LIPOPROTEINS IN PRIMARY BILIARY CIRRHOSIS [1]

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Evidence gradually accumulated by moving boundary electrophoresis (1–4), chemical fractionation, (5–7) using method of Cohn and his associates (8), zone electrophoresis in a starch-supporting medium (9), and the ultracentrifuge (10) has indicated that plasma lipids in the acute stage of infectious hepatitis, in biliary cirrhosis, and in some cases of biliary obstruction are atypically combined with peptides in the form of abnormal lipoproteins.

This is a report of an investigation to explore further by chemical, physical-chemical, and immunochemical means the composition and distribution of abnormal lipoproteins in a case of primary biliary cirrhosis and to compare their properties with lipoproteins from plasma of a normal woman of like age.

METHOD

Plasma was first fractionated according to method number 10 of Cohn and his associates (8) with minor modifications adapted by this laboratory (11). Three lipoprotein-bearing fractions, Fractions IV + V, VI, and I + III were obtained from biliary cirrhotic plasma. No lipoproteins are recovered in Fraction VI of normal plasma (8). Alpha and beta lipoproteins designated also respectively as high and low density lipoproteins were separated from all fractions and unfractionated plasma by ultracentrifugation in a Spinco Model L preparative ultracentrifuge after their solution densities had been adjusted to 1.063 − 1.068 with sodium chloride. The ultracentrifugally separated low density ($p < 1.063$) lipoproteins were further separated in a density gradient tube as recently described by Oncley and Mannick (12) and chemically characterized. Immunochemical examinations were made of Fraction IV + V, VI, and I + III, and ultracentrifugal analyses for S_f components were carried out on both unfractionated plasma and the fractions.

Preparation of unfractionated plasma. Five ml. of biliary cirrhotic ACD [2] plasma and 9 ml. of normal ACD[2] plasma were dialyzed against several changes of citrate-sodium chloride reagent [3] for 48 hours at 0° C. Densities of the dialyzed solutions were adjusted to approximately 1.065 with 4.0 M NaCl and distilled water until a final volume of 11.0 ml. was obtained for the biliary cirrhotic and 13.0 ml. for the normal plasma. Nine-ml. aliquots of each were placed in 13 × 85 mm. plastic centrifuge tubes and ultracentrifuged at 30,000 rpm (80,000 G) for 18 to 20 hours at 5° C. The top 3.0 cm. of solution were separated with a tube-slicing device. From the upper and lower cuts containing low and high density lipoproteins, respectively, 100 λ samples were removed for determination of solution density. The upper cut of the biliary cirrhotic plasma was diluted to 6.0 ml. with 2.3 M NaCl and from the normal plasma with 2.0 M NaCl. An aliquot of 4.5 ml. was removed from each into 13 × 85 mm. plastic tubes for density gradient studies (described presently); the remainder was dialyzed together with both lower cuts (high density lipoproteins) against 0.15 M NaCl for 24 hours at 0° C. The dialyzed solutions were then analyzed for lipids, nitrogen, and dry weight.

A linear density gradient as described by Linderstrøm-Lang and Lanz (13, 14) and Anfinsen (15) was made of the diluted upper cuts as follows: 4.5 ml. 0.1 M NaCl were layered over the 4.5-ml. aliquot of low density lipoproteins that had been previously placed in a 13 × 85 mm. plastic tube. A simply-constructed wire stirrer with a spiral button tip was gently inserted to the interface of the two solutions, from which point gentle vertical strokes of increasing 0.75 cm. distances were made, keeping the plane of the spiral tip as level as possible. The manipulation usually required four strokes each down and up from the interface. The tubes were then ultracentrifuged at 30,000 rpm for 18 to 20 hours at 5° C. Lipoproteins concentrate in such gradients at layers where the lipoprotein density is equal to the density of the sodium chloride solution. The resulting gradients were partitioned into 8 or 9 cuts, 0.75 cm. each, either by slicing with a tube-slicing device or by aspirating with a fine-tipped pipette. From the volume of each cut (approximately 1.0 ml.) a 100 λ sample was removed for determination of solution density. To the remainder of each cut was added an equal volume of 0.15 M NaCl; each

[2] ACD (acid citrate-dextrose) is the special anticoagulant solution used when fractionating plasma proteins by the Cohn method (8).

[3] Citrate-sodium chloride reagent consisted of 0.02 M trisodium citrate and 0.07 M sodium chloride.

dilution was placed in Visking cellophane tubing (1 cm. in diameter) and dialyzed against 0.15 M NaCl for 24 hours at 5° C. The contents of each bag were removed as quantitatively as possible, the volumes measured, and the solutions analyzed for nitrogen, lipids, and dry weight.

Preparation of plasma fractions. In each of six tubes, 5-ml. samples of plasma (6 × 5 ml.) were fractionated simultaneously. Fractions from 1 × 5 ml. plasma were used solely for protein and lipid analyses as described in the quantitative method of Cohn and his associates (8).

Fraction IV + V obtained as a zinc proteinate paste from Fraction IV + V + VI of 3 × 5 ml. of biliary cirrhotic plasma or 5 × 5 ml. normal plasma were dissolved in 13.5 ml. of citrate-sodium chloride reagent and for complete removal of zinc ions dialyzed 24 hours at 0° C. against the same reagent (11). The density of the entire dialyzed solution was adjusted to 1.064 with 3 to 4 M NaCl yielding a final volume of 20.5 ml. From this mixture two 9-ml. portions were removed for ultracentrifugal separation of low and high density lipoproteins as described for unfractionated plasma. Absence of low density lipoproteins in normal plasma was ascertained by lipid analyses of the upper cut. The pooled upper cuts from biliary cirrhotic plasma were diluted to a volume of 12.0 ml. with 2.0 M NaCl, 4.5 ml. of which were removed for density gradient studies as already described; the remainder was aliquoted for (a) examination of S_f classes of lipoproteins (approximately 10 mg.), (b) immunochemical reactions, and (c) analytic purposes. The lower cut pools and aliquot (c) of the upper cuts were then dialyzed and analyzed for chemical components as described for unfractionated plasma.

Fraction VI, obtained as a supernatant of the Fraction IV + V paste just described for biliary cirrhosis, was concentrated by precipitation of the proteins with $NH_4Cl - NH_4OH$ buffer to a pH of 7.6 (16). The resulting paste was dissolved in 7.5-ml. acetate buffer $\Gamma/2 = 0.5$, pH 4.5 followed by dialysis against the same buffer for 24 hours at 0° C. to remove zinc ions. The density of the entire solution was adjusted to 1.066 by addition of 4.0 M NaCl and distilled water so as to obtain a final volume of 20.0 ml. From this solution two 9-ml. portions were removed for analysis as described in Fraction IV + V.

