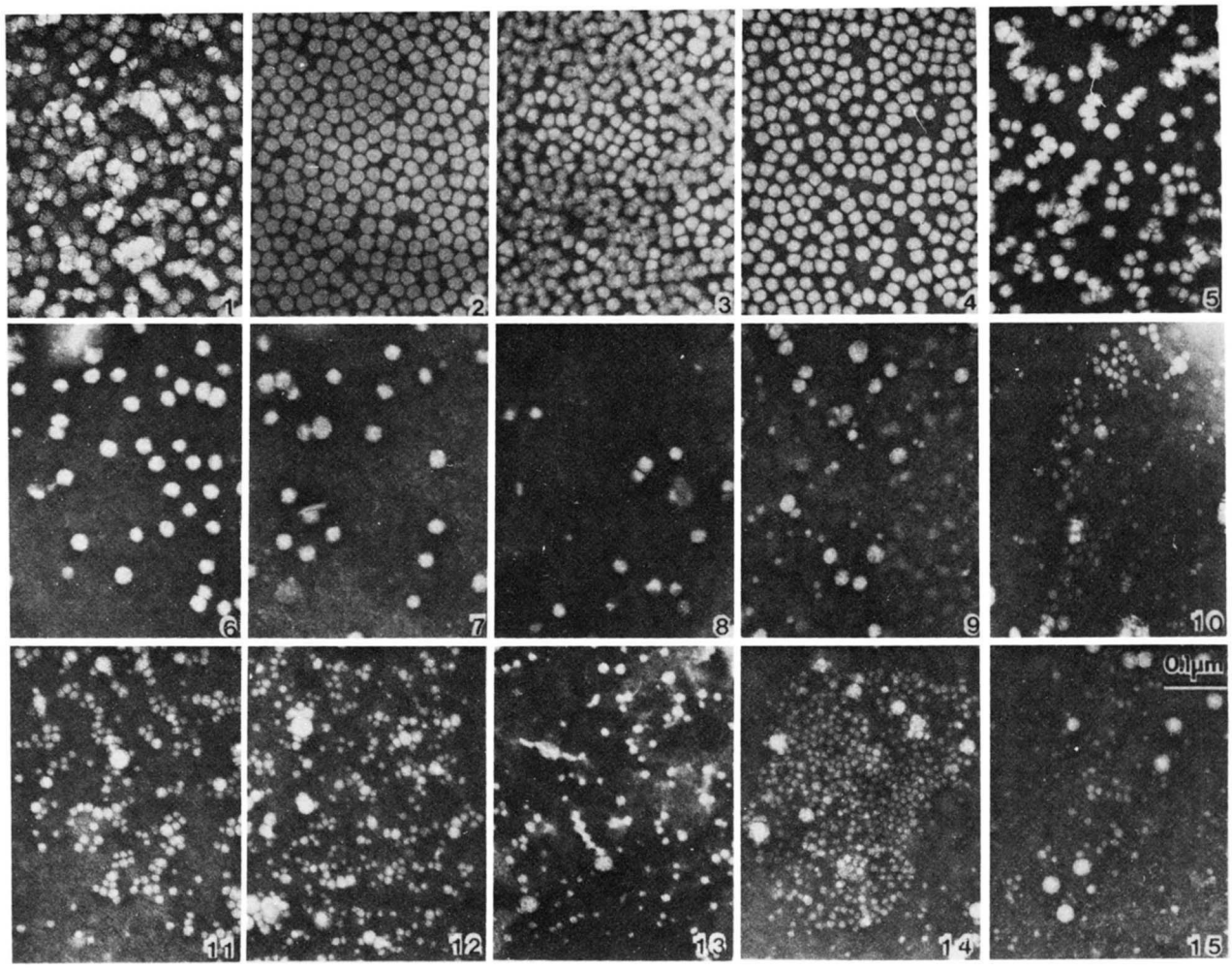

Fig. 9. Electron micrographs of negatively stained samples of the density gradient fractions 1–15 shown in Fig. 8 for patient No. 1 on day 18. Final magnification 100,000×. Particle diameters were not measured.

LCAT activity and hepatic triglyceride lipase activity, this VLDL cannot be converted into the usual EC-rich LDL. Thus a "TG-rich LDL" accumulates.

We have previously demonstrated by sequential ultracentrifugation that an apoE-rich HDL (d 1.063–1.21 g/ml), containing some apoA-I, accumulates in the plasma of patients with alcoholic hepatitis. In the present study we have shown that this particle is contained primarily in the HDL_2 region (d 1.063–1.125 g/ml) of our density gradient. This lipoprotein is similar to an apoE-rich lipoprotein secreted from normal rat liver when perfused with a solution containing an LCAT inhibitor

TABLE 3. Long-term recovery plasma parameters of alcoholic hepatitis patients

Patient No.	Time Period[a]	Plasma CH	Plasma % CH Esterified	Plasma TG	HDL-CH	Plasma apo A-I	Total Bilirubin	Alkaline Phosphatase	SGOT	SGPT
	months	*mg/dl*		*mg/dl*	*mg/dl*	*mg/dl*	*mg/dl*	*mU/ml*	*mU/ml*	*mU/ml*
1	10	195	74	51	43	132	2.4[b]	271[b]	75[b]	31[b]
2	7.5	169	76	57	75	147	1.97[b]	93[b]	53[b]	42[b]
3	6.5	239	74	80	108	186	0.7	126[b]	19	5
4	6.0	162	64	98	71	144	3.25[b]	99[b]	131[b]	88[b]

[a] Time between first sampling during acute alcoholic hepatitis study and most recent sample.
[b] Outside normal range.

