Intestinal apoB synthesis, lipids, and lipoproteins in chylomicron retention disease

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Abstract Chylomicron retention disease is characterized by fat malabsorption, hypocholesterolemia, normal fasting triglycerides, and marked intestinal steatosis despite the presence of both plasma and intestinal apoprotein B. The defect remains unknown but presumably involves the synthesis or secretion of chylomicrons. The present investigation examines this hypothesis by studying the biosynthesis of chylomicrons in cultured jejunal explants and by defining the quantitative and qualitative abnormalities of plasma lipids and of circulating lipoproteins. Following 2-3 years of a low fat diet supplemented with medium chain triglycerides, six patients with chylomicron retention disease had significantly higher triglyceride (TG) levels coupled with a decrease in both free (FC) and esterified cholesterol (EC) as well as in essential fatty acids and phospholipids (PL) when compared to healthy controls. The low total plasma cholesterol was largely accounted for by low levels of both low density (LDL) and high density lipoprotein (HDL) cholesterol. VLDL and LDL were characterized by a diminished percentage of CE with an increase of TG while HDL contained relatively more FC as well as PL and less CE. The diameter of VLDL was larger whereas those of LDL and HDL were smaller than in normal controls. Jejunal explants, when incubated with [^{14}C]palmitate, were capable of normal biosynthesis of TG, diglycerides, PL, and CE. These lipids, however, except for PL, were retained in the tissue and could not be secreted into the culture medium. Incubation of intestinal biopsies with [^{3}H]leucine and [^{14}C]mannose resulted in normal protein synthesis and reduced glycosylation. The presence of intestinal apoB-48 was confirmed by immunoblot using 2D8 antibodies. These data suggest that the intestinal defect in this disease results from a disorder of the final assembly of chylomicrons or in the mechanism of their exocytosis.—**Levy, E., Y. Marcel, R. J. Deckelbaum, R. Milne, G. Lepage, E. Seidman, M. Bendayan, and C. C. Roy.** Intestinal apoB synthesis, lipids, and lipoproteins in chylomicron retention disease. *J. Lipid Res.* 1987. **28:** 1263–1274.

Supplementary key words chylomicron • VLDL • LDL • HDL • apoB-48 • intestinal explant • glycosylation

Abetalipoproteinemia and homozygous hypobetalipoproteinemia are characterized by severe fat malabsorption and by neuromuscular and ocular manifestations (1, 2). In view of the absence of apoprotein B in the intestine and liver, chylomicrons (CM) cannot be synthesized and very low density (VLDL) as well as low density lipoprotein (LDL) are undetectable in the plasma (3).

We have recently described another defect of lipoprotein metabolism associated with hypocholesterolemia, i.e., chylomicron retention disease (CRD). This condition is characterized by malabsorption, normal plasma fasting triglycerides, hypocholesterolemia, reduced plasma concentration of apoprotein B, and the absence of chylomicrons after a fat meal test (4). Steatosis of the enterocytes was marked even in the fasting state. CM-like particles were identified by electron microscopy (EM) in the endoplasmic reticulum, within dilated vesicles, but were essentially absent from both the intercellular spaces and lacteals. The purpose of the present study was to characterize the lipid profile and the lipoprotein pattern as well as to investigate the capacity of jejunal explants to esterify lipids, synthesize and glycosylate proteins, and to examine these explants for the presence of apoB-48.

METHODS

Six cases of chylomicron retention disease (CRD) with a mean (± SD) age of 8 ± 3 yr were subjects of this study. The patients were investigated 2 to 3 yr after having being placed on a diet restricted in long-chain triglycerides (± 20 g/day) and supplemented with medium-chain

Abbreviations: CRD, chylomicron retention disease; LPL, lipoprotein lipase; LCAT, lecithin:cholesterol acyltransferase; PC, phosphatidylcholine; SP, sphingomyelin; HL, hepatic lipase; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; CM, chylomicrons; EM, electron microscopy; TLC, thin-layer chromatography; TG, triglycerides.

triglycerides. During that period of time, a distinct improvement in their clinical condition had been observed. They were compared to ten age-matched healthy children and adolescents. Informed consent was obtained from the patients or their parents and permission to conduct the study was obtained from the Ethics Committee of l'Hôpital Sainte-Justine.

Oral fat loading test

Study of intestinal fat absorption was carried out after the patients, fasted for 12 hr, ingested 50 g of fat per 1.73 m^2 surface area (flavored commercial cream). The plasma cholesterol and triglycerides were measured 2, 3, and 5 hr following the fat meal (1) and lipoprotein electrophoretograms were performed as described previously (5).

Isolation of lipoproteins

Blood was collected after a 12-hr fast in 1 mM disodium EDTA and 0.05% sodium azide. Plasma was separated by low speed centrifugation (3,000 rpm, 30 min) at 4°C. The lipoprotein fractions were isolated by sequential ultracentrifugation according to Havel, Eder, and Bragdon (6) with a Ti-50 rotor in a Beckman Model L5-65 ultracentrifuge. VLDL and LDL were separated at densities of 1.006 g/ml and 1.063 g/ml, respectively, centrifuging at 100,000 *g* for 18 hr at 5°C. The high density lipoprotein fraction (HDL) was obtained by adjusting the LDL infranatant to 1.21 g/ml and by centrifuging for 48 hr. Each lipoprotein fraction was washed by its equilibrium density and dialyzed exhaustively against 0.15 M NaCl, 0.001 M EDTA, pH 7.0, at 4°C. In a second phase of this study, LDL and HDL were isolated and subfractionated by zonal ultracentrifugation at 40,000 rpm in a continuous gradient of NaBr, spanning the density range 1.00–1.40 g/ml. Using a Beckman Ti-14 zonal rotor in a Beckman L5-50 ultracentrifuge (15°C), the runs lasted 140 min and 22 hr for LDL and HDL, respectively. The rotor effluent monitored continuously at 280 nm and 250-ml fractions were collected. For analysis, fractions under a given peak were pooled, concentrated by vacuum dialysis, and extensively dialyzed.