Fraction I + III, obtained as a paste from the Cohn fractionation of 3 × 5 ml. biliary cirrhotic plasma or 5 × 5 ml. normal plasma, was dissolved with 2.0 M NaCl and the density adjusted to 1.064 with distilled water and 2.0 M NaCl until a final volume of 20.0 ml. was obtained. From this solution two 9-ml. aliquots were removed for analyses as described for Fractions IV + V and VI.

ANALYTICAL PROCEDURES

Unfractionated plasma and all Cohn fractions were analyzed for concentrations of cholesterol, phospholipids, and protein and for distribution of electrophoretic components (moving boundary and filter paper methods) as previously described (8, 11, 17).

In addition, the following lipid procedures were employed. Micro or semi-micro adaptations were applied to the *free and esterified cholesterol* method of Sobel and Mayer (18) and *phospholipid* method of Stewart and Hendry (19). *Acetal phospholipids* by the Korey-Wittenberg procedure (20). *Total fatty acids* by variation of a standard gravimetric method as follows: 3:1 alcohol-ether extracts of plasma and fractions were evaporated to dryness in a 125-ml. soil digestion flask and saponified by refluxing gently with 10 per cent KOH in 95 per cent alcohol for one hour. The contents were cooled, diluted with water to bring the alcohol concentration to 40 per cent, transferred to a separatory funnel, and extracted three times with petroleum ether to remove the unsaponified material. The remaining soaps were acidified with concentrated HCl to liberate fatty acids which were then extracted three times with petroleum ether. The extracts were centrifuged to remove any traces of insoluble matter, quantitatively transferred to a tared flask, evaporated to dryness and desiccated to constant weight.

Fatty acids as neutral fat (triglycerides + free fatty acids) were estimated by subtracting the fatty acids of the predetermined ester cholesterol and phospholipids from the gravimetric value for total fatty acids according to the formula

$$NFFA = TFA - \{ester\ cholesterol \times 0.72 + Lipid\ P\ [0.8 \times 17.9 + 0.2 \times 8.95]\}$$

Neutral fat was calculated by multiplying the NFFA value by 1.05. In these calculations it is assumed that 80 per cent of the phospholipids exist as lecithin and cephalin and 20 per cent as sphingomyelin, that the average molecular weight of a fatty acid chain in ester cholesterol, phospholipid, and neutral fat is 277, and that the neutral fat contains three fatty acid chains.

Nitrogen analyses were made by a modification of the Conway (21) diffusion system following digestion with 0.5-ml. acid digestion mixture [4] in special micro Kjeldahl flasks.[5] The digest was diluted to 10 ml. with water and 0.5-ml. aliquots were removed for ammonia determination in 45 mm. Conway dishes. One-ml. saturated potassium metaborate was used to liberate the ammonia into 0.2 ml. 2 per cent boric acid which in turn was titrated with 0.005 N HCl. Concentrations of lipoprotein in solution were estimated from *dry weight measurements* according to Armstrong, Budka, Morrison, and Hasson (22) using the following variation. Protein solutions were dialyzed 48 hours at 0° C. against several changes of 0.15 M NaCl. Micro aliquots of the dialyzed material and final dialysate were dried to constant weight *in vacuo* at 38° C. for one week. The weight of protein was obtained from the difference in weight between the protein solution and that of the dialysate.

Densities were determined by weighing protein solu-

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[4] Acid digestion mixture consisted of a 1:1 mixture of concentrated H_2SO_4 and saturated $KHSO_4$ containing 0.2 per cent $CuSO_4$.

[5] Micro Kjeldahl flasks, graduated to 5.0 and 10.0 ml.

TABLE I

Lipid analysis of plasma and lipoprotein fractions in biliary cirrhosis compared to normal

	Concentration as mg./100 ml. plasma in										
	Unfractionated plasma			Fractions							
				IV+V+VI			IV+V	VI	I+III		
	11/29 B.C.	1/5 B.C.	Norm.	11/29 B.C.	1/5 B.C.	Norm.	1/5 B.C.	1/5 B.C.	11/29 B.C.	1/5 B.C.	Norm.
Cholesterol, Total (Bloor)	1,620	1,565	196	1,223	1,300	53.4	443	780	237	264	134
Phospholipids	3,480	3,496	226	2,786	2,896	96.8	1,206	1,580	413	456	112
C/Pl RATIO	0.47	0.45	0.87	0.45		0.55	0.37	0.49	0.58		1.19
Cholesterol, Free (S-S)	1,595	1,750	44	1,232	1,440	10.0			165	192	34
Cholesterol Ester (S-S)	286	235	215	94	0	57.0			136	176	177
F/TC RATIO	0.90	0.93	0.26	0.96	1.0	0.23			0.67	0.68	0.24
Fatty Acids, Total		2,920	330		2,095	143.0				755	212
Neutral Fat (FFA + Trigl.)		626	120		253	63.0				427	71
Total Lipids		6,107	605		4,589	227.0				1,245	394
Acetal Phospholipids								0			

tions and water of same temperature in 100 λ pycnometers[6] on a micro analytic balance. For approximation of density as in adjustment of solution densities prior to ultracentrifugal separation of low and high density lipoproteins, a specific gravity spindle[7] was employed.

Analyses of S_t classes of lipoproteins[8] were made of the low density lipoproteins which were floated from plasma and concentrated fractions after dialysis against a solvent density of 1.063. All samples were then analyzed according to the method of Gofman and his associates (23, 24), using a Spinco Model E analytic ultracentrifuge.

Immunochemical analyses[8] of the lipoprotein fractions were carried out by the precipitin reaction described by Kabat and Mayer (25) using an antiserum which reacts with all low density lipoproteins from normal plasma as described by Levine, Kauffman, and Brown (26).

RESULTS

Lipid and protein concentrations in plasma and. fractions

In Table I, concentrations of lipids in unfractionated plasma and Cohn fractions from primary biliary cirrhosis are compared with values of a normal woman. Immediately apparent is the

[6] 100 λ pycnometers obtained from Micro Specialties Co., Berkeley, California.

[7] Special micro spindles having a displacement of 5.0 ml. in a 10 ml. cylinder obtained from R. C. Ewald Co., Middle Village, L. I.