(35–37) and may represent human nascent HDL. Marcel et al. (38) have succeeded in isolating two "nascent" HDL particles from alcoholic hepatitis plasma by heparin affinity chromatography. One contained primarily apo-E with only traces of apoA-I while the other contained primarily apoA-I with traces of apoE. We can designate these as E "nascent" HDL and A-I "nascent" HDL. Both of these fractions were rich in unesterified cholesterol and phospholipid. Analyses of density gradient fractions 15–17 (d = 1.158–1.21 g/ml, Fig. 4A) indicated the presence of a small concentration of apoA-I and UC along with appreciable levels of PL. It is possible that this material is similar to the A-I "nascent" HDL isolated by Marcel et al. (38). Mitchell et al. (39) have employed a combination of heparin-Sepharose and con A-agarose chromatography to separate an A-I species of "nascent" HDL and an apoE-rich species from the plasma of patients with familial LCAT deficiency. These results, those of Marcel et al. (38), and our own would seem to leave little doubt that separate apoA-I and apoE forms of nascent HDL occur in LCAT deficiency, whether familial or secondary to liver disease.

Tada, Fidge, and Nestel (40) have reported the occurrence of mixed disulfide complexes of apoE with apoA-II in HDL from patients with alcoholic hepatitis. They postulate that perhaps the complexes of apoE render E "nascent" HDL less reactive with apoE receptor sites and thus prevent immediate uptake upon secretion. Our data and that of Marcel et al. (38) do not support these findings. In our density gradient, E and A-II do not co-distribute and the E-HDL from alcoholic hepatitis purified by Marcel et al. (38) contains very little apoA-II.

An interesting observation which we report here is that, during recovery from alcoholic hepatitis, lipoproteins with the density characteristic of "light" HDL reappear before "heavy" HDL. This material is similar to HDL in that it is rich in PL and apoA-I and contains more cholesteryl esters than unesterified cholesterol. However, since the overall lipid and apoprotein pattern of these patients is still distinctly abnormal, even after clinical recovery from hepatitis, this "light" HDL probably is not similar to lipoproteins present in normolipidemic subjects. The mechanism of this apparent preferential recovery of material having HDL_2 density characteristics is not known, but may arise from the action of LCAT on either Lp-X-like material peaking at fraction 8 in the density gradient (41) or "nascent" HDL-like material isolated in fractions 9–13 (d 1.063–1.125 g/ml).

The long-term recovery of three out of four of the patients' HDL levels may be related to their post-illness alcohol consumption. Elevations in HDL levels have been noted in chronic alcoholics. During alcohol abstinence, HDL levels decline to normal within 2 weeks. The increase in HDL reported by Danielsson et al. (42), as studied by rate zonal ultracentrifugation, was heterogeneous with changes in HDL_2 as well as HDL_3 subfractions. Our observation of increased HDL levels with increased lipoproteins isolated in the HDL_2 density range is interesting in light of the preferential recovery of this same density range during partial recovery from alcoholic hepatitis. Presumably, if the patients continued to drink, their livers would still be partially damaged and their HDL composition possibly abnormal. The mechanism of this remarkable elevation of material isolable in the HDL density range in these patients is the subject of current investigations in this laboratory.

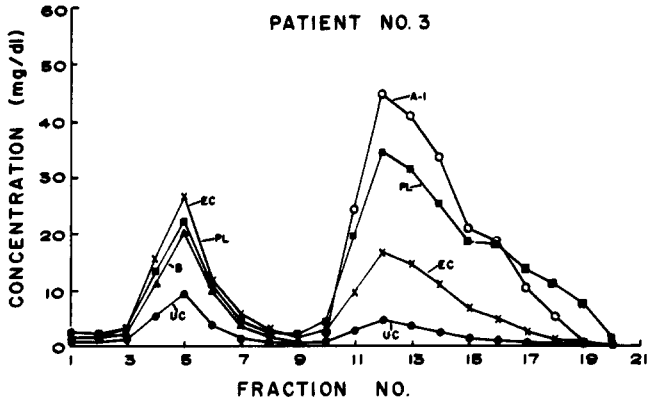

Fig. 10. Distribution of lipids and apoproteins in the density gradient fractionation of the d > 1.006 gm/ml plasma fraction of an alcoholic hepatitis patient 6.5 months after her initial sampling at the onset of her illness. Symbols same as Fig. 4.

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