Analyses

Lipids were extracted with chloroform–methanol (7). Total phospholipid content and individual phospholipid subclasses on TLC were determined by the Bartlett method (8). Lipoprotein-protein was measured according to Lowry et al. (9) with bovine serum albumin as a standard. TG were quantitated by enzymatic methods and total and free cholesterol by the oxidase-esterase method (10) using the Boehringer-Mannheim kit (Montreal). Esterified cholesterol was calculated as the difference between total and unesterified cholesterol and cholesteryl ester mass was taken to be 1.7 × esterified cholesterol mass. HDL-cholesterol was measured after precipitation of very low and low density lipoproteins with phosphotungstic acid (11). LDL-Cholesterol was directly determined using polyvinylsulphate (Boehringer-Mannheim, Montreal, Quebec) (12). VLDL cholesterol was calculated from the difference between cholesterol in the polyvinylsulphate supernatant and HDL-cholesterol (HDL-C). The apolipoprotein C content was examined by tetramethylurea gel electrophoresis (TMU gel) (13). Electron microscopy of lipoprotein particles was performed on a Zeiss EM-10 microscope, using negative staining with 1% phosphotungstic acid (pH 7.2), as described previously (14). The fatty acid composition was determined with an improved method described previously (15).

Lipolytic activity was measured with an emulsion of glycerol tri[1-^{14}C]oleoylglycerol as substrate (16). Hepatic triglyceride lipase was assayed in the presence of protamine sulfate which was verified to completely inhibit peripheral lipoprotein lipase. Extraction of free fatty acids (FFA) was performed by the procedure of Belfrage and Vaughan (17) as previously described (18).

The plasma LCAT activity was measured following the original procedure of Stokke and Norum (19).

Explant cultures of jejunal biopsies

Fasting intestinal biopsies were obtained with a Carey capsule at the ligament of Treitz from patients and from controls investigated for growth failure but without digestive manifestations. The specimens were immediately placed in RMPI-1640 culture medium (Gibco Inc., Grand Island, NY) containing 10% inactivated and dialyzed human lipoprotein-deficient serum (LPDS). This medium was saturated with 95% O_2 and 5% CO_2. Cultures were set up by methods previously described (20) within 20 min of taking the biopsies. Small, well-laid-out pieces of intestinal tissue, mucosal side up, were placed on a stainless-steel wire screen over the middle well in sterile plastic culture dishes (Falcon Plastics, Los Angeles, CA). Filter paper soaked in sterile distilled water was placed around the well to keep the enrivonment saturated with moisture. The tissue culture medium (0.8 ml) consisted of leucine-free RPMI-1640 with LPDS as well as with gentamycin (100 μg/ml) and soybean trypsin inhibitor (60 μg/ml). The medium was also supplemented with 8 mM sodium taurocholate, 20 mM palmitic acid, and 10 mM *sn*-2-monooleoylglycerol to stimulate intracellular lipid esterification. Lipid synthesis and secretion by explants was studied by adding 10 μCi of [^{14}C]palmitic acid (sp act 40–60 mCi/mmol, New England Nuclear, Montreal, Quebec). The Petri dishes were thereafter placed in anaerobic jars, sealed, gassed with 5% CO_2, 95% O_2, and incubated for 8–18 hr at 37°C. The pH of the medium was maintained between 7.2–7.4 as indicated by phenol red.

Following incubation, the explants were sonicated (Kontes Microultrasonic Cell Disruptor, Ultrasonics) at a

setting of 4 for 3–10-sec bursts. In experiments with [^{14}C]palmitate, the biopsy homogenates and their respective incubation medium were lipid-extracted with chloroform–methanol 2:1 (v/v) (7). Small amounts of lipid standards were added to the samples before separation of individual lipid classes by one-dimensional TLC (silica gel, Eastman Kodak, Rochester, NY) as described previously (21). The developing solvent system was heptane–diethylether–glacial acetic acid 80:20:3 (v/v/v). The radioactivity of the separated fractions was measured in a Beckman liquid scintillation spectrometer. Quenching was corrected using computerized curves generated with external standards. An aliquot of the tissue homogenate was used for protein determination. In experiments carried out to measure [^{3}H]leucine incorporation, radioactivity (CPM) was measured in the 10% trichloroacetic precipitable protein fraction of a tissue homogenate diluted 1:1 with 0.2% BSA. Tritium counts were also quantitated in aliquots of the CM-like fraction isolated by centrifuging sonicated explants at 25,000 rpm for 30 min. This fraction was used for SDS gel electrophoresis and for Western blots (22).

Statistical methods

All results are expressed as means ± SD. The differences between means were assessed using the two-tailed Student's *t* test.

RESULTS

Response to the oral fat load

A representative response to the oral fat load (case 1) is illustrated in **Fig. 1.** The electrophoretic agarose gel demonstrated a total absence of CM at 1, 3, and 5 hr postprandially and an abnormal lipoprotein pattern was visualized. In contrast, control subjects responded normally to the fat meal with the peak of TG absorption at 3 hr.

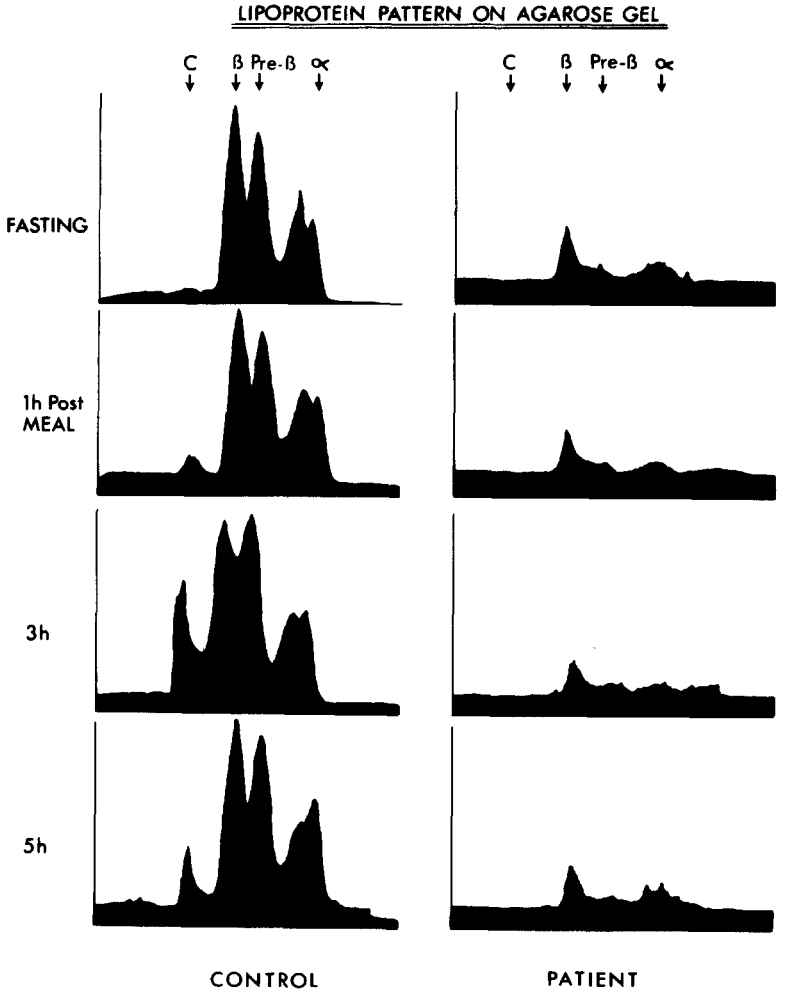

Fig. 1. Agarose electrophoresis scan of plasma from one CRD patient (case 1) and one control. With this technique, the chylomicrons (C) were not visualized in the patient after the fat meal. By comparison with the control, an abnormal lipoprotein mobility (beta, pre-beta, and alpha) was observed.