[8] We are indebted to Dr. Ray K. Brown, Division of Laboratories and Research, New York State Department of Health, Albany, N. Y., for these determinations.

unusual and characteristic distribution of the enormous quantities of the plasma lipids. Approximately 75 per cent of the total lipids and total fatty acids are recovered in Fraction IV + V + VI, where the cholesterol-phospholipid ratio is normal but the cholesterol is almost entirely unesterified. Inspection of the remaining plasma lipids recovered in Fraction I + III reveals a cholesterol-phospholipid ratio of 0.58 or about half of the ratio normally found for this fraction; the cholesterol is approximately 33 per cent esterified, and about two-thirds of the plasma neutral fat is recovered herein. Fraction VI contains approximately one-half of the plasma cholesterol and phospholipid, with only 91 mg. per cent protein as measured by the biuret method. Normally, only traces of lipids and approximately 50 mg. per cent protein are found in Fraction VI. No acetal phospholipid is detected in Fraction VI. The wash from Fraction I + III of any occluded Fraction IV + V + VI is normally devoid of lipids but in the biliary cirrhotic plasma 117 mg. per cent phospholipids and 145 mg. per cent total fatty acids were recovered.

Electrophoretic studies

Moving boundary electrophoretic patterns of unfractionated plasma and Cohn fractions in biliary cirrhosis are compared to a normal in Fig-

ure 1. In biliary cirrhosis we find, as others have, a marked decrease in plasma albumin and an increase of beta-1, beta-2, and gamma globulin peaks. By fractionation we are able to demonstrate unusual appearance of a beta-2 peak in Fraction IV + V; and a moderate increase in beta-1 and beta-2 peaks in Fraction I + III and in gamma globulin in Fraction II.

Filter paper electrophoretic analyses of the plasma and Cohn fractions in biliary cirrhosis indicate that lipids of Fraction IV + V and I + III migrate with both beta-1 and beta-2 globu-

lins, while in Fraction VI a general dragging of lipids is apparent from the origin through the beta-2 globulin area.

Density gradient analyses

In Figure 2, results obtained from analysis of each cut in the density gradient of low density (p < 1.063) lipoproteins floated from unfractionated plasma of a normal and a biliary cirrhotic are plotted against respective solution densities. In this and in Figures 3 and 4, weight ratios are given for cholesterol to phospholipids (C/PL)
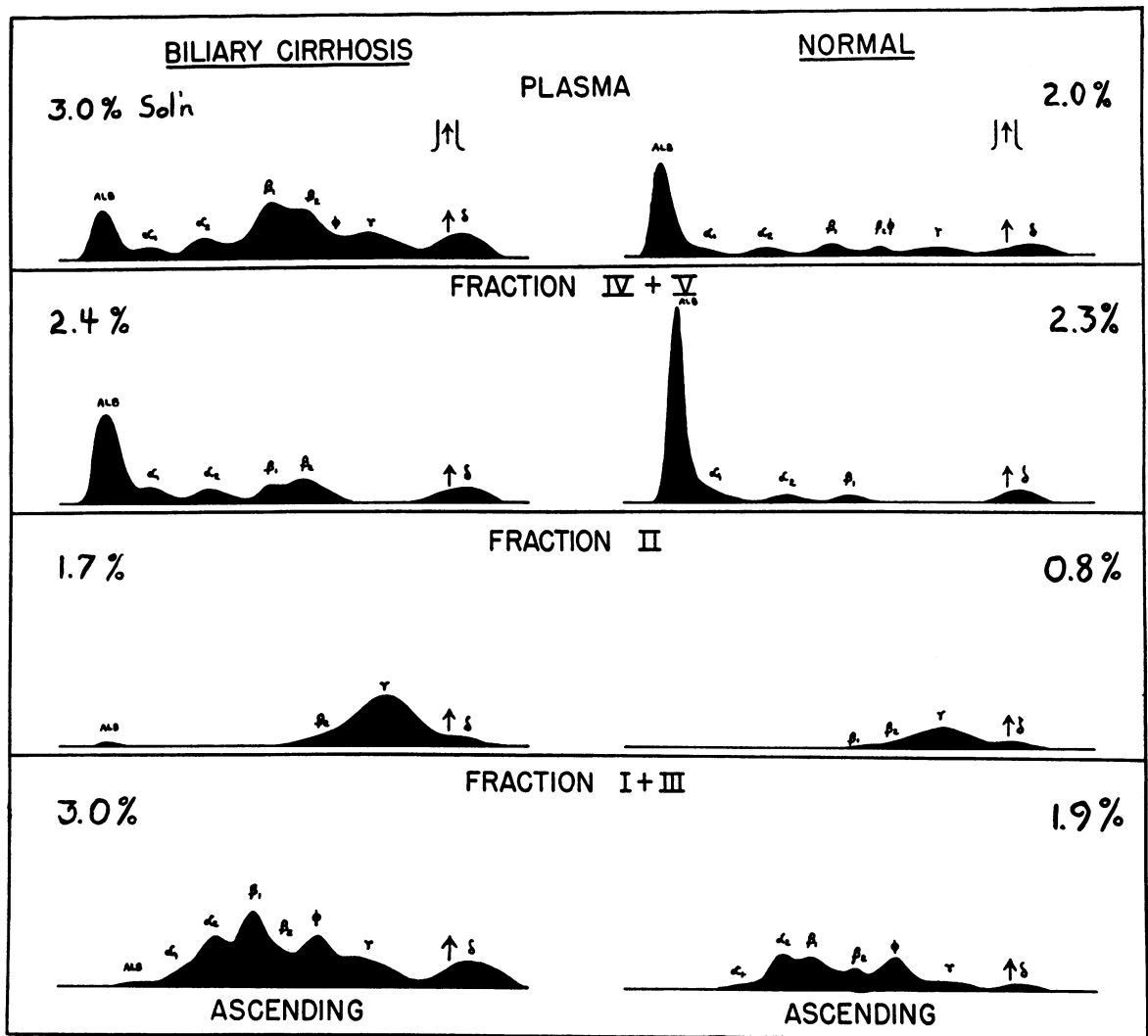

FIG. 1. ELECTROPHORETIC COMPONENTS IN UNFRACTIONATED PLASMA AND COHN FRACTIONS IN PRIMARY BILIARY CIRRHOSIS COMPARED TO NORMAL

Analyses were made of protein solutions differing in concentration as indicated to the sides of each pattern. Veronal-citrate buffer pH = 8.6, $\Gamma/2 = 0.1$, T = 2° C.