TABLE 1. Plasma and lipoprotein cholesterol concentrations of chylomicron retention disease (CRD) and controls

Patients	Age	Plasma			Lipoprotein Cholesterol		
		TG	TC	(CE as % of total)	VLDL	LDL	HDL
	yr				*mg/dl*		
CRD							
Case 1	7	138	64	(71.8)	15	37	12
Case 2	4	187	60	(54.2)	21	29	10
Case 3	11	77	44	(71.4)	27	6	11
Case 4	6	108	96	(73.3)	21	62	13
Case 5	9	158	92	(65.8)	35	42	15
Case 6	13	140	113	(79.5)	45	52	16
Mean ± SD	8.3 ± 3.3	135 ± 38[b]	78 ± 26[b]	(69.2 ± 2.3)[a]	27.3 ± 11.0[a]	38.0 ± 19.4[a]	12.8 ± 2.3[b]
Controls (n = 6)							
Mean ± SD	8.6 ± 3.2	65 ± 17	183 ± 35	(80.3 ± 2.0)	13.0 ± 5.7	110.8 ± 20.5	59.0 ± 11.3

TG, triglycerides; TC, total cholesterol; CE, cholesteryl ester.
CRD versus controls: [a]$P < 0.05$; [b]$P < 0.001$.

Plasma lipid and lipoprotein-cholesterol levels

Plasma lipids and lipoprotein cholesterol concentrations in all study subjects are given in **Table 1**. In the CRD group, plasma cholesterol and TG ranged between 44 and 113 mg/dl and 77 and 187 mg/dl, respectively. The mean (± SD) plasma cholesterol (78 ± 26 mg/dl) was less than half of the levels in normal controls (183 ± 25 mg/dl) but the average TG levels were twofold higher (135 ± 38 vs. 65 ± 17 mg/dl). The cholesterol distribution among the lipoprotein classes showed an increase of twice the absolute amount of cholesterol in the VLDL range relative to normal. Thus, in this disease, a larger fraction of plasma cholesterol (1/3) is carried by triglyceride-rich lipoproteins. A decrease was observed in both LDL-C (38.0 ± 19.4 vs. 110.8 ± 20.5 mg/dl) and HDL-C (12.8 ± 2.3 vs. 59.0 ± 11.3 mg/dl). The percentage of cholesteryl ester was significantly reduced in patients when compared to controls (69.2 ± 2.3 vs. 80.3 ± 2.0%).

From **Table 2,** it is apparent that total PL values in CRD were significantly below those of controls (125.8 ± 28.8 vs. 183.8 ± 12.7 mg/dl). However, no important variation in PL composition was noted and the PC/SP ratio was similar to that of controls. Likewise, red cell PL distribution in two patients (cases 1 and 2) was quite similar to controls and dissimilar to the disturbed PL composition which characterized two abetalipoproteinemia subjects. The mean PC/SP ratio was 1.14 for CRD patients, 0.91 for controls, and 0.24 for abetalipoproteinemia subjects. Therefore, it is apparent that plasma and erythrocyte PL composition are indeed affected in abetalipoproteinemia but are unaltered in CRD subjects. **Table 3** documents the presence of essential fatty acid deficiency in CRD patients. The polyunsaturated fatty acids, linoleic and arachidonic, were lower when expressed either in absolute values or as a % of total fatty acids. The C18:1/C18:2 ratio, an index of essential fatty acid deficiency, was found to be higher than in controls

TABLE 2. Plasma phospholipid composition in chylomicron retention disease (CRD) and controls

	Total mg/dl	PC	PE	SP	PC/SP
		% of total mass			
CRD					
Case 1	112.44	72.4	16.4	11.2	6.46
Case 2	126.73	67.7	18.0	14.4	4.70
Case 3	78.84	58.9	14.0	27.0	2.18
Case 4	151.44	66.8	9.2	24.0	2.78
Case 5	143.41	57.6	17.5	25.0	2.30
Case 6	146.23	74.3	4.5	21.2	3.50
Mean ± SD	126.51 ± 27.44	66.28 ± 6.84	13.27 ± 5.37	20.46 ± 6.3	3.65 ± 1.65
Controls					
Mean ± SD	183.80 ± 12.66	71.44 ± 6.47	7.7 ± 4.7	16.82 ± 3.09	4.38 ± 0.98

Data are mean ± SD of the six patients with CRD and seven controls. Differences between phospholipids of CRD and controls are significant at a level of $P < 0.001$. PC, phosphatidylcholine; PE, phosphatidylethanolamine, SP, sphingomyelin.

TABLE 3. Plasma fatty acid composition in chylomicron retention disease (CRD)

Fatty acids	Controls (n = 6)		CRD (n = 6)	
	μg/ml	*wt %*	*μg/ml*	*wt %*
C14:0	23.0 ± 3.7	1.0 ± 0.2	37.7 ± 14.0[a]	1.9 ± 1.0[a]
C15:0	8.4 ± 1.5	0.4 ± 0.2	8.3	0.4
C16:0	510.3 ± 58.8	21.4 ± 1.2	517.2 ± 156.5	27.6 ± 3.7[b]
C16:1	33.3 ± 18.4	1.4 ± 0.7	131.1 ± 66.6[b]	6.0 ± 2.2[c]
C18:0	184.2 ± 23.5	7.7 ± 0.2	156.1 ± 41.6	7.4 ± 1.0
C18:1	584.2 ± 67.1	24.6 ± 2.2	710.3 ± 216.5	33.1 ± 5.1[b]
C18:2	734.1 ± 101.4	30.8 ± 1.7	250.5 ± 36.2[c]	12.4 ± 2.7[c]
C20:3	35.4 ± 6.4	1.5 ± 0.2	31.6 ± 11.0	1.5 ± 0.5
C20:4	203.8 ± 45.1	8.5 ± 1.2	132.2 ± 24.0[b]	6.4 ± 1.2[a]
C22:0	ND[d]	0.7	8.2	0.3
C22:6	42.9 ± 18.4	1.8 ± 0.5	32.2 ± 8.1	1.6 ± 0.7
C24:1	19.4 ± 3.4	0.8 ± 0.2	21.1 ± 4.4	1.0 ± 0.2
Total	2381.5 ± 268.9	99.9	2105.6 ± 468.8	99.6
C18:1/C18:2	0.8 ± 0.09		2.82 ± 0.83[c]	
C18:2/C20:4	3.71 ± 0.86		1.92 ± 0.29	
Nonsaturated	69.4 ± 2.44		63.1 ± 4.89[c]	

Data are mean ± SD. Fatty acids contributing less than 0.2% of the total have been omitted from the Table.
CRD versus controls: [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$.
[d]ND, Nondetectable.

while the C18:2/C20:4 ratio, indicative of fatty acid elongation, was lower. Similar results were observed when fatty acid patterns of the FFA and of individual lipoprotein fractions (VLDL, LDL, HDL) were examined **(Table 4)**.

Lipoprotein composition

When the lipid composition of lipoproteins was studied in CRD, all fractions were PL-enriched and cholesterol-depleted. This is best illustrated by plotting each lipid constituent as a percentage of the total lipoprotein mass on triangular coordinates **(Fig. 2)**. TG-enrichment was also observed in VLDL and LDL fractions. To determine whether the decreased cholesterol content was due to a decline in free (FC) or cholesteryl ester (CE), the percentage esterification was estimated **(Table 5)**. CE was depressed in all lipoprotein classes while FC was relatively lower in VLDL, showed no change in LDL, and was higher in HDL.

The ratio of core constituents (CE and TG) to surface constituents (FC, PR and PL) can be used to make an inference on the size of spherical lipoprotein particles. Since the volume of the sphere increases with the cube of the radius, whereas the surface area increases with the square of the radius, a higher ratio of core/surface constituents would imply a population of larger particles. The (insert formula) ratio was raised by 9% in VLDL and reduced by 32% in LDL and by 67% in HDL than normals. This indicates that larger VLDL and smaller LDL and HDL are present in CRD patients than in normals. Electron microscopy of CRD plasma lipoproteins (Table 5) confirmed the data calculated from compositional analyses. Electron micrographs of the isolated lipoprotein fractions are shown in **Fig. 3**. Generally, a spherical appearance was observed and only a few discoidal particles were visible in the HDL fraction. Zonal ultracentrifugation elution profiles of HDL in CRD showed that most HDL consisted of HDL_3. A smaller subpopulation with an elution profile of HDL_4 similar to the one described in abetalipoproteinemia (23) was eluted at a higher density than normal HDL_3**(Fig. 4)**. LDL was heterogeneous and contained at least two distinct subpopulations. The major LDL peak eluted later than normal LDL. This is consistent with a smaller particle population.

The SDS- (4% and 10%) and TMU-PAGE showed in CRD an apoprotein distribution of apoproteins which did not differ from that obtained from control subjects (figures not shown here). Moreover, the patients' VLDL and LDL reacted with a monoclonal antibody 2D8 thereby demonstrating the presence of normal apoB-100. These results, therefore, confirm our previous data (4).

LCAT activity

To determine whether the low percentage of cholesterol present as cholesteryl ester observed in the plasma and lipoprotein fractions was due to a drop of LCAT activity, a quantification of the latter was carried out. It was found that plasma of the six CRD patients only contained 59.3 to 70.9% of the activity of control plasma **(Table 6)**. These results suggest that a diminished enzyme activity is associated with inefficient cholesterol esterification.

TABLE 4. Essential fatty acids in plasma FFA and lipoproteins

	FFA			VLDL			LDL			HDL		
	Total (μg/ml)	18:2 (%)	18:1/18:2	18:2/20:4	18:2 (%)	18:1/18:2	18:2/20:4	18:2 (%)	18:1/18:2	18:2/20:4	18:2 (%)	18:1/18:2	18:2/20:4
CRD (n = 6) Mean ± SD	314.2 ± 127.1	8.6 ± 1.9	3.8 ± 1.5	1.7 ± 0.2[b]	10.5 ± 3.2[b]	4.1 ± 1.5[a]	3.2 ± 0.4[b]	15.6 ± 3.7[b]	1.9 ± 0.9[a]	2.1 ± 0.2[b]	13.2 ± 2.7[b]	2.0 ± 0.5[b]	1.4 ± 0.2[b]
Controls (n = 5) Mean ± SD	341.7 ± 125.4	14.9 ± 0.2	2.3 ± 0.2	3.7 ± 0.9	18.9 ± 1.9	2.1 ± 0.2	6.1 ± 1.4	35.7 ± 1.9	0.6 ± 0.2	4.7 ± 1.4	31.0 ± 1.7	0.6 ± 0.2	2.9 ± 0.7

Results are expressed as means ± SD.
CRD versus controls: [a]$P < 0.05$; [b]$P < 0.001$.

LPL activity

To assess the role of LPL activity in the abnormal TG observed in plasma and lipoprotein fractions, lipolytic activity was measured in the plasma of CRD patients. The hypertriglyceridemia, described above, was associated with a 65.8% decrease of total postheparin lipolytic activity **(Table 7)**. Lipoprotein lipase as well as hepatic lipase activities were affected. A decrease of 68.9% was noted for LPL and of 73.4% for HL.

Synthesis and release of labeled lipids in jejunal explants

To determine whether the defect might be related to defective synthesis or release of either lipid or protein components, we studied these processes in cultured jejunal explants. The biosynthesis and release of labeled lipid classes are shown in **Table 8**. The incorporation of [^{14}C]palmitic acid into TG, DG, and CE was only moderately decreased in CRD jejunal explants and contrasts with the markedly impaired secretion of these lipids in the culture medium when compared to controls. The discrepancy between synthesis of newly formed lipids and their secretion became particularly evident when expressed as a medium/tissue ratio. It should be noted that the ratios for glycerides and CE were much lower than for PL which appeared to be freely released from the explant into the culture medium. These results suggest that while the intestine in CRD cannot release TG-rich lipoprotein particles, it can secreted PL-rich, but neutral lipid-poor particles, similar in composition to nascent or small spherical HDL.

Incorporation of [^{14}C]mannose into newly synthesized proteins

The radioactivity incorporated into the protein fraction of chylomicrons isolated from CRD jejunal explants amounted to 70% of the leucine **(Table 9)**. However, upon submitting the chylomicron-like fraction to SDS-PAGE, radioactivity was found at the apoB-100 and B-48 positions **(Fig. 5)**. This finding constitutes evidence for the capacity of the intestine to synthesize apoB. Upon incubating explants with mannose, only 20% of incorporation observed in controls was found. The decrease in glycosylation was similarly evident ($P < 0.05$) when expressed as the ratio of leucine/mannose incorporation. These results imply that while lipid synthesis is normal in the intestine of CRD, chylomicron apoprotein synthesis is impaired in view of the altered glycosylation. This latter finding may contribute to the inability to release chylomicrons in this condition.