UNFRACTIONATED PLASMA

NORMAL

ρ GM/ML	MG/ML	DRY WT	C/PL	C/N (Molar)	F/TC
1.027		2.90	0.47	0.17	0.40
1.028		1.22	1.02	0.21	—
1.029		1.62	1.05	0.22	—
1.030		2.26	1.04	0.19	0.32
1.034		2.94	1.22	0.29	0.24
1.038		3.64	1.23	0.29	0.26
1.040		2.88	1.28	0.26	0.31
1.042		2.27	1.14	0.19	0.27
1.043		2.84	1.06	—	—

ρ	(MG/4.5 ML)			
	CHOL	PL	N	DRY WT
(β-Lipoproteins) U.C. < 1.063	4.6	4.1	—	21.1
(α-Lipoproteins) L.C. > 1.063	2.4	5.1	—	—

PRIMARY BILIARY CIRRHOSIS

ρ GM/ML	MG/ML	DRY WT	C/PL	C/N (Molar)	F/TC
1.030		—	0.50	—	—
1.031		2.2	0.48	0.33	—
1.033		3.8	0.43	0.86	1.00
1.035		5.8	0.47	1.01	1.00
1.041		9.8	0.45	1.13	0.94
1.042		14.0	0.44	0.78	0.97
1.044		6.2	0.46	0.68	0.99
1.047		3.8	0.56	0.17	1.00
1.050		6.8	0.65	0.06	—

ρ	(MG/4.5 ML)			
	CHOL	PL	N	DRY WT
(β-Lipoproteins) U.C. < 1.063	10.3	22.1	43	51.0
(α-Lipoproteins) L.C. > 1.063	0.5	1.5	6.33	88.5

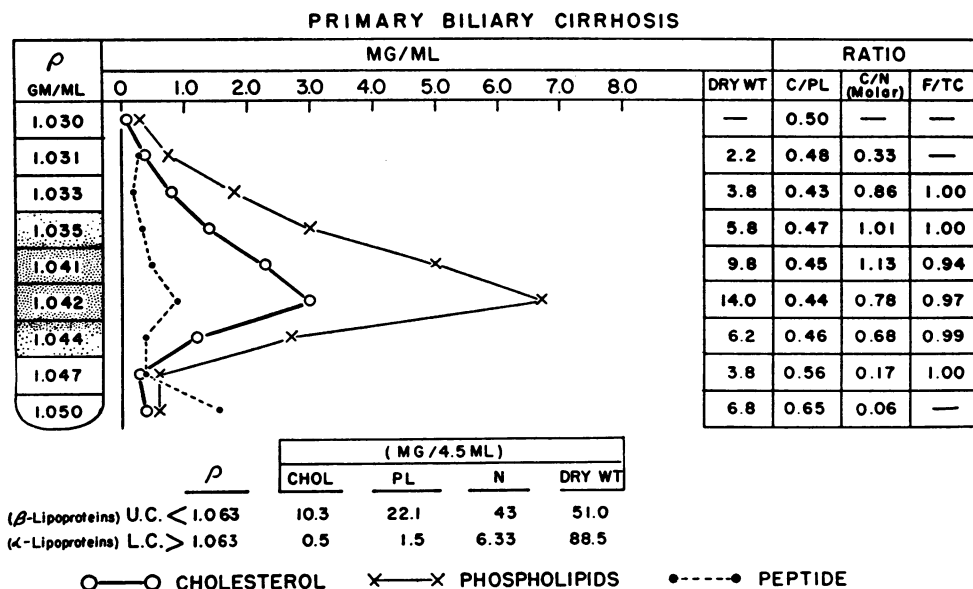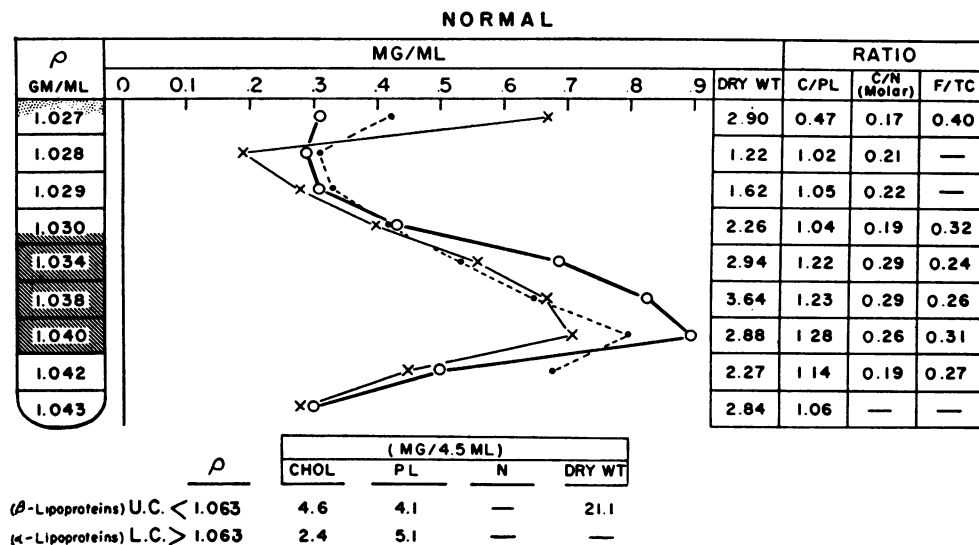O——O CHOLESTEROL   X——X PHOSPHOLIPIDS   •-----• PEPTIDE

FIG. 2. ANALYSES AND PATTERNS OBTAINED FROM DENSITY GRADIENTS OF LOW DENSITY LIPOPROTEINS IN UNFRACTIONATED PLASMA OF A NORMAL AND A PRIMARY BILIARY CIRRHOTIC

ρ represents solution densities.

and free to total cholesterol (F/TC) and molar ratios for cholesterol to nitrogen (C/N). Also shown are analyses of (a) upper cuts designated as (U.C.) containing all low density (p < 1.063) or beta lipoproteins from which density gradients were made; and (b) lower cuts (L.C.) containing all high density (p > 1.063) or alpha lipoproteins if any were present.