Identification of apoB-48 in jejunal explants

The chylomicron-like fraction that floats upon centrifugation of sonicated jejunal explants was isolated, delipidated, and submitted to SDS-gel electrophoresis as

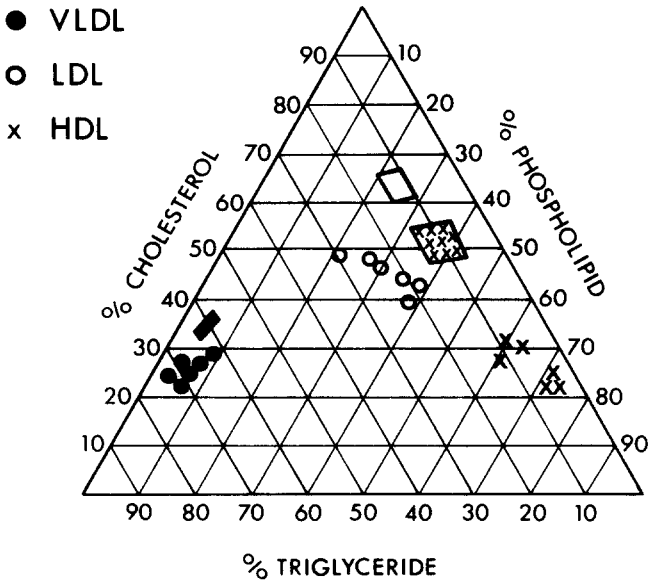

Fig. 2. Percentage composition of VLDL (●), LDL (○), and HDL (x) in CRD patients. The ± 2 SD ranges in control subjects are outlined in boxes.

described under Methods. The proteins thus separated were immunoblotted with a monoclonal antibody 2D8 which reacts with both B-100 and B-48 (22). In each of the explants from three different CRD patients, antibody 2D8 reacted with a protein band that comigrated with a standard of apoB-48 **(Fig. 6)** and which demonstrated the presence of apoB-48 in the intestinal cells of the patients. This is in contrast with the biopsies of two abetalipoproteinemia patients where no signal for 2D8 was noted and which served as negative controls. Of interest, in the jejunal explant from one of the patients, antibody 2D8 not only showed the presence of apoB-48 but also reacted with a protein comigrating with apoB-100.

DISCUSSION

A number of rare disorders of chylomicron synthesis have been described, including abetalipoproteinemia and the homozygous form of hypobetalipoproteinemia. The purpose of the present study was to characterize the distinguishing features of CRD (4), as well as to shed light on its pathogenesis. Our results demonstrate that patients with CRD, also referred to as Anderson's disease (24), have the apoprotein species essential for chylomicron synthesis, including apoA-I, apoA-IV (4), and apoB-48. This disorder causes extensive changes in plasma lipids and lipoproteins secondary to a defective release of chylomicrons which have undergone normal esterification by enterocytes. However, CRD leads to extensive changes in

TABLE 5. Chemical lipid composition and weight ratios in chylomicron retention disease (CRD)

	% Composition				Weight Ratios			Diameter Å
	TG	FC	CE	PL	TG/PR	CE/PR	$\frac{CE + TG}{PL + PR + FC}$	
VLDL								
CRD	69.25 ± 3.57[a]	3.75 ± 1.08[a]	5.85 ± 0.63[a]	21.12 ± 2.89[b]	8.79 ± 2.68[b]	0.74 ± 0.21[b]	0.28 ± 0.04[a]	412 ± 11[a]
C	62.34 ± 3.31	6.39 ± 1.10	12.92 ± 3.66	18.39 ± 0.93	5.83 ± 1.76	1.21 ± 0.45	0.70 ± 0.20	309 ± 59
LDL								
CRD	24.20 ± 4.32[a]	12.18 ± 5.05	17.94 ± 5.32[a]	45.38 ± 6.17[a]	0.66 ± 0.22[a]	0.58 ± 0.21[a]	0.34 ± 0.09[a]	207 ± 45[a]
C	10.7 ± 1.6	12.20 ± 2.75	44.32 ± 4.53	33.0 ± 3.14	0.29 ± 0.02	1.22 ± 0.22	1.36 ± 0.30	219 ± 14
HDL								
CRD	7.48 ± 3.29	9.05 ± 2.76[b]	10.58 ± 1.83[a]	72.90 ± 4.94[a]	0.10 ± 0.02	0.19 ± 0.09[a]	0.16 ± 0.04[a]	89 ± 17[a]
C	9.20 ± 3.57	5.81 ± 0.72	36.24 ± 3.70	45.79 ± 4.26	0.11 ± 0.04	0.45 ± 0.01	0.83 ± 0.11	106 ± 4

Data are mean ± SD of the six CRD patients and nine controls (C) who participated in the study. TG, triglyceride; CE, cholesteryl ester; FC, free cholesterol; PL, phospholipid; PR, protein. Differences between CRD-lipoproteins and normal lipoproteins are significant at levels of [a]$P < 0.001$ and [b]$P < 0.025$.

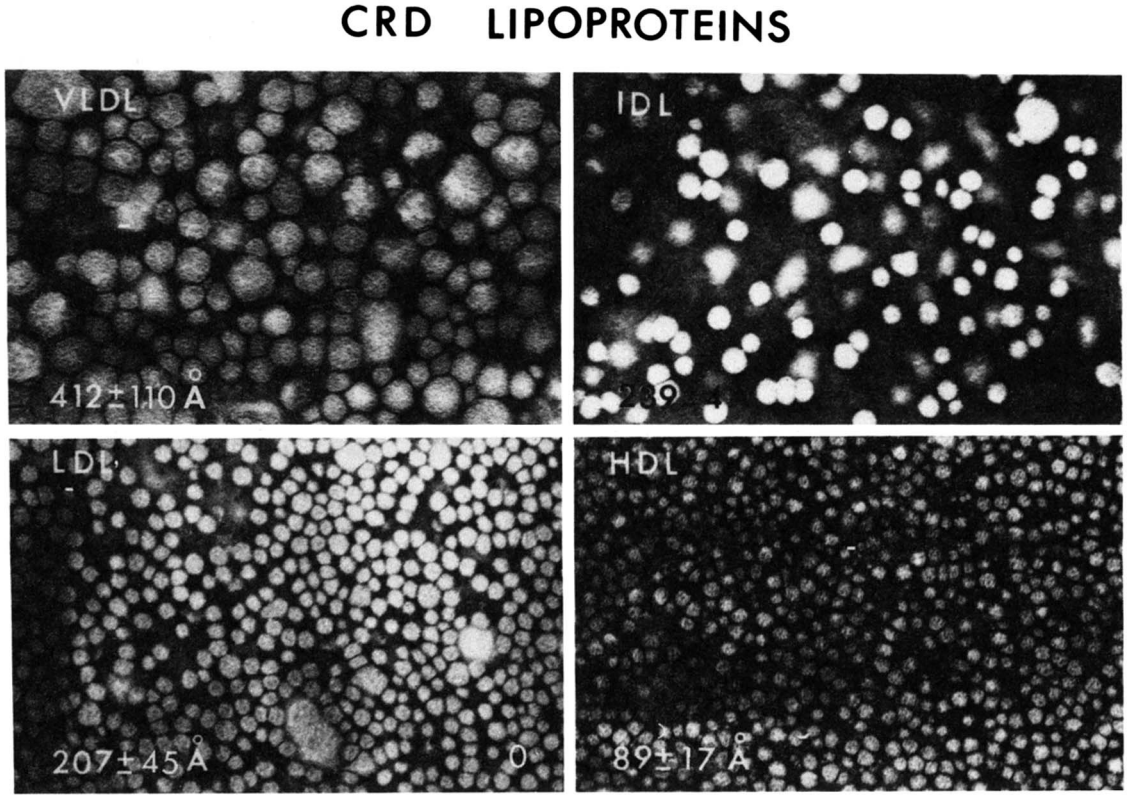

Fig. 3. Representative electron photomicrographs of negatively stained plasma lipoproteins in one CRD patient. The mean ± SD of particle diameters are those of the six CRD patients except for the intermediate density lipoprotein (IDL) fraction which was isolated from only one patient.

plasma lipids and lipoproteins secondary to the defective cellular release of lipids which undergo normal in vitro esterification by enterocytes.

Relationship between diet and triglyceride fatty acids

The mean triglyceride level found in this study was significantly higher than those observed in our initial report (4). It is possible that the difference may be the result of the high carbohydrate diet on which these children had been maintained for the past few years. Presumably, increased lipogenesis from carbohydrates could be responsible for the increased TG and saturated fatty acids (Tables 1, 3, 4). A compounding factor leading to impairment of triglyceride clearance may be attributed to a decreased postheparin lipolytic activity in CRD (Table 6). The present data support our earlier documentation about defective hydrolysis (4). At this time, we have no evidence for the cause of the decreased LPL activity. We can only suggest that it may be related to the nutritional status of the patients and in particular to their essential fatty acid deficiency (25).

Hypocholesterolemia in CRD

Severely depressed levels of plasma cholesterol are always present in abetalipoproteinemia (1) and in homozygous hypobetalipoproteinemia (26). Although present, hypocholesterolemia is less severe in CRD (4). The present data provides information about the underlying changes in plasma lipoproteins. The lipoprotein fractions LDL and HDL were dramatically affected, given that

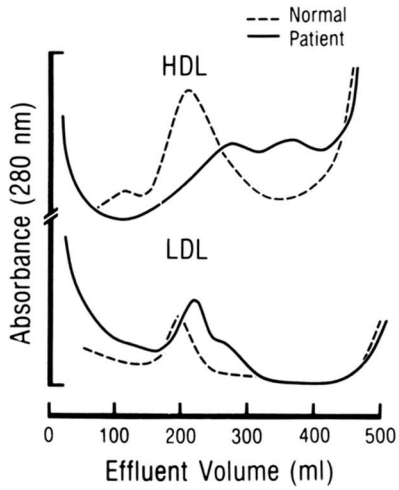

Fig. 4. Zonal elution profile of lipoproteins from a CRD patient and one normal subject.

TABLE 6. Plasma activity of lecithin:cholesterol acytransferase

Subject	LCAT	
	% · hr^{-1}	% of Control Value
CRD		
Case 1	4.48	70.9
Case 2	4.22	66.7
Case 3	3.82	60.3
Case 4	4.33	68.4
Case 5	3.75	59.3
Case 6	3.80	60.0
Mean ± SD	4.07 ± 0.31[a]	64.3 ± 5.0
Controls (n = 6)		
Mean ± SD	6.33 ± 1.68	100

The plasma LCAT activity was measured following the technique described by Stokke and Norum (19). The reaction was stopped with chloroform–methanol 2:1 and the extracted lipids were separated by TLC. The determination of the amount of radioactivity in each spot permitted the calculation of the fractional esterification rate (% · hr^{-1}) which expressed the percentage of labeled free cholesterol esterified in the plasma sample per hr.

[a] $P < 0.01$.

their content of cholesterol represented only 20–30% of normal values. The noticeable increase of VLDL-C is of significant interest and might be a reflection of increased delay of plasma-accumulated VLDL for the reduced lipolytic activity. The LPL enzyme is normally responsible for hydrolysis of TG-rich lipoproteins.

LCAT catalyzes the removal and transfer of the *sn*-2 acyl chain from phosphatidylcholine to cholesterol (27). This reaction, activated by apoA-I, occurs preferentially on the surface of the smaller, spherical HDL subclasses and nascent discoidal HDL (28, 29). In view of the marked decrease of apoA-I (4), HDL-C (Table 1), and PL (Table 2) in CRD patients, one might predict a diminished LCAT activity because of a low substrate and activator concentration. The patients' low esterification capacity could be explained in part by the relative decrease of HDL in which cholesterol esterification takes place, since the Stokke and Norum method (19) does not effectively measure absolute levels of LCAT activity independently of its major substrate. Furthermore, the main product of the LCAT reaction in plasma is cholesteryl linoleate; therefore, the relative deficiency of essential polyunsaturated fatty acids in our patients (Table 3) might constitute a limiting factor for LCAT activity.

Phospholipids in CRD

Even though CRD shares many clinical features with the two forms of abetalipoproteinemia, it is easily distinguishable in that acanthocytosis is rare and only minimally present. PL were reduced but they had a composition similar to that of controls and the ratio of phosphatidylcholine (PC) to sphingomyelin (SP) was normal. In view of the minimal stomatocytic changes in circulating erythrocytes (4), and their normal PC/SP ratio, it is evident that CRD is different from abetalipoproteinemia (25).