It should be borne in mind that the first cut could contain unbound lipid such as triglycerides and that in the last cut there could exist lipid free proteins or possibly lipoproteins of which the fatty acids may have undergone oxidation, thus giving rise to heavier components. Such factors could influence observed densities and dry weight values presented as an estimation of the
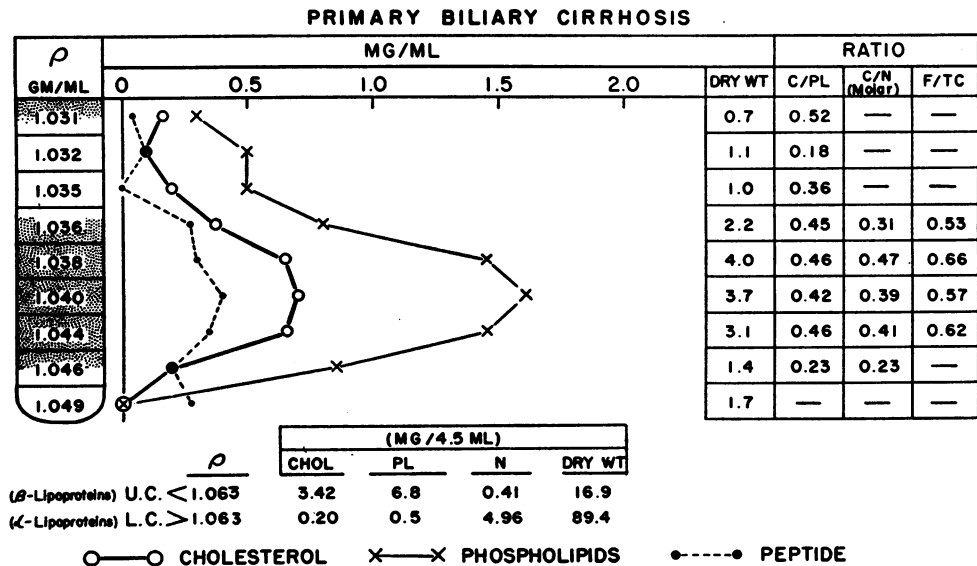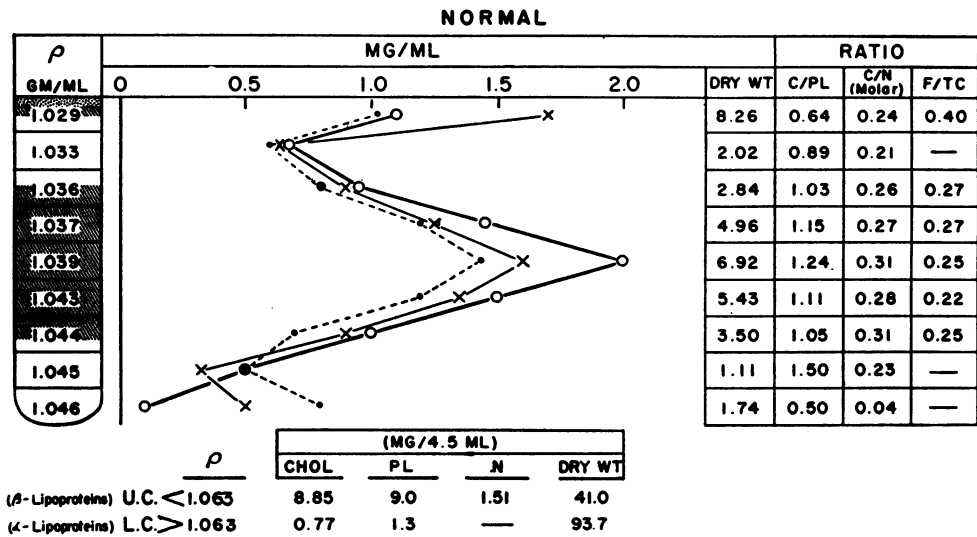
concentration of lipoproteins per cut. Nitrogen figures are corrected for phospholipid nitrogen, assuming a 1 : 1 ratio of nitrogen to phosphorus [9] Peptide is calculated from the corrected N × 6.25. Turbidity is depicted by dotted and yellow pig-

[9] Studies by Petersen (27) have shown that the enormous increases in plasma phospholipids in biliary cirrhosis are due entirely to accumulation of lecithin and cephalin.

mentation by shaded areas on the diagrams of the tubes.

In the normal unfractionated plasma approximately one-half of the total lipids and peptide are distributed between the densities 1.034 − 1.041 where ratios of the various constituents are essentially homogeneous throughout: average values for weight ratios of C/PL being 1.25, of F/TC 0.27 and for molar ratios of C/N being 0.26.

FRACTION I + III

NORMAL

ρ GM/ML	MG/ML					DRY WT	C/PL	C/N (Molar)	F/TC
1.029						8.26	0.64	0.24	0.40
1.033						2.02	0.89	0.21	—
1.036						2.84	1.03	0.26	0.27
1.037						4.96	1.15	0.27	0.27
1.039						6.92	1.24.	0.31	0.25
1.043						5.43	1.11	0.28	0.22
1.044						3.50	1.05	0.31	0.25
1.045						1.11	1.50	0.23	—
1.046						1.74	0.50	0.04	—

ρ	(MG/4.5 ML)			
	CHOL	PL	N	DRY WT
(β-Lipoproteins) U.C. <1.063	8.85	9.0	1.51	41.0
(κ-Lipoproteins) L.C. >1.063	0.77	1.3	—	93.7

PRIMARY BILIARY CIRRHOSIS

ρ GM/ML	MG/ML					DRY WT	C/PL	C/N (Molar)	F/TC
1.031						0.7	0.52	—	—
1.032						1.1	0.18	—	—
1.035						1.0	0.36	—	—
1.036						2.2	0.45	0.31	0.53
1.038						4.0	0.46	0.47	0.66
1.040						3.7	0.42	0.39	0.57
1.044						3.1	0.46	0.41	0.62
1.046						1.4	0.23	0.23	—
1.049						1.7	—	—	—

ρ	(MG/4.5 ML)			
	CHOL	PL	N	DRY WT
(β-Lipoproteins) U.C. <1.063	3.42	6.8	0.41	16.9
(κ-Lipoproteins) L.C. >1.063	0.20	0.5	4.96	89.4

O——O CHOLESTEROL   X——X PHOSPHOLIPIDS   •----• PEPTIDE

FIG. 3. ANALYSES AND PATTERNS OBTAINED FROM DENSITY GRADIENTS OF LOW DENSITY LIPOPROTEINS IN FRACTION I + III OF A NORMAL AND A PRIMARY BILIARY CIRRHOTIC

ρ represents solution densities.
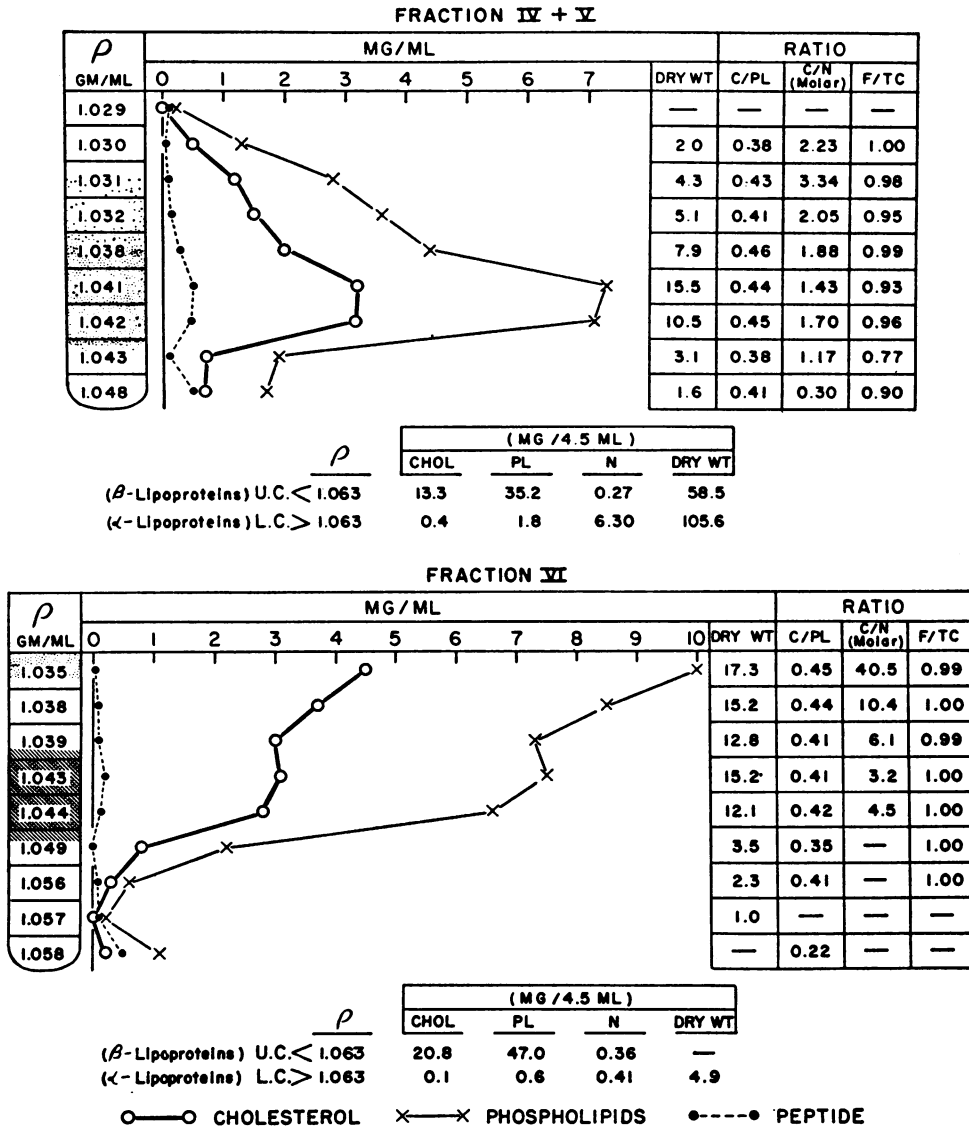
PRIMARY BILIARY CIRRHOSIS

FRACTION IV + V

ρ GM/ML	MG/ML	DRY WT	RATIO C/PL	RATIO C/N (Molar)	RATIO F/TC
1.029		—	—	—	—
1.030		2 0	0.38	2.23	1.00
1.031		4.3	0.43	3.34	0.98
1.032		5.1	0.41	2.05	0.95
1.038		7.9	0.46	1.88	0.99
1.041		15.5	0.44	1.43	0.93
1.042		10.5	0.45	1.70	0.96
1.043		3.1	0.38	1.17	0.77
1.048		1.6	0.41	0.30	0.90

ρ	CHOL (MG /4.5 ML)	PL	N	DRY WT
(β-Lipoproteins) U.C.< 1.063	13.3	35.2	0.27	58.5
(α-Lipoproteins) L.C.> 1.063	0.4	1.8	6.30	105.6

FRACTION VI

ρ GM/ML	MG/ML	DRY WT	RATIO C/PL	RATIO C/N (Molar)	RATIO F/TC
1.035		17.3	0.45	40.5	0.99
1.038		15.2	0.44	10.4	1.00
1.039		12.8	0.41	6.1	0.99
1.043		15.2	0.41	3.2	1.00
1.044		12.1	0.42	4.5	1.00
1.049		3.5	0.35	—	1.00
1.056		2.3	0.41	—	1.00
1.057		1.0	—	—	—
1.058		—	0.22	—	—

ρ	CHOL (MG /4.5 ML)	PL	N	DRY WT
(β-Lipoproteins) U.C.< 1.063	20.8	47.0	0.36	—
(α-Lipoproteins) L.C.> 1.063	0.1	0.6	0.41	4.9

O———O CHOLESTEROL   X———X PHOSPHOLIPIDS   ●----● PEPTIDE

FIG. 4. ANALYSES AND PATTERNS OBTAINED FROM DENSITY GRADIENTS OF LOW DENSITY LIPO-
PROTEINS IN FRACTIONS IV + V AND VI IN A PRIMARY BILIARY CIRRHOTIC

ρ represents solution densities.

Studies from several other normals have shown that as much as three-quarters of the total lipo-proteins in the gradient may be similarly distrib-uted and that the contour of the patterns is the same. Outside the density range 1.034 − 1.041 the lipoproteins appear to have variable composi-tion but appear to be similar in all the normals examined thus far.

In the unfractionated plasma of biliary cirrhosis as in the normal, about 70 per cent of the total concentration of lipids and peptide appear to pile up into one large peak between the densities 1.033 − 1.044, where the composition of lipo-proteins is essentially uniform. In contrast to the normal in this range, however, the ratios for C/PL average only 0.45, for F/TC indicate little if any esterification of the cholesterol, and for C/N are much higher (.68 − 1.01).

In Figure 3 are shown density gradient patterns and chemical ratios of low density lipoproteins in Fraction I + III of normal and biliary cirrhotic plasmas. Each may be compared with its respective unfractionated plasma. In the normal, inspection of gradient patterns and analytic results reveals little if any difference. In biliary cirrhosis, however, the only similarities between Fraction I + III and the unfractionated plasma are the general contour of the pattern and the C/PL ratios. Throughout the one peak area of lipoprotein concentration Fraction I + III is unlike the unfractionated plasma in that C/N ratios are lower, averaging 0.36, and F/TC ratios indicate that the cholesterol is about 40 per cent esterified. In contrast to the normal, the bulk of the lipoproteins are concentrated in a slightly higher density range (1.036 − 1.046) where average ratios for C/PL are lower (0.45), F/TC higher (0.59), and C/N higher (0.39).