Lipoprotein composition

In the fasting state, the biochemical and EM studies directed to lipoproteins identified several abnormalities. VLDL and LDL were both TG-enriched. Despite that observation, only VLDL particles were larger by EM and by the various weight ratios of VLDL constituents. Conversely, the LDL particles were smaller by the same criteria. The decreased proportion of CE and higher PL were likely responsible for the change in size of LDL. A decrease in the size of HDL was also observed and the EM measurements were confirmed by changes in the relative proportions of HDL lipid components. Clearly they contained more PL and less CE than controls. These observations are likely due to the predominance of the smaller and denser HDL_3 subpopulation demonstrated by zonal ultracentrifugation. By contrast, abetalipoproteinemia patients have larger HDL particles secondary to the presence of a larger HDL_2 population (23).

It was not considered necessary to examine the composition of lipoproteins after the fat meal in view of the total lack of response as measured by triglyceride levels, lipoprotein patterns on agarose gel (Fig. 1), and by apoA-I concentrations (4).

Intestinal defect

In our previous study (4), we showed that following the administration of fat, there was no detectable TG increase in plasma, and biopsies exhibited small fat particles vesiculating the endoplasmic reticulum. These particles, morphologically similar to CM, were also noted to be enclosed in Golgi vesicles but were absent from the intracellular spaces and the lacteals. In the present study, the decreased ability of jejunal explants from CRD patients

TABLE 7 Postheparin lipolytic activity

Subject	Total PHLA	LPL	HL
	μmol FFA × ml^{-1} × hr^{-1}		
CRD			
Case 1	4.02	1.45	2.57
Case 2	4.04	0.92	3.12
Case 3	5.04	2.10	2.94
Case 4	4.25	1.75	2.50
Case 5	5.23	1.33	3.90
Case 6	3.60	1.19	2.41
Mean ± SD	4.36 ± 0.64[a]	1.46 ± 0.42[a]	2.19 ± 0.56[a]
Controls (n = 9)			
Mean ± SD	12.76 ± 0.43	4.70 ± 1.20	8.25 ± 1.16

PHLA, postheparin lipolytic activity; LPL, extrahepatic lipoprotein lipase; HL, hepatic lipase.

[a] $P < 0.01$.

TABLE 8. Incorporation of [^{14}C]palmitic acid into the TG, PL, DG, and CE of explants and their release in the culture medium

Lipids	Tissue				Medium				Medium/Tissue			
	Case 2	Case 4	Control 1	Control 2	Case 2	Case 4	Control 1	Control 2	Case 2	Case 4	Control 1	Control 2
	cpm/mg of protein of the explants											
TG	22,306	27,238	29,835	38,482	393	1,516	110,733	147,708	0.02	0.05	3.71	3.83
DG[a]	2,238	1,812	3,733	2,956	95	325	9,770	8,950	0.04	0.18	2.62	3.02
PL[b]	23,788	34,188	61,161	93,212	19,233	25,617	40,193	59,892	0.8	0.75	0.66	0.64
CE	1,924	2,113	2,124	3,992	454	512	13,793	18,821	0.23	0.24	6.49	4.71

Jejunal biopsies were cultured for 8 h in a medium containing 10 μCi of [^{3}H]palmitic acid. The lipid fractions were extracted as described in Methods. TG, triglyceride; DG, diglyceride; FA, fatty acid; PL, phospholipid; CE, cholesteryl ester.

[a]Results are mean of two CRD subjects and one control.

[b]The PL fraction could also include trace amounts of MG in view of the close juxtaposition between these two lipid classes.

to secrete TG in the medium was investigated with the technique of intestinal organ culture, as described recently by Rachmilewitz, Sharon, and Eisenberg (30). This model provided us with a tool to demonstrate the defective delivery of CM observed following an oral fat load. Although the sum of radioactivity incorporated into the explants and secreted into the medium was about 4 times less than in controls, this difference can be accounted for almost entirely by the lack of secretion into the medium. It is our contention that the reason why enterocytes from CRD patients could not incorporate as much as in controls is due to the large amount of fat present in the fasting state (4). Therefore, it is suggested that the impairment of secretion is the primary phenomenon. Although the esterification step was demonstrated to be an active cellular process in the intestinal mucosa of CRD patients, especially noticeable was the presence of PL in the incubation media, which represented the bulk of the synthetic and secreted product. Given the strong evidence that the small intestine serves as an important source of de novo nascent HDL (31), and our recent demonstration that in CRD there is an abundance of intestinal apoA-I (4), the present observations showing that PL are normally released suggest that the synthesis of PL-rich but neutral lipid-poor particles is not impaired. These particles might be similar in composition to nascent or small spherical HDL. Furthermore, it is of interest to note that CRD patients have a plasma HDL fraction of small spherical particles eluted in the HDL_4 range previously described by Deckelbaum et al. (23). In their study of abetalipoproteinemia, the presence of HDL_4 of intestinal origin was suggested (23, 31, 32). Although a preliminary observation suggests that HDL_4 in CRD share with abetalipoproteinemia patients a high % protein composition, we do not have strong evidence that HDL_4 originates from the intestine.

Protein synthesis is believed to be crucial in lipoprotein formation and release. Glickman, Perrotto, and Kirsch (33) demonstrated an impairment of lipid transport with numerous CM-sized particles in the Golgi apparatus of the enterocytes following colchicine administration. Rachmilewitz et al. (30) also found inhibition of lipoprotein secretion by human jejunal explants following the addition of colchicine. Our experiments on protein biosynthesis, measured by [^{3}H]leucine incorporation in the protein fraction of chylomicrons isolated from cultured jejunal explants of CRD patients, gave results approximating those obtained in controls, thereby showing that protein synthesis is not defective. However, the finding of a glycosylation defect may be pertinent to the hitherto unidentified basic defect associated with this disease. It is of interest to note that Hoffman, Child, and Kuksis (34) found a blockage of lipoprotein transport by isolated rat jejunal enterocytes following the inhibition of glycosylation.

Patients identified as suffering from Anderson's disease or CRD have been recently studied by others (35). These patients were also found to have hypobetalipoproteinemia with accumulation of apoprotein B-like protein in intestinal cells identified as apoB-48 (35). The epitope for antibody 2D8 which used to identify apoB-48 has been mapped to the thrombolytic fragment T3 of apoB-100 and is localized within 64 Kda of the N-terminal end of T3 (36). The epitope for 2DB is also expressed on apoB-48 (18) and on a newly discovered mutant form of apoB, called apoB-37, which represents in all probability the N-terminal portion of B-100 and B-48 (37). Therefore, the expression of the well-characterized epitope for 2D8 on a protein comigrating with apoB-48 in the intestinal explants of each CRD patient that was tested, demonstrates

TABLE 9. Synthesis of protein and glycosylation in jejunal explants

	[^{3}H]Leucine	[^{14}C]Mannose	[^{3}H]Leucine / [^{14}C]Mannose
	cpm/h · mg of protein		
Controls (4)	51,410 ± 5,967	21,479 ± 6,819	3.02 ± 0.74
CRD (6)	35,638 ± 6,230	4,343 ± 514[a]	8.40 ± 1.23[a]

Jejunal biopsies were cultured in the presence of [^{3}H]leucine and of [^{14}C]mannose. After 18 hr of incubation, the explants were gently homogenized and chylomicrons were separated by ultracentrifugation (25,000 rpm, 30 min). Protein synthesis and glycosylation were assessed after trichloroacetic acid treatment.