In Figure 4 are shown density gradient patterns and chemical ratios of low density lipoproteins in Fraction IV + V and in Fraction VI of the biliary cirrhotic. Since analysis of equivalent fractions from normal plasma showed no beta or low density lipoproteins, normal gradients are omitted from Figure 4. It should be noted also that in the biliary cirrhotic exceedingly low lipid values of the lower cuts (L.C.) of Fractions IV + V and VI indicate mere traces of high density ($p >$ 1.063) or alpha lipoproteins.

Fraction IV + V shows one peak concentration of lipoproteins having essentially uniform chemical composition between the densities 1.041 − 1.042. In this area 78 per cent of the total lipids and peptides in the gradient are concentrated, C/PL ratios average 0.45, cholesterol is almost entirely unesterified, and C/N ratios average 1.7.

In Fraction VI two peaks of lipid concentration are apparent; a peak in the density range 1.035 − 1.038 contains 46 per cent of the cholesterol and phospholipids of the entire gradient; the other in the range 1.039 − 1.049 contains 50 per cent of the total. In both, C/PL ratios are similar to those in Fractions IV + V and I + III. The cholesterol, however, is entirely unesterified. The C/N ratios in the density range $> 1.039 − <$ 1.049 are high (3.2 − 6.1). In the density range $<$ 1.035 − 1.038, however, the total nitrogen values after correction for phospholipid nitrogen are barely significant, and it appears that with decrease in density the increasing concentrations of lipids are almost devoid of peptides.

A summation of the concentrations of lipids, nitrogen, and dry weights in all gradients indicated good recoveries when compared to total concentrations in the 4.5-ml. aliquots of upper cuts (U.C.) from which the gradients were made. Repeat density gradient studies have shown identical patterns and analyses.

S_f classes of lipoproteins

Ultracentrifuge patterns and analysis of the relative concentrations of S_f classes of lipoproteins in unfractionated plasma and Cohn fractions from the biliary cirrhotic are shown in Figure 5. A baseline is photographically superimposed. For comparison of relative amounts of S_f 10–20 classes the pattern of a patient with long-standing atherosclerosis and coronary artery disease and a normal are given.

In biliary cirrhosis the unfractionated plasma reveals three species of lipoproteins: S_f 13 representing two-thirds of the total with the remaining being equally divided between S_f 10 and S_f 7. Fraction VI also shows three species but all have considerably faster flotation rates: S_f 25 and S_f 29 molecules comprise 65 per cent of the total, S_f 16 the remainder. Fraction IV + V consists of two species: S_f 13 predominating (90 per cent) and a remaining small amount of S_f 6. Fraction I + III contains S_f 5 molecules almost entirely.

Of particular interest is the relatively small amount of S_f 10–20 molecules seen in plasma of the atherosclerotic compared to exceedingly large concentrations of S_f 10 and S_f 13 molecules in the biliary cirrhotic.

Immunochemical analysis of fractions

Precipitin reactions were carried out on each of the fractions with antiserum reacting with all the low density lipoproteins of normal plasma. A positive reaction was obtained only for Fraction I + III.

<div align="center">DISCUSSION</div>

The present study was carried out on plasma of a single case of xanthomatous biliary cirrhosis.
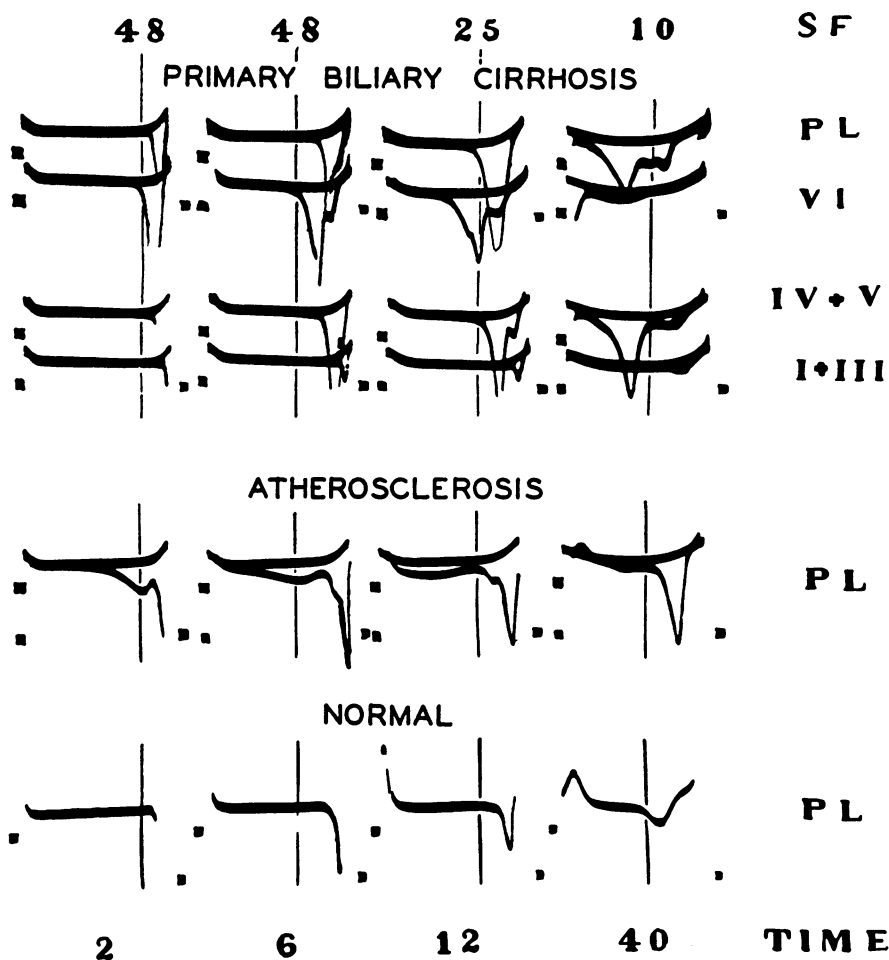
FIG. 5. ULTRACENTRIFUGAL ANALYSES OF S_f MOLECULES IN UNFRACTIONATED PLASMA AND COHN FRACTIONS OF A PRIMARY BILIARY CIRRHOTIC COMPARED TO THE PLASMA OF AN ATHEROSCLEROTIC AND A NORMAL

Primary Biliary Cirrhosis

Plasma	S_f 13 = 63%	S_f 10 = 19%	S_f 7 = 18%
Fraction VI	S_f 29 = 19%	S_f 25 = 46%	S_f 16 = 35%
Fraction IV+V	S_f 13 = 87%		S_f 6 = 13%
Fraction I+III	S_f 5 = approximately 100%		

Atherosclerosis

Plasma: S_f 20–74 = 31%; S_f 10–20 = 13%; S_f 2–10 = 56%

Similar observations on additional subjects might disclose minor variations. Analysis of plasma reveals only traces of alpha lipoprotein. Immunological tests indicate persistence of beta lipoproteins which are antigenically similar to normal beta lipoproteins but which account for only a small part of the enormous accumulation of plasma lipids. Most of the lipids are combined with peptides and with each other in atypical forms, the nature of which has been partially illuminated by

the various physical chemical examinations of the plasma protein fractions.