[a]$P < 0.05$.

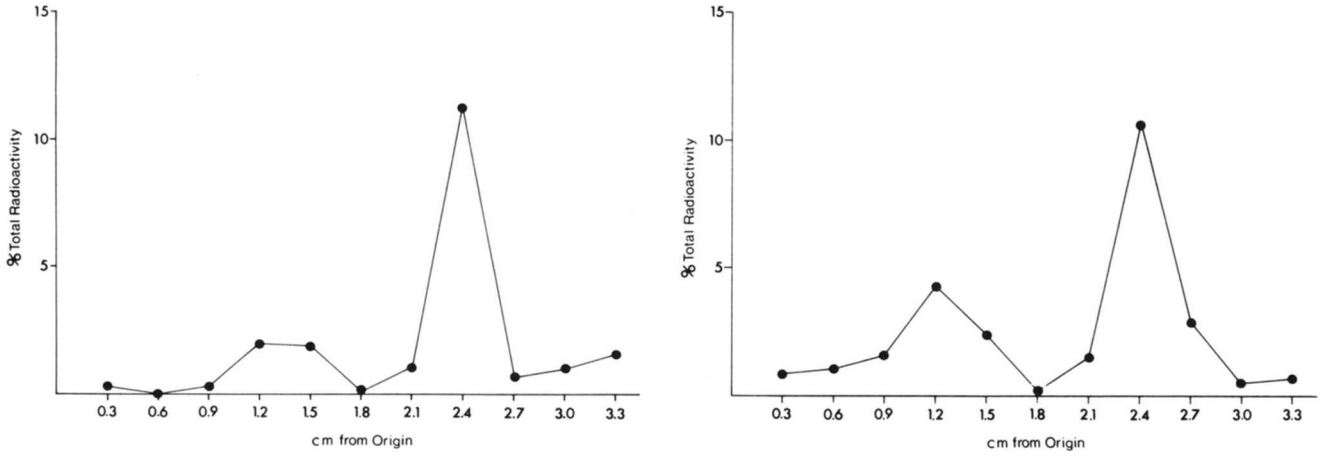

Fig. 5. Radioactive incorporation of [^{3}H]leucine into chylomicron-like fractions. Cultured intestinal explants of two CRD patients were incubated with [^{3}H]leucine. Samples from floated tissue lipoprotein fractions (25,000 rpm, 30') were mixed with small amounts of B-100 and B-48 apoprotein standards before being subjected to 3–15% SDS-PAGE. The radioactivity in each 3-mm slice was determined. The points on the curves represent the percent of the applied total protein radioactivity recovered in the corresponding region of the gel.

unambiguously that apoB-48 is present in the intestine of these patients. Furthermore, we now have preliminary evidence in two patients that de novo synthesis of apoB-48 is intact as measured by the incorporation of tritiated leucine into this apoprotein, whereas abetalipoproteinemia patients were unable to demonstrate the same process (Levy, E., et al., unpublished results). The identification of apoB-100 in the intestinal biopsies of some of the patients is intriguing and requires further study. Indeed, others have recently shown that fetal intestine but not adult intestine could synthesize apoB-100 while both could synthesize apoB-48 (38). In both cases, only one large mRNA was detected when a probe from the 3' end of the B-100 gene was used. In addition to this large message, a smaller message was detected with probes from the 5' end of the apoB gene in adult intestinal cells which was not detected in adult liver cells (39). In spite of the production of B-100 mRNA in adult intestinal cells, the synthesis of apoB species other than B-48 remains to be definitively demonstrated. In conclusion, the pathogenesis of CRD is not related to the absence of apoB-48 or to its defective synthesis which we recently demonstrated in abetalipoproteinemia (Levy, E., et al., unpublished results). Thus it is apparent that the CM secretion is impaired, suggesting that the defect probably involves the final assembly of chylomicrons or their delivery. Our data from cultured jejunal explants showing normal esterification and protein synthesis but impaired glycosylation and minimal release of glycerides are consistent with this hypothesis.

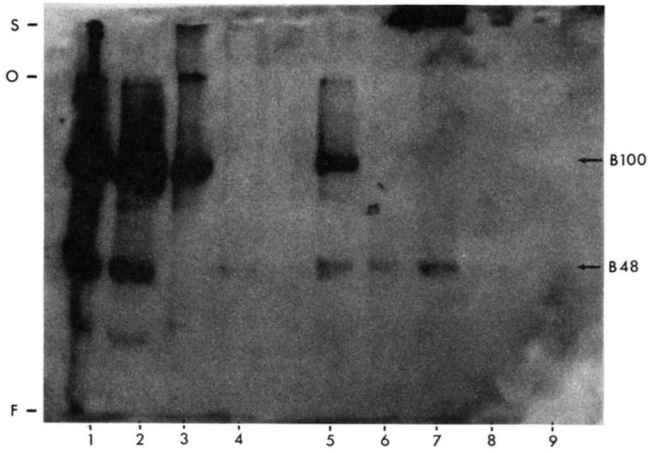

Fig. 6. Immunoblot with antibody 2D8 of the proteins from chylomicron-like fractions isolated from jejunal explants and separated by SDS-gel electrophoresis. On the left side, S – indicates the stacking gel, 0 – , the origin of the running gel and F – , the front of migration. On the right side, the positions of apoB-100 and apoB-48 are indicated. The following fractions were applied to the different lanes: 1 and 2, VLDL and LDL from type III hyperlipoproteinemia (as respectively positive controls) containing B-100 and B-48; 3, apoB-100 standard; 4, apoB-48 standard; 5, 6, and 7, biopsies from three different CRD patients; 8 and 9, biopsies from two abetalipoproteinemia patients (as negative controls).

We thank Dr. Vijay L. Grey for her advice in intestinal explant culture and we appreciate Sylvie Tassé's assistance in manuscript preparation. This project was supported by grants from the Medical Research Council of Canada. E. L. and R. M. are the recipients of a postdoctoral fellowship and of a scholarship, respectively, from le Fonds de la Recherche en Santé du Québec.

Manuscript received 3 February 1987 and in revised form 30 April 1987.

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