It appears that there are in biliary cirrhosis at least three atypical lipoproteins or groups of lipoproteins. In each the lipoproteins migrate electrophoretically as beta globulins; the content of peptide in proportion to analyzed lipid is smaller than is found in normal beta lipoproteins; and mean density as observed in density gradient analyses is somewhat higher than Oncley and

Mannick (12, 28, 29) reported in their density gradient studies of beta-1 lipoprotein prepared from Fraction III — O from normal, pooled Red Cross plasma.

One of the atypical lipoproteins is recovered in Fraction IV + V. It has an electrophoretic mobility of a beta globulin and appears in the density gradient tube in a density range of 1.041 — 1.042. It is characterized by the following approximate ratios: cholesterol-phospholipid of 0.45, free to total cholesterol of 0.95, (molar) cholesterol to nitrogen of 1.5, and peptide to analyzed lipid of 1 to 50.

In Fraction VI there is another lipoprotein with electrophoretic mobility of a beta-2 globulin, appearing in the density gradient tube at a somewhat higher density range (1.043 — 1.044), possessing a similar cholesterol-phospholipid ratio, slightly higher free to total cholesterol ratio, and a much higher cholesterol to nitrogen ratio. The relation of peptide to analyzed lipid is about 1 to 80.

These lipoproteins do not react with antibodies to the protein part of normal beta lipoproteins, suggesting that the protein here is antigenically dissimilar. It is possible, however, that lipids block the antigenic site.

Neither of these atypical lipoproteins recovered in Fractions IV + V and VI is identical with another found in Fraction I + III. The major lipoprotein of Fraction I + III appears in the density gradient tube at a density of 1.040, where analyses in this range indicate a cholesterol to phospholipid ratio of 0.45, free to total cholesterol ratio of 0.63, molar cholesterol to nitrogen ratio of about 0.43, and a peptide to analyzed lipid ratio approximately 1 to 7. These values deviate widely from those of normal beta lipoproteins. Since normal beta lipoproteins occur in the same density range and since the immunological tests indicate the presence of beta lipoproteins antigenically similar to the normal, the possibility that the chemical analyses could represent mixtures must be considered. Thus the analytic data cannot be taken as definitive of the atypical lipoproteins in Fraction I + III.

In addition to these recognizable lipoproteins, there is in the density gradient analysis of Fraction VI a large accumulation of lipids at a density of 1.038 and less, which is almost devoid of pep-

tides. Repeat studies of the plasma made several months apart have confirmed the low peptide content and have shown identical gradient patterns and analyses. Although the paucity of peptides in the mixture might discredit the existence of definite lipoprotein molecules in this density range, other explanations must be considered. It is possible that some extremely labile lipoproteins may have been destroyed by exposure to fairly concentrated alcohol buffers in the early steps of the Cohn fractionation.

It is also possible that in assuming that phospholipids are chiefly in the form of lecithin and cephalin and therefore calculating a nitrogen-phosphorus ratio of 1:1 we have overestimated the lipid nitrogen which is subtracted from total nitrogen and thus erroneously have derived peptide concentrations that appear scarcely significant. Because of this assumption and also because the density of the high lipid-low peptide mixture in Fraction VI is higher than might be expected, acetal phospholipid analyses were undertaken but with negative results.

A word may be added concerning the distribution of fatty acids. In biliary cirrhosis, fatty acids recovered in Fraction IV + V + VI and representing three-quarters of the plasma concentration are derived almost exclusively from the enormous quantities of phospholipids. In Fraction I + III, however, they are derived equally from the phospholipids and neutral fat (triglycerides + FFA). In normal plasma, the greatest concentration of total fatty acids is in Fraction I + III where they are derived equally from cholesterol esters, phospholipids, and neutral fats.

Difficulty is encountered in correlating peptide-lipid combinations detected by analysis of density gradients with the lipoproteins indicated by Gofman flotation rates. Density gradient separation of the lipoproteins floated from the unfractionated plasma in biliary cirrhosis fails to resolve the atypical lipoproteins, apparently because of general aggregation of lipoprotein molecules in the density range 1.035 — 1.044. Similar analyses made on the Cohn fractions afford closer inspection and better characterization. Study of flotation rates of lipoproteins in both unfractionated plasma and Cohn fractions provides additional properties. In biliary cirrhosis the major concentration of molecules is in the S_f 10–20 cate-

gory. Analytic data obtained from previous Cohn fractionation (5) and the present density gradient studies indicated, however, that the lipoproteins differ chemically from those in a disease such as atherosclerosis, which is also characterized by large concentrations of S_f 10–20 molecules. The discrepancy offers additional evidence that no one method of analysis suffices for characterization of lipoproteins.

SUMMARY

Lipoproteins in the plasma of a woman with biliary cirrhosis have been compared by chemical, physical-chemical, and immunochemical methods to those found in the plasma of a normal woman of like age.

Preliminary fractionation of plasma by Cohn method number 10 and subsequent examination of density gradients made of the lipoproteins floated from the fractions have permitted separation and analysis of several atypical protein-lipid combinations not distinguishable by examination of the unfractionated plasma.

In biliary cirrhosis only traces of high density or alpha lipoproteins are found. The presence of normal low density or beta lipoproteins can be inferred only from immunologic tests and if present account for only a relatively small part of the enormously augmented plasma lipids. Most of the lipids are combined atypically with beta globulins as low density lipoproteins.

Three separate abnormal beta lipoproteins have been partially characterized. In all of them the peptide in proportion to analyzed lipid is less than that found in normal beta lipoproteins. The cholesterol-phospholipid ratios are less than normal, and concentration of esterified cholesterol is low or even absent.

Gofman flotation analysis of plasma in biliary cirrhosis shows that molecules within the S_f 10–20 classes form the major portion of the spectrum and in far greater concentrations than seen in atherosclerosis. However, despite similar flotation rates, chemical analyses indicate the lipoproteins in biliary cirrhosis differ from those in atherosclerosis.

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